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Construction of the Silkworm, *Bombyx mori*, with a Green Fluorescence by *Autographa californica* Nuclear Polyhedrosis Virus

Byung Rae Jin[1,2,*], Eun Young Yun[2], Seok Woo Kang[2], Hyung Joo Yoon[2], Keun Young Kim[2], Ho Rak Kim[2], Yeon Ho Je[3] and Seok Kwon Kang[3]

[1]*College of Natural Resources and Life Science, Dong-A University, Pusan 604-714, Korea.*
[2]*Department of Sericulture and Entomology, The National Institute of Agricultural Science and Technology, RDA, Suwon 441-744, Korea.*
[3]*College of Agriculture and Life Sciences, Seoul National University, Suwon 441-100, Korea.*

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We have constructed a recombinant baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcNPV), containing green fluorescent protein (GFP) gene from the jellyfish, *Aequorea victoria*, and transferred it into the domestic silkworm *Bombyx mori* larvae for the production of visible transgenic silkworm of living organism. When one day-old fifth instar female larvae were injected with the recombinant AcNPV of 1×10^5 plaque forming units, the bright glow of GFP was detected in the recombinant AcNPV-infected larvae and in the newly hatched larvae of the next generation. Our findings demonstrate that the viral replication was detected in the silkworm treated with the recombinant AcNPV and the *gfp* gene was expressed under the transcriptional control of the polyhedrin gene promoter. Furthermore, the *gfp* gene was transmitted to the next generation, suggesting that this system can be applied for the development of transgenic silkworms.

Key words : Baculovirus, *Bombyx mori*, Green fluorescent protein

Introduction

Because of its economic importance in silk production, the domestic silkworm, *Bombyx mori*, has been domesticated for thousands of years. Silkworm larvae offer a number of advantages over other insect larvae, such as larvae size, ease of handling, nonallergenic to human, and well-characterized genetics (Choudary *et al.*, 1995; Maeda, 1994). Furthermore, silkworms are very useful as host for the production of heterologous proteins by baculovirus expression vector system (Choudary *et al.*, 1995; Jin *et al.*, 1998; Maeda *et al.*, 1985; O'Reilly *et al.*, 1992). However, gene expression by baculovirus expression vectors is transient, because the infected insects and cells ultimately die from the virus replication. For these reasons, silkworm is deeply being studied for the development of transgenic insects. *Autographa californica* nuclear polyhedrosis virus (AcNPV) (Mori *et al.*, 1995; Yamao *et al.*, 2000) or PiggyBac (Tamura *et al.*, 2000) has been utilized for stable transformation of silkworms and constitute expression of foreign genes. On the other hand, transgenesis of *Drosophila melanogaster* is routinely accomplished by use of the P-element transposon and this has facilitated the analysis of developmental regulation of gene expression (Rubin and Spradling, 1982). Recently, dipteran insects, Mediterranean fruit fly and mosquitoes, have been genetically transformed with insect transposable elements (Handler *et al.*, 1998; Jasinskiene *et al.*, 1998; Zwiebel *et al.*, 1995; Coates *et al.*, 1998).

Although AcNPV can replicate in silkworm, the AcNPV-infected silkworm larvae survive and grow (Mori *et al.*, 1995; Yamao *et al.*, 2000). The previous studies reported that AcNPV could be utilized as a vector for the transovarian transmission of foreign genes in the silkworm. Luciferase (Mori *et al.*, 1995) gene was introduced into the AcNPV genome, female fifth instar silkworm larvae were inoculated with the recombinant baculovirus, and foreign gene product activity was detected in the larvae of subsequent generations, indicating that the foreign

*To whom correspondence should be addressed.
College of Natural Resources and Life Science, Dong-A University, Pusan 604-714, Korea. Tel: 82-51-200-7594; Fax: 82-51-200-7594; E-mail: brjin@mail.donga.ac.kr

gene had been vertically transmitted. AcNPV is also demonstrated to be a gene targeting vector for transgenesis of silkworm (Yamao *et al.*, 2000).

There are some reports of transovarian transmission of AcNPV in a non-target insect, silkworm (Mori *et al.*, 1995; Yamao *et al.*, 2000). The previous report introduced a recombinant AcNPV containing luciferase gene under the control of the *Drosophila* heat shock protein gene into the fifth instar larvae of silkworm (Mori *et al.*, 1995). Silkworm with luciferase gene as marker is transient, because the insects and cells ultimately die from the substrates for the detection. Because the detection of intracellular green fluorescent protein (GFP) requires only irradiation by near ultraviolet or blue light at 395 or 470 nm, however, it is not limited by the availability of substrates (Chalfie *et al.*, 1994). A GFP has been developed as a visible marker for monitoring gene expression and protein localization in living organisms (Plautz *et al.*, 1996). In this report we have constructed a recombinant AcNPV containing *gfp* gene under the transcriptional control of the polyhedrin gene promoter and transferred it into silkworm larvae for the production of visible transgenic silkworm of living organism.

