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Immobilization of foreign protein into polyhedra of *Bombyx mori* nucleopolyhedrovirus (BmNPV)*

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Abstract: In the late phase of *Bombyx mori* nucleopolyhedrovirus (BmNPV) infection, a large amount of polyhedra appear in the infected cell nucleolus, these polyhedra being dense protein crystals protecting the incorporated virions from the harsh environment. To investigate whether the foreign protein could be immobilized into the polyhedra of BmNPV, two recombinant baculoviruses were generated by a novel BmNPV polyhedrin-plus (polh$^+$) Bac-to-Bac system, designated as vBmBac(polh$^+$)-enhanced green fluorescent protein (EGFP) and vBmBac(polh$^+$)-LacZ, which can express the polyhedrin and foreign protein simultaneously. Light microscopy analysis showed that all viruses produced polyhedra of normal appearance. Green fluorescence can be apparently detected on the surface of the vBmBac(polh$^+$)-EGFP polyhedra, but not the BmNPV polyhedra. Fluorescence analysis and anti-desiccation testing confirmed that EGFP was embedded in the polyhedra. As expected, the vBmBac(polh$^+$)-LacZ polyhedra contained an amount of LacZ and had a higher β-galactosidase activity. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were also performed to verify if the foreign proteins were immobilized into polyhedra. This study provides a new inspiration for efficient preservation of useful proteins and development of new pesticides with toxic proteins.

Key words: *Bombyx mori* nucleopolyhedrovirus, Polyhedra, Foreign protein, Protein immobilization
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1 Introduction

Bombyx mori nucleopolyhedrovirus (BmNPV) is a member of *Baculoviridae*. Members of the *Baculoviridae* are characterized by having enveloped, rod-shaped virions with large circular double-stranded DNA genomes. During the typical biphasic infection cycle, two types of progeny viruses, budded virus (BV) and occlusion-derived virus (ODV), are produced in different infection phases with different infective bioactivities. ODV is transmitted from insect to insect via oral infection, whereas BV spreads infection to neighboring cells (Keddie *et al*., 1989; Zhao *et al*., 2008). In the late phase of infection, the virus produces large, proteinaceous particles called polyhedra, which occlude progeny virions. The polyhedron can protect the incorporated virus from desiccation, high temperature, even acid cauterization, and allow the virus to survive for several years in soil (Rohrmann, 1986; Slack and Arif, 2006).

Many baculoviruses can produce polyhedra during the late phase of viral infection. At present, the structure of polyhedra of *Bombyx mori* cytoplasmic polyhedrosis virus (BmCPV) has been studied in great details. It reveals that polyhedron is a trimeric building block connected by extensive interactions (Coulibaly *et al*., 2007). The Japanese researchers demonstrated that the N-terminal 75 amino acids of BmCPV turret protein (VP3) can function as a polyhedrin recognition signal, leading to the incorporation of foreign proteins into polyhedra. Enhanced green fluorescent protein (EGFP) and a variety of human

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proteins tagged with the VP3 polyhedrin recognition signal were incorporated into polyhedra by co-expression with polyhedrin in insect cells. The proteins immobilized into the polyhedra were protected from dehydration and stabilized against higher temperature (Ikeda *et al*., 2006). Subsequently, researchers confirmed that the polyhedrin H1-helix also functioned as a polyhedrin recognition signal and can be used like the VP3 N-terminal sequence to target foreign proteins into polyhedra (Ijiri *et al*., 2009). Fibroblast growth factor-2 (FGF-2), FGF-7, and epidermal growth factor (EGF) were successfully immobilized into polyhedra with H1 signal (Ijiri *et al*., 2009). Recently, Ji *et al*. (2010) have determined the structure of polyhedra of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), revealing a highly symmetrical covalently cross-braced robust lattice, the subunits of which possessed a flexible adaptor enabling this supramolecular assembly to specifically entrap massive baculoviruses. Coulibaly *et al.* (2009) revealed that, although the baculovirus and cypovirus polyhedra had nearly identical unit cell dimensions and shared I23 symmetry, the polyhedrin molecules were structurally unrelated and packed differently in the crystals. Therefore, we wanted to test if the polyhedra of BmNPV can be used for encapsulating and stabilizing bioactive proteins and development of high-throughput protein microarrays.

