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Expression and Characterization of Fusion Protein with *Autographa californica* Nuclear Polyhedrosis Virus Polyhedrin and Green Fluorescent Protein in Insect Cells

곤충세포주에서 *Autographa californica* 핵다각체병 바이러스의 다각체 단백질과 초록색 형광 단백질의 융합단백질 발현 및 특성

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Abstract - We have now constructed a novel recombinant baculovirus producing fusion protein with *Autographa californica* nuclear polyhedrosis virus (AcNPV) polyhedrin and green fluorescent protein (GFP). The fusion protein expressed by the recombinant baculovirus in insect cells was characterized. The GFP gene was introduced under the control of polyhedrin gene promoter of AcNPV, by fusion in the front or back of intact polyhedrin gene. The recombinant baculoviruses were named as Ac-GFPPOL or Ac-POLGFP, respectively. As expected, the 56 kDa fusion protein was expressed in the recombinant virus-infected cells. Interestingly, however, the fluorescence of GFP in the cells infected with Ac-POLGFP was only detected within the nuclei, and that was observed as polyhedra-like granular particles. In the microscopy of cells infected with Ac-GFPPOL, furthermore, GFP was detected in both cytoplasm and nuclei although most of GFP were present within the nuclei. However, fusion protein produced by recombinant virus did not form polyhedra although the fusion protein was fused with polyhedrin and GFP. It is suggested that difference of GFP location in the infected cells appear to be involved in the region of polyhedrin in the fusion protein, and the polyhedrin in the fusion protein might be responsible for the polyhedra-like granular particles present within nuclei.

Key Words - *Autographa california* nuclear polyhedrosis virus, Polyhedrin, Green fluorescent protein, Polyhedra-like granular particles

초 록 - *Autographa californica* 핵다각체병 바이러스(AcNPV)의 다각체 단백질과 초록색 형광 단백질의 융합단백질을 생산하는 새로운 재조합 바이러스를 제작하고, 곤충세포주(*Spodoptera frugiperda* 9)에서 발현된 융합단백질의 특성을 분석하였다. 초록색 형광 단백질 유전자는 AcNPV의 완전한 다각체 단백질 유전자의 앞쪽과 뒷쪽에 융합하여 다각체 단백질 유전자의 프로모터 조절하에 도입하였다. 이렇게 작성된 재조합 바이러스를 각각 Ac-GFPPOL 또는 Ac-POLGFP이라고 명명하였다. 이들 재조합 바이러스에 의해 감염된 곤충세포주에서는 56 kDa의 융합단백질이 발현되었다. 한편, 흥미롭게도 재조합 바이러스 Ac-POLGFP에 의해 감염된 세포주에서는 초록색 형광이 핵내에서만 다각체 유사 granular particle 형태로 관찰되었다. 반면에 Ac-GFPPOL에 의해 감염된 세포주에서는 대부분 핵내에 존재하였지만, 세포질과 핵 모두에서 초록색 형광을 관찰할

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수 있었다. 그러나 발현된 융합단백질은 분명히 다각체단백질을 포함하고 있음에도 다각체는 형성하지 않았다. 이러한 결과들은 융합단백질에서 다각체단백질의 위치와 관련이 있는 것으로 보여진다.

검색어 - *Autographa california* 핵다각체병 바이러스, 다각체단백질, 초록색 형광 단백질, 다각체 유사 입자

Baculoviruses are excellent eucaryotic expression vectors because they possess characteristics such as high-level expression, authentic biological and immunological activity, and post-translational modifications (Luckow and Summers, 1988; Maeda, 1989; O'Reilly *et al.*, 1992). In addition, baculoviruses have an attractive potential as biological control agents for insect pest management owing to their host specificity, efficacy and stability (Granados and Federici, 1986; Maeda, 1995).

The occlusion bodies of nuclear polyhedrosis viruses (NPVs) are polyhedral, contain virus particles and transmit the virus in nature. Polyhedrin is of utmost importance to the NPVs, since it forms the occlusion body matrix in which mature infectious virus particles are found at late stages of infection (Carstens *et al.*, 1986). Ultrastructural studies on the morphogenesis of occlusion body indicated that occlusion of enveloped virus occur by condensation of fibrous material in nuclei to form a lattice which surrounds the virus bundles (Chung *et al.*, 1980; Yamamoto *et al.*, 1981). Moreover, molecular and ultrastructural features of polyhedra morphology mutants has been reported (Chung *et al.*, 1980; Yamamoto *et al.*, 1981; Duncan *et al.*, 1983; Carstens *et al.*, 1986). However, mechanism of polyhedrin conformation and polyhedra morphogenesis has not yet been estabilished.