Materials and Methods

Cell line and virus

Spodoptera frugiperda (Sf9) cells (Vaughn *et al.*, 1977) were maintained at 27°C in TC100 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) as described by standard methods (O'Reilly *et al.*, 1992). Recombinant AcNPV expressing GFP (Jin *et al.*, 1998) was propagated and titred in Sf9 cells. The titer was expressed as a plaque forming units (pfu) per ml (O'Reilly *et al.*, 1992).

Injection of larvae with AcNPV

The silkworm eggs were obtained from Department of Sericulture & Entomology, National Institute of Agricultural Science & Technology, Korea and the silkworms were reared on mulberry leaves. The 1 day-old female larvae were injected with 20 μl containing 1×10^5 pfu of the recombinant AcNPV expressing GFP by percutaneous inoculation. The fatbody collected from the newly hatched silkworm larva expressing GFP was visualized with light and fluorescent microscope (Nikon).

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

Hemolymph proteins were collected from the control silkworm or silkworm infected with recombinant AcNPV at 7 days p.i. For SDS-PAGE, hemolymph proteins were mixed with an equal amount of 2× 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 prepared samples were subjected to 10% SDS-PAGE (Laemmli, 1970), electroblotted and incubated with GFP antibody (Clontech) (Towbin *et al.*, 1979). SDS-PAGE molecular weight standards were purchased from BioRad Laboratories.

Genomic DNA extraction

Genomic DNA was extracted from the silkworms by Wizard™ genomic DNA purification kit, according to the manufacturers instructions (Promega).

PCR analysis

Genomic DNA extracted from the silkworms was used as templates. 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. The resultant DNA was electrophoresed on 1% agarose gel and visualized by EtBr staining under ultraviolet light. The *gfp* gene from the genomic DNA of the silkworms was amplified using a synthetic primer set, 5'-TATCGTGTTCGCCATTAGGGCAG-3' for the 5' coding region and 5'-GCTGTATTTGTACGTGAGCGTAC-3' for the 3' coding region of the *gfp* gene (Chalfie *et al.*, 2000). The primer for the amplification of AcNPV polyhedrin gene promoter region was used 5'-CTGATATCATGGAGATAA-3' (Possee *et al.*, 1991).

Results and Discussion

Twenty silkworm strains were initially inoculated with the recombinant AcNPV expressing GFP by hemocoelic injection with the virions. From 4 days after infection, we observed clear green fluorescence in the thoracic leg of the infected larvae by the irradiation of ultraviolet light. The infected larvae clearly revealed the green fluorescence of GFP from the whole body, especially in the abdominal leg, at 7 days p.i. (Fig. 1). However, such a phenomenon was not observed for larvae of all tested silkworm strains (data not shown). Among these strains, Daiseongjam (Jam 125× Jam 126) which is F1 hybrid silkworm of Japanese and Chinese races was effective for the expression of GFP under the transcriptional control of the polyhedrin gene promoter by the replication of AcNPV (Mori *et al.*, 1995; Huang *et al.*, 1997). Differences in the hemolymph protein pattern by SDS-PAGE were not observed between the control silkworm and silk-

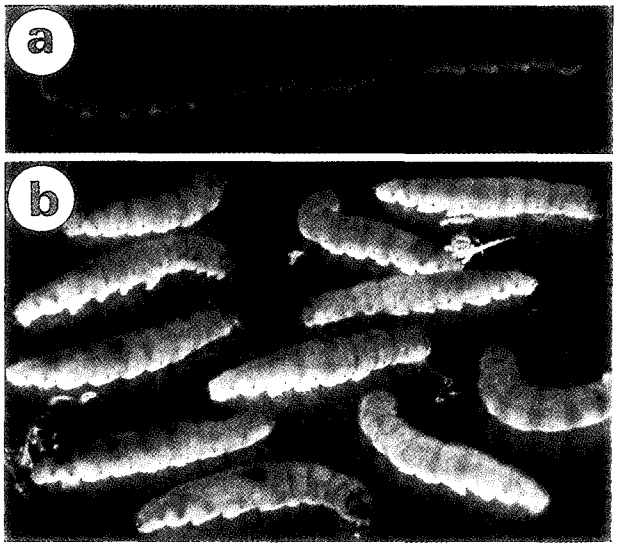

Fig. 1. Silkworms with green fluorescence. Photographs of the non-infected (a) and infected silkworm larvae (b) by the irradiation of ultraviolet light.