Two recombinant viruses were generated by the BmNPV polyhedrin-plus (polh$^+$) Bac-to-Bac expression system, designated as vBmBac(polh$^+$)-EGFP and vBmBac(polh$^+$)-LacZ, which can express the polyhedrin and foreign protein simultaneously. There is no significant difference between the polyhedra of recombinant virus and the wild-type. In this paper, we investigated if both the foreign proteins could be immobilized into respective polyhedra.

2 Materials and methods

2.1 Materials

The BmNPV polh$^+$ Bac-to-Bac expression system was constructed by our laboratory (Xiang *et al*., 2010). The donor plasmid pFastBacHTs and Cellfectin® reagent for transfection were purchased from Invitrogen (USA). EGFP antibody was available from BioVision (USA), and LacZ antibody was purchased from Calbiochem (Germany).

Bombyx mori BmN cells were maintained at 27 °C in TC-100 medium supplemented with 10% fetal calf serum (FCS). FCS and culture medium TC-100 for cultured cells were purchased from GibcoBRL (USA). BmNPV was stored in our laboratory.

2.2 Construction of the recombinant virus

The *egfp* and *lacZ* genes were cloned by polymerase chain reaction (PCR) using pEGFP-N3 (Clontech, USA) and pBlueBacHisB (Invitrogen) as templates, respectively (Zhang *et al.*, 1996; Nordström *et al.*, 1999). The amplified fragments of *egfp* and *lacZ* were digested with *Bam*HI and *Hin*dIII, respectively, and cloned into the corresponding sites of pFastBacHTa to construct the final recombinant donor vectors. The recombinant donor vectors were confirmed by PCR amplification and restriction digestion.

Using BmNPV polh$^+$ Bac-to-Bac expression system, the recombinant donor vector was transformed into DH10BmBac (polh$^+$) competent cells, incubated at 37 °C for 4 h, serially diluted using SOC medium, and spread evenly on plates containing kanamycin (50 μg/ml), gentamicin (7 μg/ml), tetracycline (10 μg/ml), X-Gal (100 μg/ml), and isopropyl β-D-1-thiogalactopyranoside (IPTG; 40 μg/ml). After 48 h incubation at 37 °C, the white colonies verified by PCR were picked and cultured overnight in medium containing kanamycin. Bacmids DNA was subsequently extracted and transfected into BmN cells to generate recombinant baculoviruses (Cao *et al*., 2006). The recombinant viruses were designated as vBmBac(polh$^+$)-EGFP and vBmBac(polh$^+$)-LacZ.

2.3 Purification of polyhedra

BmN cells were inoculated with the recombinant virus, collected at 96 h post-infection, washed with phosphate buffered saline (PBS; 20 mmol/L NaH_2PO_4, 20 mmol/L Na_2HPO_4, 150 mmol/L NaCl, pH 7.2), and fragmented by ultrasonic wave. We then collected the precipitate by centrifugation at 15000×*g* for 10 min. The pellets containing polyhedra were further purified by Percoll density gradient centrifugation at 15000×*g* for 20 min. A nine-to-one ratio of Percoll to PBS was employed for this purpose. The purified polyhedra were washed several times with PBS and finally suspended in PBS buffer.

2.4 Measurement of fluorescence by the EGFP immobilized into polyhedra

Equal amounts of purified BmNPV and vBmBac (polh$^+$)-EGFP polyhedra were suspended in 50 mmo/L carbonate buffer (Na_2CO_3-$NaHCO_3$, pH 11.0). After 30 min incubation at 30 °C, EGFP fluorescence was measured by excitation light source at 395 nm and emission light source at 510 nm using an F-4600 fluorescence spectrophotometer. We repeated this experiment three times, and took the average.