To understand some features of polyhedra, therefore, polyhedrin gene was fused with green fluorescent protein (GFP) gene. Because the detection of intracellular GFP requires only irradiation by near UV or blue light, it is not limited by the availability of substrates. Thus, it should provide an excellent means for monitoring gene expression and protein localization in living cells (Chalfie *et al.*, 1994). In this study we have now constructed a novel recombinant baculovirus producing fusion protein with AcNPV polyhedrin and GFP. The fusion protein expressed by the recombinant baculovirus in insect cells was characterized.

Materials and Methods

Cell lines and viruses

Spodoptera frugiperda (Sf9) cells used in this study were maintained at 27°C in TC-100 medium (Sigma) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS, Gibco). Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant virus were propagated in Sf9 cells. The titer of viruses was determined by a plaque assay in Sf9 cells as described previously (Summers and Smith, 1987; O'Reilly *et al.*, 1992).

Construction of baculovirus transfer vector

The *Xho* I-*Sna* BI fragment containing AcNPV polyhedrin gene was inserted into the baculovirus transfer vector pBacPAK8 to yield pBacPAK-AcPOL. The GFP gene was excised from plasmid pGFP (Clontech). The GFP gene on a 730 bp fragment was inserted into the *Bam* HI and *Pst* I sites of pBacPAK8 (Clontech) to yield baculovirus transfer vector pAc-GFP (Jin *et al.*, 1997). In the second step, GFP gene was inserted into the *Xho* I site in the front of polyhedrin gene of pBacPAK-AcPOL to yield pAc-GFPPOL. In addition, GFP gene was inserted into the *Hind* III site in the back of polyhedrin gene of pBacPAK-AcPOL to yield pAc-POLGFP.

PCR

The introduction of fusion gene under the control of polyhedrin gene promoter was analyzed by PCR using recombinant virus genomes and synthetic primers (5'-ACCATCTCGCAAATAAATAAG-3'; 5'-GCGATCTA-AGACACGCAACA-3') (Clontech). Viral DNA used as template was purified from supernatant of Sf9 cells infected with recombinant viruses. After a 35-cycle amplification (94°C for 1 min; 55°C for 1 min; 72°C for 1 min), PCR products were ethanol precipitated, centrifuged at 10,000 × g for 30 min, and rinsed with 70% ethanol. These DNAs were analyzed by agarose gel elec-

trophoresis.

Construction of recombinant AcNPV

The 35-mm diameter cell culture dish seeded with 1 – 1.5 × 10^6 Sf9 cells was incubated at 27°C for 1 hr. One microgram of BacPAK6 viral DNA, 5 μg of pAc-POLGFP or pAc-GFPPOL DNA in 20 mM HEPES buffer and sterile water to make a total volume of 50 μl were mixed in a polystylene tube. Fifty microliters of 100 μg/ml Lipofectin ™ (Gibco) were gently mixed with the DNA solution, and the mixture was incubated at room temperature for 30 min. The cells were washed twice with 2 ml serum-free TC-100 medium. Serum-free TC-100 (1.5 ml) was added to each dish. The Lipofectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubating at 27°C for 5 hr, 1.5 ml TC-100 containing antibiotics and 10% FBS was added to each dish and the incubation at 27°C continued. At 5 days postinfection (p.i.), the supernatant was harvested, clarified by centrifugation at 2,000 rpm for 5 min, and stored at 4°C before plaquing on Sf9 cells. Recombinant AcNPV was plaque purified in Sf9 cells (Summers & Smith, 1987; O'Reilly *et al.*, 1992).

Microscopy

Microscopy of Sf9 cells infected with recombinant virus was performed using a light and fluorescent microscope (Axiophot Universal Microscope, Zeiss).

SDS-PAGE and Western blot

Sf9 cells were mock infected or infected with wild-type or recombinant AcNPV in a 35-mm diameter dish (1.0 × 10^6 cells) at a MOI of 5 PFU per cell. After incubation at 27°C, cells were harvested at 2 days p.i. For SDS-PAGE of cell lysates, Sf9 cells infected with or without virus were washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) and mixed with a sample buffer (5% SDS, 10% β-mercaptoethanol, 0.02% bromophenol blue, 20% glycerol). Samples were boiled for 5 min and clarified by centrifugation (10,000 × g for 1 min). The total cellular lysates were subjected to 10% SDS-PAGE (Laemmli, 1970), electroblotted and incubated with AcNPV polyhedrin or GFP antibody (Towbin *et al.*, 1979).

