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# The Transgenic BmN Cells with Polyhedrin Gene: A Potential Way to Improve the Recombinant Baculovirus Infection *Per Os* to Insect Larvae

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**Abstract** The principle of baculovirus expression system is that substitute exogenous gene for polyhedrin (*polh)* gene, and the recombinant baculovirus lacks the ability to infect insect larvae by oral inoculation. In this study, we cloned the *polh* gene with immediate early gene 1 (*ie1*) promoter of *Bombyx mori* nucleopolyhedrovirus (BmNPV) into transposon pigA3GFP vector, transported it into BmN cells by lipofectamine and obtained the transgenic BmN cell line. The mRNA transcription of the polyhedrin gene was demonstrated by reverse transcription-polymerase chain reaction. Then the *polh* gene negative viruses (BmPAK6 and BmGFP), infected the transgenic BmN cells and Polyhedrin-like structures were observed in the infected cells. Subsequently, the viruses (vBmPAK6 and vBmGFP) from infected cells were used to orally inoculated the fifth instar larvae of *B. mori*, respectively. The results showed that *B. mori* larvae could be infected *per os* with the recombinant baculoviruses vBmPAK6 and vBmGFP, respectively. These results suggest that the products of *polh* gene expressed in the transgenic BmN cells could package the recombinant baculoviruses when the viruses infected the cells and raise the pathogenicity of the recombinant virus in orally infected *B. mori* larvae.

**Keywords** Baculovirus · Polyhedrin · Transgenic · *Bombyx mori* · Infection

## Introduction

Baculoviruses comprise a large family of viruses, which naturally infect many different insect species. In its wild type form, which produce two virus phenotypes: the budded virus (BV) and the occlusion-derived virus (ODV) [1]. BVs are produced first in the infection cycle and transmit virus infection within the insect host tissues or in insect cell cultures. ODVs are produced later in infection and are occluded in polyhedral or granular occlusion bodies (OBs) [2].

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Recombinant baculoviruses have become widely used as vectors to express heterologous genes in cultured insect cells and insect larvae. The baculovirus expression vector system (BEVS) has a number of unique features, such as the capacity for large inserts of DNA and a high yield of recombinant protein. Proteins produced in the BEVS are very similar to naturally occurring human proteins in terms of post-translational modifications (e.g., phosphorylation), biological activity, and protein stability. For this reason the BEVS is widely used in academia and industry [3–5]. However, BEVS lacks the *polh* gene and does not produce OBs. Thus, these kinds of recombinant baculovirus had to be inoculated to insect larvae. It made the larvae wounded (even infected by other microorganisms, such as bacteria) in the process of infection. In current study, there was the desire to develop a new method to improve the recombinant baculovirus infection *per os* to insect larvae [6].

Unlike late and very late promoters, the promoter from the *ie1* gene is expressed immediately after infection and in uninfected cells, in the absence of viral transcription factors [7, 8]. Thus, we inserted the fragment of *polh* gene under control of *ie1* promoter of *Bombyx mori* nucleopolyhedrosis virus (BmNPV) into transposon pigA3GFP vector [9, 10], and transported it into BmN cells by lipofectamine with the helper vector pHA3PIG which encodes piggyBac transposase. Then we got the stable transgenic BmN cell line and tested its ability to package the virus which lacks the *polh* gene. This study showed that the products of *polh* gene from transgenic BmN cells could package the recombinant baculovirus, and raise the pathogenicity rate of virus which infected *B. mori* larvae by oral inoculation.

## Materials and Methods

### Cells and Media

BmN cells were propagated in TC-100 medium (Gibco) supplemented with 10% fetal bovine serum [11]. Larvae of the silkworm were reared on mulberry leaves [12]. The wild type (wt) BmNPV, and the recombinant baculoviruses BmGFP and BmPAK6 were collected from the culture medium at 96-h post-infection, respectively [13].