2.5 Analysis of the stability of the EGFP immobilized into polyhedra

Equal amounts of purified vBmBac(polh$^+$)-EGFP polyhedra were collected. One was dried 30 min at room temperature after centrifugation, and the other was suspended in PBS buffer (pH 7.2) for 30 min, and then collected and resuspended in carbonate buffer (pH 11.0). After 30 min incubation at 30 °C, EGFP fluorescence was measured as above.

2.6 Analysis of β-galactosidase activity

Polyhedra of BmNPV and vBmBac(polh$^+$)-LacZ were mixed with X-Gal (100 μg/ml). The β-galactosidase activity of polyhedra was detected using the β-galactosidase enzyme assay system (Promega) as described by Hitchman *et al*. (2010). Briefly, reporter lysis buffer (200 μl) was added to a cell pellet and incubated at room temperature for 15 min, vortexed and centrifuged at 8000×*g* for 2 min. The supernatant was then transferred to a fresh tube. Assays were carried out in standard assay format, where dilutions of cell lysate were prepared in reporter lysis buffer in a total volume of 150 μl. A total of 150 μl of 2× assay buffer was then added to the plate and mixed by vortexing, before incubating the plate at 37 °C for 30 min. Each plate also contained a negative control (reporter lysis buffer only) and a standard dilution of β-galactosidase (1:10000). The reaction was stopped with 500 μl of 2 mol/L sodium carbonate and the absorbance was read immediately at 410 nm.

2.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

The polyhedra purified from BmN cells infected with vBmBac(polh$^+$)-EGFP and vBmBac(polh$^+$)-LacZ were electrophoresed in 12% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membrane with a Bio-Rad liquid transfer apparatus, according to the manufacturers' recommended protocols. The vBmBac(polh$^+$)-EGFP polyhedra were probed by rabbit monoclonal EGFP antibody (1:1000) as a primary antibody, and goat anti-rabbit IgG antibody (1:1000) as a secondary antibody, while the vBmBac(polh$^+$)-LacZ polyhedra were probed with the primary antibody (1:10000) to mouse monoclonal β-galactosidase antibody followed by a secondary goat anti-mouse IgG antibody (1:1000). 3,3′,5,5′-Tetramethylbenzidine (TMB) was used to display the color.

3 Results

3.1 Observation of polyhedra under microscopy

At 96 h post infection, light microscopy analysis showed that all viruses produced polyhedra of normal appearance with no difference in shape, size, or amount (Fig. 1a). The size and shape of polyhedra were almost the same as those of the control. Further, we investigated the polyhedron production in BmN cells, and observed that equivalent polyhedra existed in BmN cells infected with vBmBac(polh$^+$)-EGFP, vBmBac(polh$^+$)-LacZ, and BmNPV at 96 h post infection (hpi) (Fig. 1b). These results indicate that the recombinant virus we constructed can form normal polyhedra in the same quantity as the control. The virions in the polyhedra were also checked in our previous study (Xiang *et al*., 2010), and we found that these polyhedra could also embed the virions, like the wild-type BmNPV.

3.2 Analysis of the EGFP in vBmBac(polh$^+$)-EGFP polyhedra

Purified polyhedra from BmN cells infected with the vBmBac(polh$^+$)-EGFP were suspended in PBS (pH 7.2) and examined by laser-scanning confocal microscopy (Fig. 2a). Green fluorescence was observed, predominantly on the surface, with polyhedra isolated from BmN cells that had been infected with vBmBac(polh$^+$)-EGFP. In contrast, there was no green fluorescence observed from the BmNPV polyhedra. Suspending the vBmBac(polh$^+$)-EGFP polyhedra in 50 mmol/L carbonate buffer (pH 11.0), green fluorescence was also analyzed in a fluorescence