Results and Discussion

Construction of recombinant AcNPV

The baculovirus transfer vector summarizing map used to generate recombinant virus expressing fusion protein with polyhedrin and GFP was described in Fig. 1A. GFP gene was inserted into the *Xho* I site in the front of polyhedrin gene of pBacPAK-AcPOL to yield pAc-GFPPOl. In addition, GFP gene was inserted into the *Hind* III site

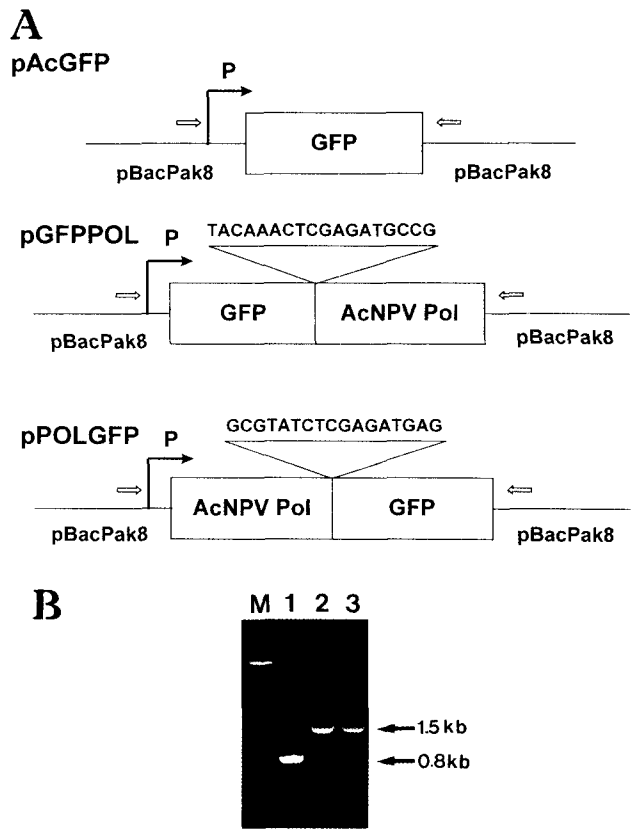

Fig. 1. The baculovirus transfer vector map used to generate recombinant virus (A) and PCR analysis of genomic DNA of recombinant virus (B). The GFP gene was introduced under the control of polyhedrin gene of the AcNPV to generate pAc-GFP. The GFP gene was introduced into the genome of the AcNPV, by fusion in the front or back of intact polyhedrin gene to yield pAc-GFPPOL or pAc-POLGFP. The introduction of fusion gene under the control of polyhedrin gene promoter was analyzed by PCR using genomic DNA extracted from the recombinant viruses, Ac-GFP (lane 1), Ac-GFPPOL (lane 2), and Ac-POLGFP (lane 3). Solid arrows indicate baculovirus polyhedrin gene promoter and orientation. Open arrows indicate primer positon of PCR.

in the back of polyhedrin gene of pBacPAK-AcPOL to yield pAc-POLGFP. The recombinant viruses produced by pAc-GFPPOL or pAc-POLGFP were named as Ac-GFPPOL or Ac-POLGFP, respectively.

To confirm the introduction of fusion gene under the control of polyhedrin gene promoter, genomic DNA extracted from the recombinant viruses was analyzed by PCR (Fig. 1B). The 1.5 kbp band expected for the fusion gene was observed in recombinant virus, Ac-GFPPOL or Ac-POLGFP.

Expression of fusion protein in insect cells

In order to examine the expression of fusion protein, Sf9 cells infected with recombinant virus was initially analyzed by SDS-PAGE and Western blot (Fig. 2). Wild-type AcNPV-infected cells showed a pattern of protein synthesis typical of AcNPV-infected cells. Fusion protein band was not detected in cells infected with wild-type AcNPV or from mock-infected cells. However, the cells infected with Ac-GFP only showed a 27 kDa GFP band. The fusion protein expressed by the fusion gene was present as a band of about 56 kDa in cells infected with recombinant viruses, Ac-GFPPOL or Ac-POLGFP, which was absent in cells infected with wild type AcNPV. That the 56 kDa band was the fusion protein with a polyhedrin and GFP was demonstrated by immunoblotting using polyhedrin or GFP antibody.