### Construction of Vector

From the genomic DNA of BmNPV, the *ie1* promoter fragment was amplified by polymerase chain reaction (PCR) using specific primers with the *Eco*R I/*Kpn* I sites, and cloned into the *Eco*R I/*Kpn* I sites of pigA3GFP vector. The primers were:

Forward: 5′-CTTGAATTCGACTTGGACTCGGCCA-3′ and
Reverse: 5′-CTCGGTACCTAGTCGTTTGGTTGTT-3′. The polyhedrin fragment was amplified by PCR using specific primers with the *Kpn* I/*Sal* I sites,
Forward: 5′-CGCGGTACCATAAATATGCCGAAT-3′ and
Reverse: 5′-CGCGTCGACCTGAAAATCATTTGA-3′, digested with *Kpn* I/*Sal* I, and cloned into the pigA3GFP (with *ie1* promoter), the resulting plasmid was named pigA3GFP–*ie1*–*polh*.

### Transfection of the Recombinant Plasmids into BmN Cells

DNA of the resulting vector plasmid (pigA3GFP–*ie1*–*polh*) and helper plasmid pHA3PIG were transfected into BmN cells by the transfection Lipofectamin 2000

(Invitrogen). Briefly, BmN cells ($1 \times 10^6$) were seeded in the wells of six-well plates and transfected via contact with the resulting vector plasmid and helper plasmid complex for 3 h at 26 °C [14]. After incubation for 72 h followed by replacement of the transfection medium with complete TC-100 medium, transgenic BmN cells were observed using fluorescent microscope equipped with green fluorescent protein (GFP) filter set (Leica).

## Detection of the Transcription of the *Polh* Gene

Total cellular RNA was isolated using Trizol reagent (Invitrogen) from the transgenic BmN cells. For reverse transcription (RT)-PCR, the first-strand cDNA was synthesized from 10 μg of total RNA with oligo(dT) primers and reverse transcriptase (Invitrogen) [15]. Subsequently, cDNA fragment was amplified by 30 cycles of PCR. In order to eliminate the possibility that the PCR product was amplified from contaminated genome DNA, the negative control without reverse transcription was routinely included in this reaction. PCR primers with a length of 700 bp were designed on the basis of the *polh* gene sequences of BmNPV. The primers were:

Forward, 5′- ATGCCGAATTATTCATACACCC - 3′, and
Reverse, 5′- ATACGCCGGACCAGTGAACAGA- 3′. The PCR products were separated in 1.0% agarose gel along with DNA marker.

## Recombinant Virus Infection in the Transgenic BmN Cells

The transgenic BmN cells were seeded in 6-well plates. To prepare occlusion bodies for the infection of the *B. mori* larvae, the transgenic BmN cells were infected with the *polh* gene negative viruses recombinant baculoviruses BmPAK6 (the mutant virus with lac Z gene instead of *polh* gene) and BmGFP (the mutant virus with *gfp* gene instead of *polh* gene) at 27 °C, respectively [16]. Seventy-two hours after infection, virus-infected cells were observed using fluorescent microscopy. After the occlusion bodies were observed, the cells were collected from culture dishes, centrifuged at 2,400×*g* for 10 min, and resuspended in distilled water.

## Recombinant Virus Infection in *B. mori* Larvae

In order to detect *per os* infection of the OBs packaged by the transgenic BmN cells, the *B. mori* larvae (50 larvae in each experiment), in the first day of fifth instar were orally inoculated with recombinant viruses (vBmGFP and vBmPAK6). We preferred this late larval stage because the fifth instar was of the largest duration (lasting for 8–10 days) so that the progress in virus infection could be monitored for several days without interference by the intervening larval molts [17]. The larvae were reared on fresh mulberry leaves. The OBs were purified and calculated. OBs, $1 \times 10^6$/mL, were used to inoculate *B. mori* orally by droplet feeding bioassay. The dead larvae were collected daily, and confirmed to be infected by the two kinds of the OBs by fluorescence observation or X-Gal staining test [18]. All bioassays were repeated three times.