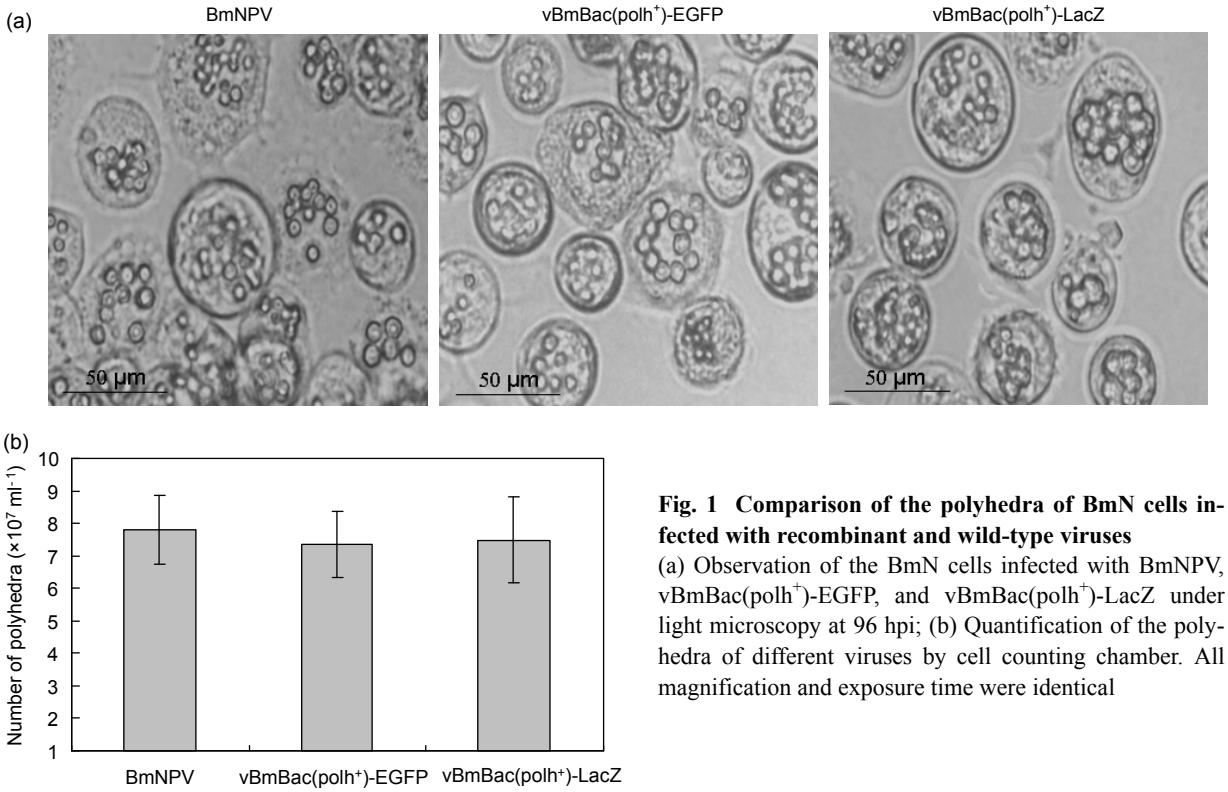

Fig. 1 Comparison of the polyhedra of BmN cells infected with recombinant and wild-type viruses
(a) Observation of the BmN cells infected with BmNPV, vBmBac(polh$^+$)-EGFP, and vBmBac(polh$^+$)-LacZ under light microscopy at 96 hpi; (b) Quantification of the polyhedra of different viruses by cell counting chamber. All magnification and exposure time were identical

spectrophotometer (Fig. 2b). Suspending the polyhedra isolated from BmN cells infected with vBmBac (polh$^+$)-EGFP in a buffer at pH 11 totally dissolved the particles, as expected, and higher levels of green fluorescence were observed. The fluorescence of vBmBac(polh$^+$)-EGFP polyhedra was six-fold higher than that of BmNPV polyhedra as negative control.

3.3 Analysis of the stability of the EGFP immobilized into polyhedra

From Fig. 2, fluorescence was observed on the surface of polyhedra, but it was not clear whether EGFP was embedded in the polyhedra. Hence, we determined the fluorescence of the lysed polyhedra (Fig. 3). The fluorescence of BmN cells infected with vBmBac(polh$^-$)-EGFP disappeared in the dehydrated state (data not shown). However, the fluorescence was detected from the suspension of polyhedra in carbonate buffer (pH 11.0), but not from suspension of polyhedra in PBS buffer (pH 7.2). The dehydrated polyhedra suspending in carbonate buffer (pH 11.0) also showed fluorescence that was weakened slightly compared to that without dehydrated treatment. The results show that the polyhedra structure can protect the inner protein from the damage of desiccation.