Microscopy of the cells infected with recombinant baculovirus

Cells infected with recombinant baculovirus were observed by light and fluorescent microscope (Fig. 3). Microscopy of cells infected with Ac-GFPPOL or Ac-POLGFP revealed that GFP in the fusion protein have their own fluorescence activity. In addition, bright glow of GFP produced by Ac-GFP was clearly appeared from the whole cells.

Interestingly, however, the fluorescence of GFP in the cells infected with Ac-POLGFP at late stages of infection was only detected within the nuclei, and that was apparently observed as polyhedra-like granular particles (Duncan *et al.*, 1983). In the cells infected with Ac-GFPPOL, furthermore, GFP was detected in both cytoplasm and nuclei although most of GFP were present with the nuclei. But cells infected with Ac-POLGFP or Ac-GFPPOL did not produce polyhedra although polyhedra-like small granular particles were observed within nuclei. The granular particles could not be purified from the infected cells by centrifugation. The polyhedra-like granular particles in the cells infected with Ac-GFP were not detected. Accordingly, these results indicated that the

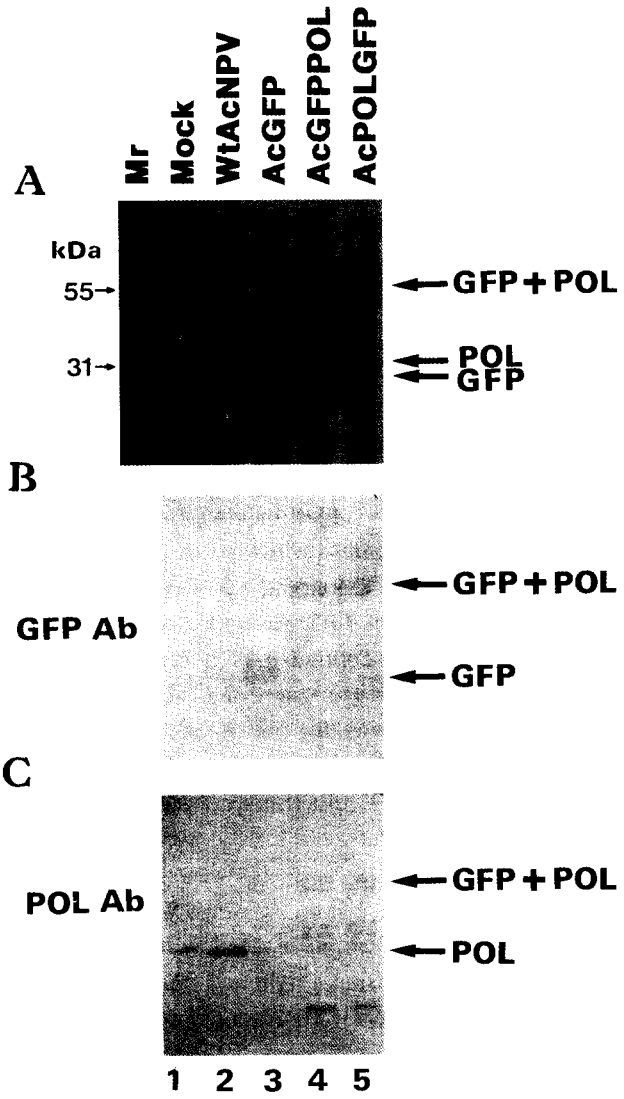

Fig. 2. SDS-PAGE and Western blot analysis of the fusion protein expression of recombinant viruses, Ac-GFPPOL or Ac-POLGFP, in Sf9 cells. Sf9 cells were mock infected (lane 1) or infected with wild-type AcNPV (lane 2), Ac-GFP (lane 3), Ac-GFPPOL (lane 4) and Ac-POLGFP (lane 5) at MOI of 5 PFU per cell. Cells were collected at 2 days p.i. Total cellular lysates were subjected to 10% SDS-PAGE (panel A), electroblotted and incubated with GFP (panel B) or polyhedrin (panel C) antibody. Fusion protein, polyhedrin, and GFP bands are indicated on the right of each panel. Molecular weight standards were used as size marker.