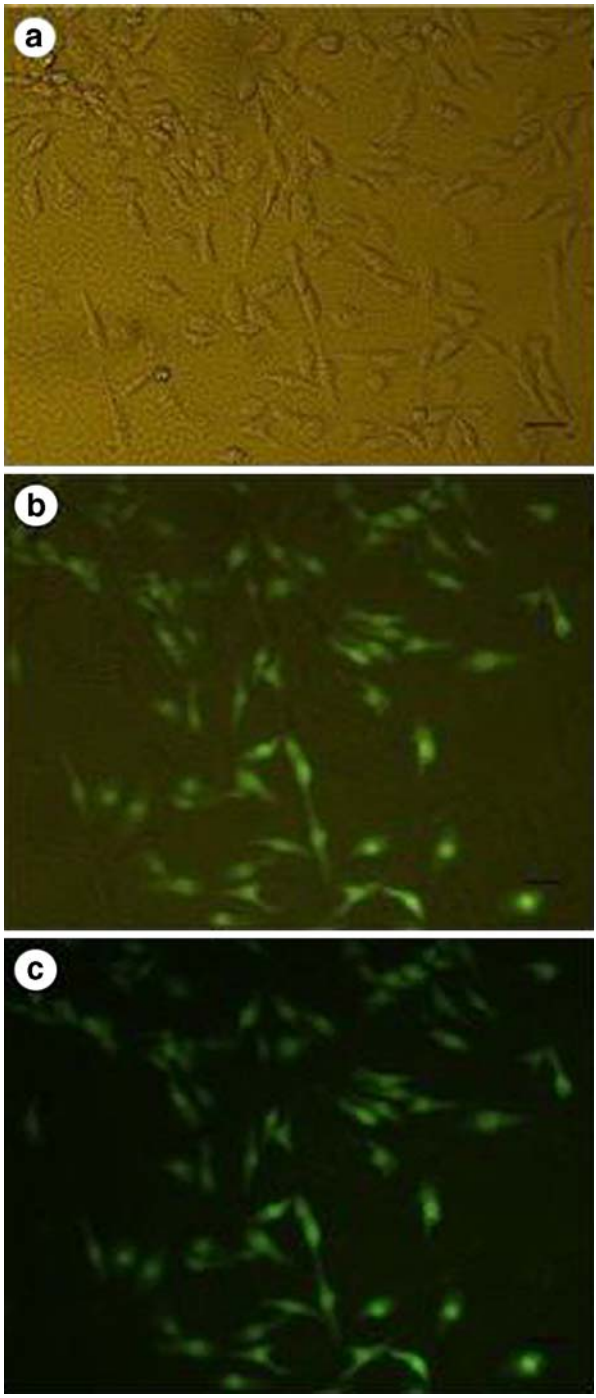


**Fig. 1** Observation of the transgenic BmN cells. For generating the transgenic BmN cells, the vector pigA3GFP–*ie1–polh* mixed with the helper plasmid transfected into BmN cell ($1 \times 10^6$ cells) by lipofection technique at 27 °C. The transgenic BmN cells were selected based on GFP expression Panels: **a** bright field; **b** mixed light field; **c** fluorescent field. The *scale bars* in the visible images are 15 μm

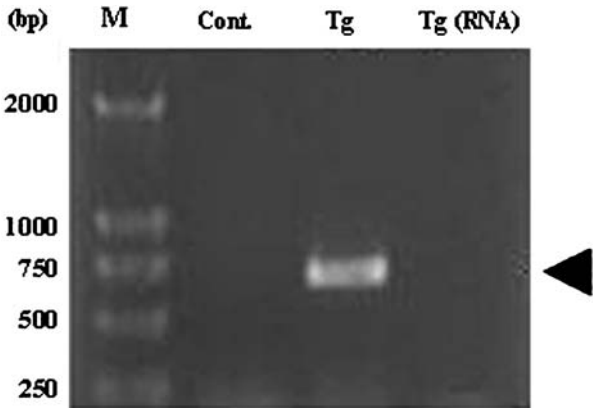


**Fig. 2** Analysis of *polh* gene expression in the transgenic cells. RT-PCR was performed using *polh*-specific primer sets and total RNA purified from the transgenic cells (Tg), untransfected BmN cells as a control (Cont.). The result of PCR when the reverse-transcription step is omitted was also shown (Tg RNA). The PCR products were fractionated on 1% agarose gel and stained with ethidium bromide