3.4 Analysis of the β-galactosidase activity of vBmBac(polh$^+$)-LacZ polyhedra

β-Galactosidase as a commonly used reporter molecule was analyzed in this study. Polyhedra were purified from the vBmBac(polh$^+$)-LacZ infected BmN cells, suspended in PBS (pH 7.2), and used to detect the activity of β-galactosidase. Using X-Gal as a substrate for color reaction, vBmBac(polh$^+$)-LacZ polyhedra, but not BmNPV polyhedra, showed β-galactosidase activity (Fig. 4a). At the same time, we measured the β-galactosidase activity of polyhedra using the β-galactosidase enzyme assay system (Fig. 4b). The results indicate that the β-galactosidase activity of polyhedra reached 1000 mU/ml, which was 50-fold higher than that of the BmNPV polyhedra. Thus, it was concluded that the polyhedra of recombinant viruses contained LacZ.

3.5 SDS-PAGE and Western blotting

In order to verify the above result, further confirmation of the foreign protein immobilized into polyhedra was performed by SDS-PAGE and Western blotting. From Fig. 5a we found that EGFP existed in polyhedra and accounted for a considerable

proportion of the polyhedra. It was further confirmed by Western blotting with the anti-EGFP antibody. The LacZ could also be detected in polyhedra by SDS-PAGE. Western blotting was also analyzed with the anti-β-galactosidase antibody (Fig. 5b). All these results showed that the foreign proteins were incorporated into the polyhedra.

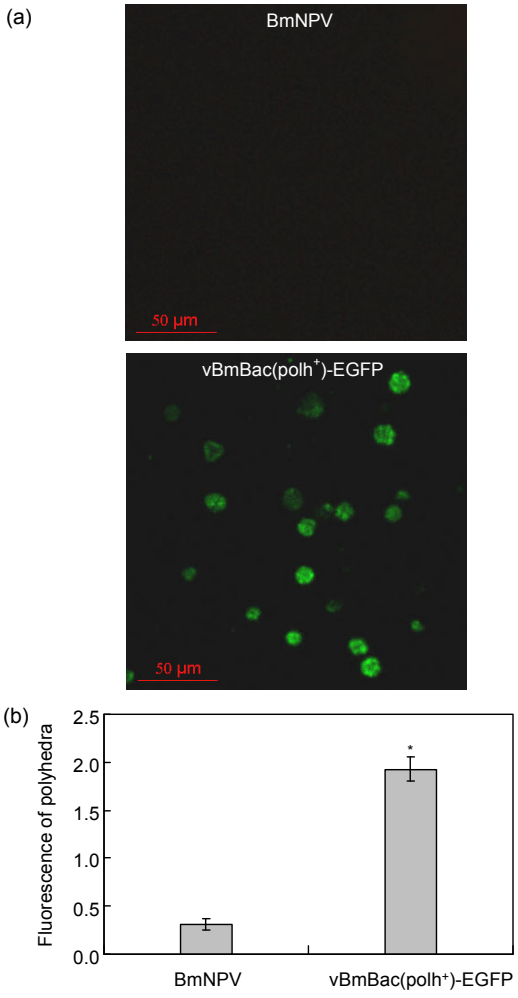

Fig. 2 Analysis of the EGFP in polyhedra produced by vBmBac(polh$^+$)-EGFP

(a) Purified polyhedra from BmN cells infected with vBmBac(polh$^+$)-EGFP at 96 hpi were observed under fluorescence microscopy, the BmNPV polyhedra being used as negative control. (b) Green fluorescence from vBmBac(polh$^+$)-EGFP polyhedra was measured by fluorescence spectrophotometry. Each green fluorescence was measured in triplicate, and results are shown as mean± standard error (SE). * P<0.01, significant differences between the vBmBac(polh$^+$)-EGFP polyhedra and BmNPV polyhedra (one-way analysis of variance (ANOVA))