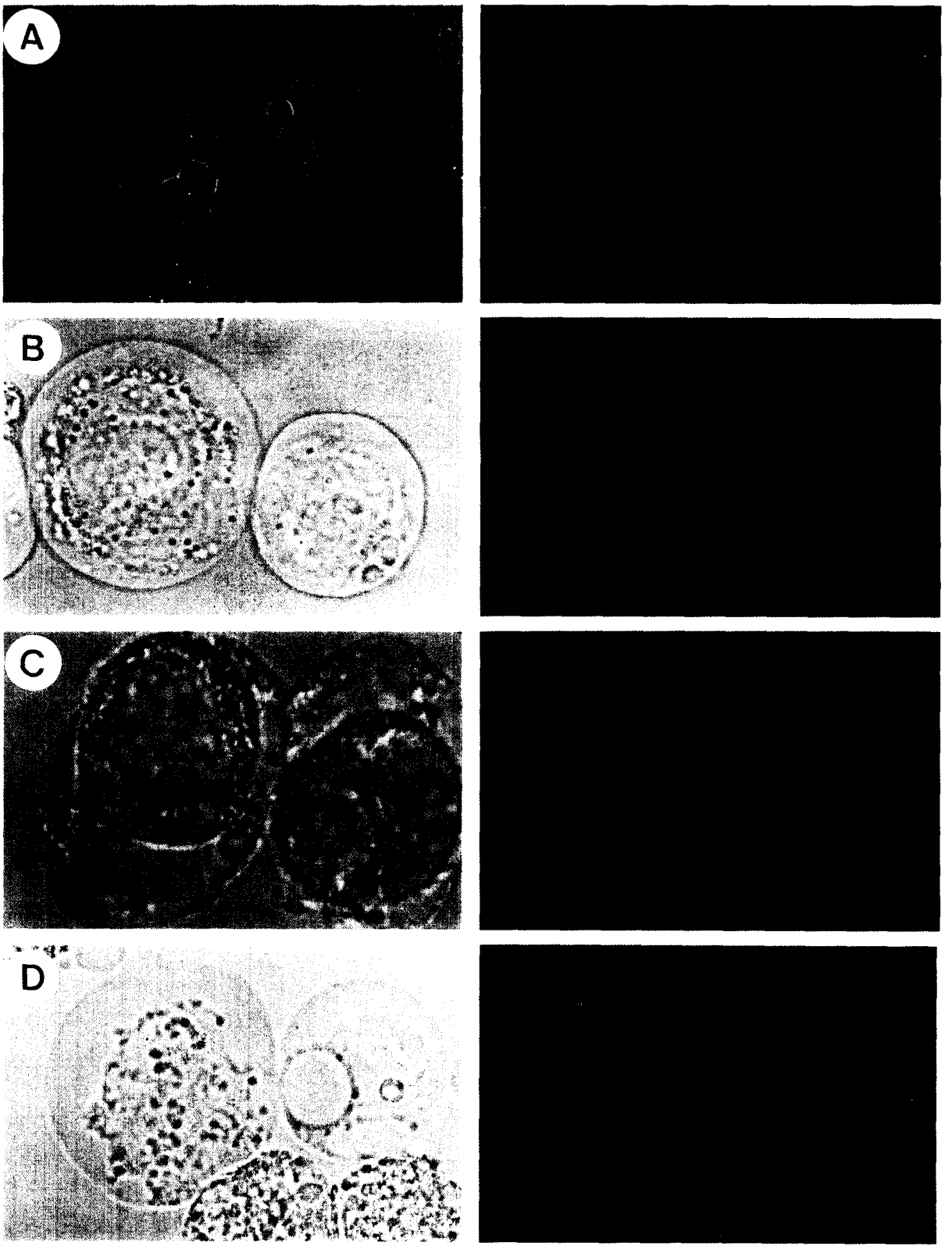

Fig. 3. Microscopy of Sf9 cells infected with recombinant viruses. Sf9 cells infected with wild-type AcNPV (A), Ac-GFP (B), Ac-GFPPOL (C) and Ac-POLGFP (D) were observed by light (Left panels) and fluorescent microscope (Right panels) (× 1,000).

polyhedrin of the fusion protein did not affect the formation of polyhedra (Je *et al.*, 1997). However, it is suggested that the polyhedrin of the fusion protein produced by Ac-GFPPOL or Ac-POLGFP might be responsible for the polyhedra-like granular particles. In the fluorescent microscopy of cells infected with Ac-POLGFP or Ac-GFPPOL, GFP location was observed in only nuclei or in both nuclei and cytoplasm, respectively.

It is also suggested that difference of GFP location in the infected cells appear to be involved in the region of polyhedrin in the fusion protein although the specific details of this association are unknown.

In conclusion, our results clearly demonstrated that GFP in the fusion protein with polyhedrin and GFP have their own fluorescence activity. Furthermore, our results in this study may improve conditions for the production of polyhedra fused with polyhedrin and GFP, and fusion polyhedrin with GFP may be useful as excellent means for monitoring gene expression and polyhedrin localization in the baculovirus-infected cells.

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