## Results

### Generation of the Transgenic BmN Cell Line

For generating the stable transgenic BmN cell line, the BmN cells were selected based on GFP expression. The presence of GFP under *B. mori* actinA3 promoter was confirmed by the observation under fluorescent microscopy. After several rounds of selecting, the number of GFP-positive cells increased significantly (Fig. 1). These results indicated that the stable transgenic BmN cells were obtained.

### Analysis of *Polh* Gene Expression

The transcription of the inserted *polh* gene in transgenic BmN cells was examined by RT-PCR. The expected fragment with 700 bp was amplified by using cDNA of the transgenic BmN cells as template (Tg), meanwhile no such fragment was amplified in the control of the total RNA (Tg RNA) directly as template and the cDNA of untransfered BmN cells

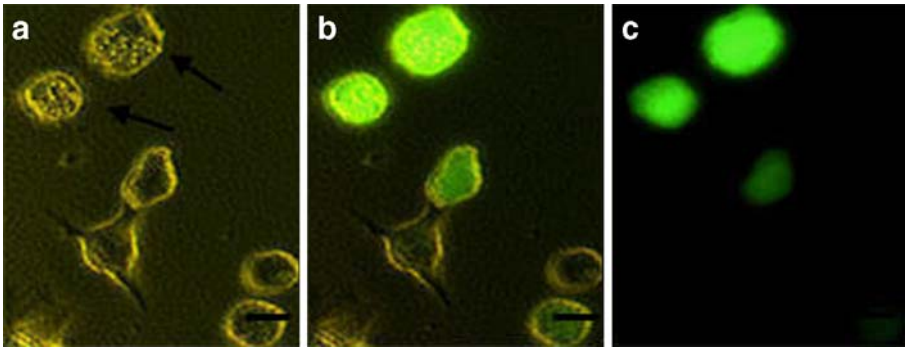


**Fig. 3** Detection of polyhedra in the transgenic BmN cells infected with BmPAK6. Virus-infected cells were visualized by microscope at 72 h infection under **a** visible bright light, **b** mixed light and **c** fluorescence. The *scale bars* in the visible images are 10 μm. The recombinant baculovirus obtained polyhedra in the transgenic BmN cells which emit fluorescence. Some of the inclusion bodies are indicated by *arrowheads*

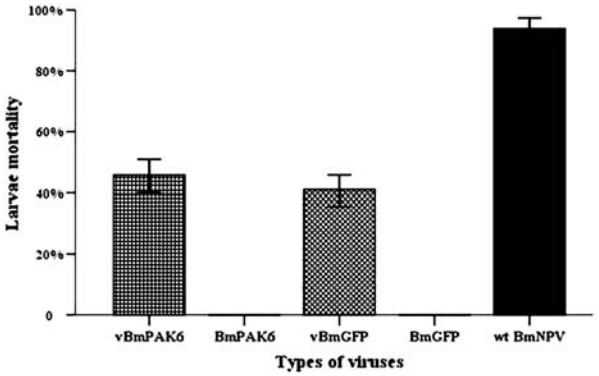


**Fig. 4** Mortality of *B. mori* larvae orally infected with vBmPAK6, vBmGFP, and BmNPV, respectively. The three types of viruses (vBmPAK6, vBmGFP, and wt BmNPV) were insufflated to mulberry leave and inoculated *per os* to *B. mori* larvae (50 larvae in each group). The dead larvae were collected daily, and confirmed to be infected with the two kinds of the OBs by fluorescence observation or X-Gal staining test. No larvae dead in the groups of the control. The mortality of *B. mori* larvae had been calculated, and standard errors are indicated

(Cont.)(Fig. 2). It indicated that mRNA of the *polh* gene was transcripted in the transgenic BmN cells.