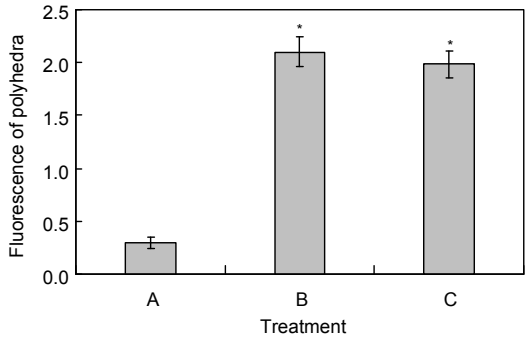

Fig. 3 Stability determination of green fluorescence in vBmBac(polh$^+$)-EGFP polyhedra

A: suspension of polyhedra in PBS (pH 7.2); B: lysates of polyhedra without dehydrated treatment in carbonate buffer (pH 11.0); C: lysates of polyhedra in carbonate buffer (pH 11.0) after dehydrated treatment for 30 min. This experiment was carried out three times, and results are shown as mean±SE. * P<0.01, values that do not differ from each other, but differ significantly from other values (one-way ANOVA)

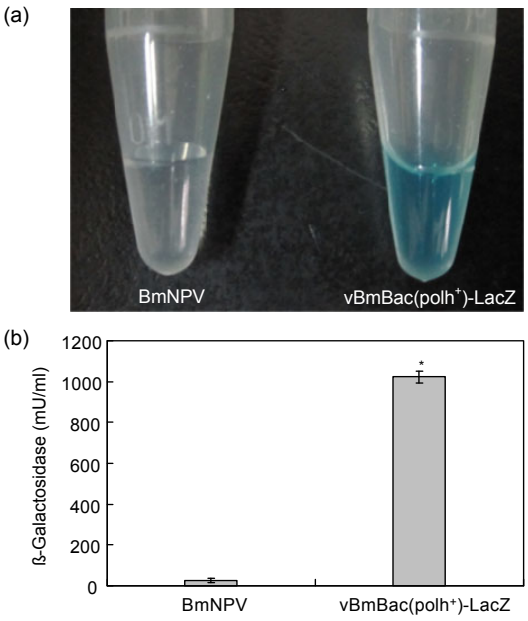

Fig. 4 Analysis of β-galactosidase in polyhedra produced by vBmBac(polh$^+$)-LacZ

(a) Polyhedra were purified from BmN cells infected with vBmBac(polh$^+$)-LacZ at 96 hpi, and equal amounts of polyhedra were taken and added into the same amounts of X-Gal. The left tube contains BmNPV polyhedra, and the vBmBac(polh$^+$)-LacZ polyhedra are in the right tube. (b) The β-galactosidase activity of polyhedra was measured by using the β-galactosidase enzyme assay system. Each assay was replicated three times, and results are shown as mean±SE. * P<0.01, significant differences between the vBmBac(polh$^+$)-LacZ polyhedra and BmNPV polyhedra (one-way ANOVA)