## Generation of the OBs of Recombinant Baculoviruses

In order to examine whether the expression product of the *polh* gene could package the recombinant virus, the transgenic BmN cells were infected with the recombinant baculoviruses without the *polh* gene, BmPAK6, and BmGFP, respectively. The OBs were observed in the nuclei of the transgenic BmN cells at 72-h post-infection (Fig. 3). However, the OB number of the recombinant baculoviruses in the transgenic BmN cells is less than that in the BmN cells which infected with wild BmNPV. The result suggests expression products of the *polh* gene in the transgenic BmN cells can package the virus particles of BmPAK6 and BmGFP. We named the OBs of BmPAK6 and BmGFP as vBmPAK6 and vBmGFP.

## Infection Test of the *B. Mori* Larvae with the OBs

To investigate the virulence of the packaged viruses, the *B. mori* larvae were orally inoculated with the OBs of the viruses (vBmPAK6 and vBmGFP), a positive control of the wt BmNPV(50 larvae in each group) and a blank control respectively. And each test was repeated three times. The dead larvae were collected daily, and confirmed to be infected with the two kinds of the OBs by fluorescence observation or X-Gal staining test. In the X-Gal staining test, the larvae mortality that infected with vBmPAK6 was 43%. And the larvae mortality that infected with vBmGFP was 39%, which were counted by fluorescence observation. The larvae mortality that infected with the control virus-wt BmNPV was 95%. No larvae dead in the groups of the control (Fig. 4). Recombinant baculoviruses that were packaged by the transgenic BmN cells (vBmPAK6 and vBmGFP) all exhibited a high infectivity phenotype relative to the origin recombinant baculoviruses, showing that the expression product of the *polh* gene did have a significant effect on the recombinant baculoviruses infectivity to *B. mori* larvae. However, the *B. mori* larvae mortality by

vBmPAK6 (or vBmGFP) compare to that by BmNPV was about 40%, suggesting that the infectivity of vBmPAK6 (or vBmGFP) was lower than that of wt BmNPV in orally infected *B. mori* larvae.

## Discussion

Polyhedrin is a protein produced by wild-type baculovirus to protect the virus from environmental pressures, such as temperature [19]. However, polyhedrin is not required for the formation of viral particles in tissue culture. In many biological studies, the *polh* gene as an unnecessary gene of NPV was substituted for foreign genes in BEVS. Hence, the recombinant viruses lacking the *polh* gene do not produce OBs and are not suitable for *per os* infection.

In order to develop a new method to improve the *per os* infection of the recombinant baculovirus, we inserted the fragment of the *polh* gene under the control of *ie1* promoter of BmNPV into transposon pigA3GFP vector, and transported the recombinant vector into BmN cells. After screening, the stable transgenic BmN cell line was obtained. Then the *polh* gene negative viruses, BmPAK6 and BmGFP, infected the transgenic BmN cells, and polyhedrin structures were observed in the infected cells (Fig. 3). The results indicated that the transgenic BmN cells could package the exogenous virus and formed occlusion bodies.

The recombinant virus, BmGFP or BmPAK6, was unable to infect the *B. mori* larvae by oral inoculation. However, in our experiments, these two viruses could infect the *B. mori* larvae *per os* inoculation through the viral package in the transgenic BmN cells with the products of the *polh* gene. The virulence of the recombinant virus had been confirmed by fluorescence observation or X-Gal staining test from the *B. mori* dead larvae. Thus, the viral package in the transgenic BmN cells did significantly affect the oral infectivity of the recombinant virus. The package of recombinant baculovirus is convenient for *per os* inoculation of *B. mori* larvae, and it could prevent bacteria infection as well.

In addition, a difference was observed in *B. mori* larvae mortality infected with wt BmNPV, vBmPAK6 and vBmGFP (Fig. 4), suggesting that the infectivity of vBmPAK6 (or vBmGFP) was lower than that of wt BmNPV in orally infected *B. mori* larvae. That might be attributed to insufficient OBs in transgenic BmN cells after infected with BmPAK6 and BmGFP.

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