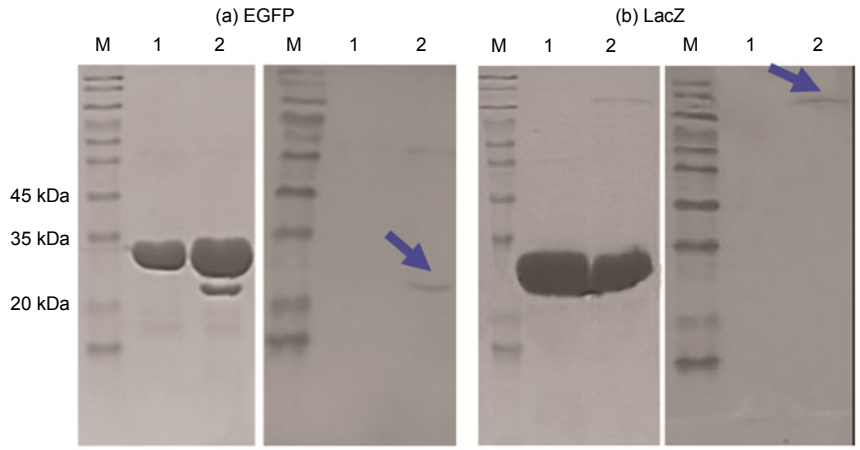

Fig. 5 Detection of the foreign protein by SDS-PAGE and Western blotting

(a) SDS-PAGE and Western blotting analyses of the vBmBac(polh$^+$)-EGFP polyhedra. The primary antibody was rabbit monoclonal EGFP antibody, and the secondary antibody was goat anti-rabbit IgG antibody. (b) SDS-PAGE and Western blotting analyses of the vBmBac(polh$^+$)-LacZ polyhedra. The primary antibody was mouse monoclonal β-galactosidase antibody, and the secondary antibody was goat anti-mouse IgG antibody. Lane M: strained protein molecular weight marker; Lane 1: BmNPV polyhedra; Lane 2: vBmBac(polh$^+$)-EGFP polyhedra (a) or vBmBac(polh$^+$)-LacZ polyhedra (b)

4 Discussion

The BmNPV/Bac-to-Bac expression system applicable to silkworm was developed based on the working principle of AcMNPV Bac-to-Bac system. In our previous study (Xiang *et al*., 2010), the BmNPV polyhedrin open reading frame (ORF) sequence was inserted downstream of the p10 promoter through homologous recombination. A gene of interest can subsequently be inserted into the new BmNPV polh$^+$ construction from a suitable donor plasmid by transposition occurring in *Escherichia coli*. Two recombinant baculoviruses were generated by this novel Bac-to-Bac system, designated as vBmBac(polh$^+$)-EGFP and vBmBac(polh$^+$)-LacZ, which can express the polyhedrin and foreign protein simultaneously.

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is stable between pH 5–12, and displays visible fluorescent light at a peak wavelength of 508 nm when excited with ultraviolet light (Reid and Flynn, 1997). According to the previous research, EGFP alone cannot resist the stimulation from dehydrated treatment, whereas the dehydrated treatment had little effects on EGFP immobilized into polyhedra (Ikeda *et al*., 2006). To determine whether the foreign protein was located in the polyhedra externally or internally, an anti-desiccation test was applied. The fluorescence of BmN cells infected with vBmBac(polh$^-$)-EGFP disappeared in dehydrated state (data not shown). However, the fluorescence could be detected from the vBmBac(polh$^+$)-EGFP polyhedra after dehydrated treatment for 30 min. The result suggested that the foreign protein was incorporated into the polyhedra and thereby protected from the dehydrated conditions. Indeed, this phenomenon might reflect the basic function of polyhedra, which is to protect the occluded virions from hostile environmental conditions and ensure insect-to-insect transmission of viral infection.

In this study, we confirmed that the foreign protein could be embedded into the polyhedra. However, the process of virion occlusion in the polyhedra is undefined and the genes that regulate the virion occlusion process have not been well investigated (Blissard and Rohrmann, 1990; Wang *et al*., 2009). BmCPV polyhedron is a simple protein crystal with only five proteins. Mori *et al.* (1993) showed that the BmCPV polyhedrin can be assembled into polyhedra in the nucleus of Sf21 and BmN cells using the baculovirus expression vector. This indicated that, during the formation of polyhedra, the overexpressed foreign protein may be embedded into the polyhedra.

We had developed the BmNPV polh$^+$ Bac-to-Bac expression system, which is an efficient system to construct recombinant baculovirus and express

foreign protein at a high level. The host of BmNPV is only limited to silkworm, which is reared indoors, having been domesticated for thousands of years. Compared with other viruses from vertebrates, the BmNPV is biologically safer (Blissard and Rohrmann, 1990). In the future, we would like to increase the ratio of foreign protein with polyhedrin in polyhedron, and subsequently detect the best way to release the incorporated foreign protein from polyhedra. Further we hope that this technique would be applied in the field of agriculture or medicine, such as developing new types of pesticide through immobilization of toxic protein into polyhedra.

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