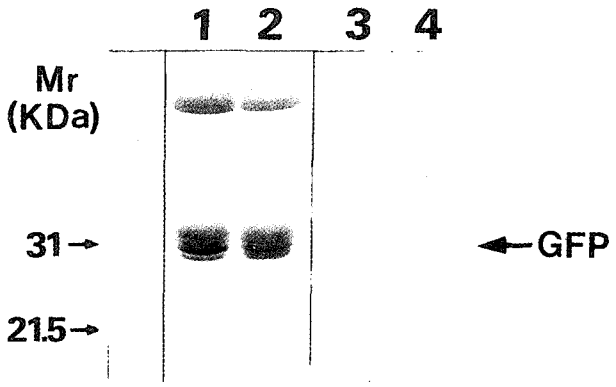

Fig. 2. SDS-PAGE and Western blot analysis. Hemolymph proteins were collected from the non-infected (lanes 1 and 3) and infected silkworm larvae (lanes 2 and 4) at 7 days p.i. The hemolymph proteins were subjected to 10% SDS-PAGE (lanes 1 and 2), electroblotted, and incubated with GFP antibody (lanes 3 and 4). The GFP band is indicated on the right of panel. SDS-PAGE molecular weight standards are indicated on the left of panel.

worm infected with the recombinant AcNPV. In the Western blot analysis, however, GFP band in the hemolymph protein of the silkworm was detected from 7 days p.i. (Fig. 2).

The pupation rate for silkworm infected with the recombinant AcNPV was approximately 53.3% compared with 94.2% for the control silkworm (data not shown). The percentage of pupation in the AcNPV-infected silkworm was relatively lower than expected. Although the nonpupated larvae did not show any typical symptom of the viral

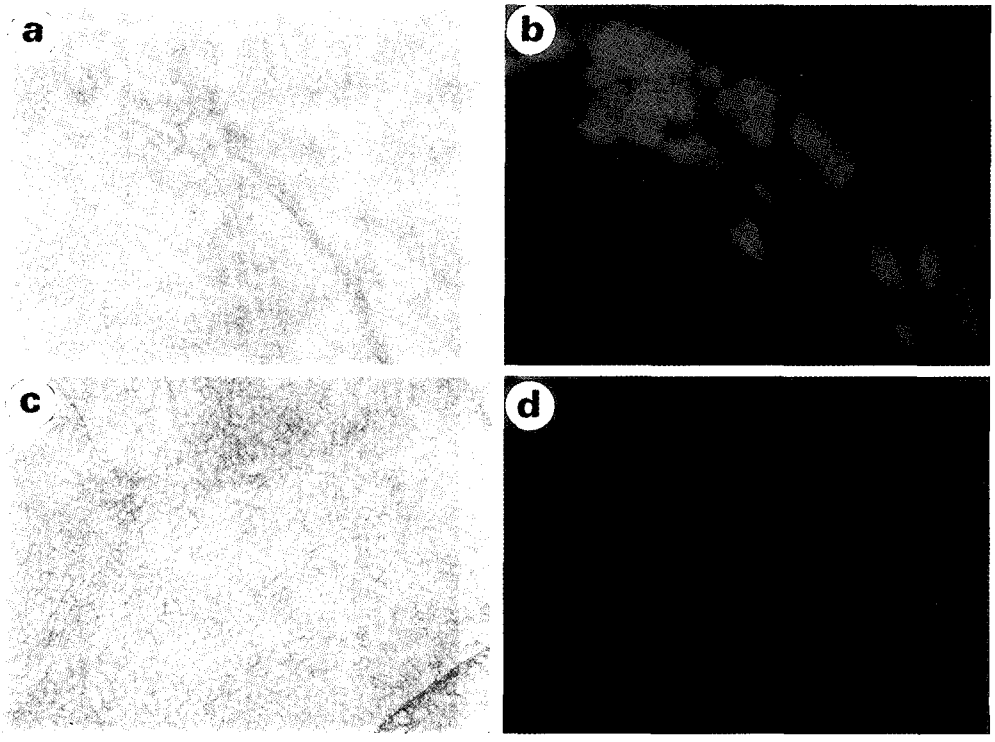

Fig. 3. Microscopy of the fat body collected from the newly hatched silkworm larva expressing GFP (a, b) or the control silkworm (c, d) was performed with light- (a, c) and fluorescent- (b, d) microscope (×200).

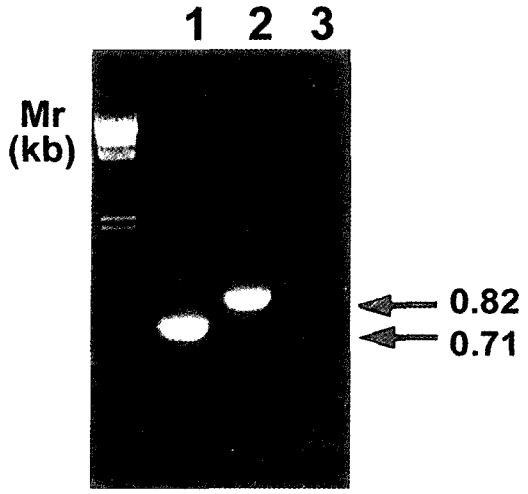

Fig. 4. PCR of genomic DNA extracted from the newly hatched silkworm larva. Genomic DNA was extracted from the newly hatched silkworm larva (lanes 1 and 2) and control silkworm larva (lane 3). The *gfp* gene was amplified with the specific primers of 5'- and 3'-coding regions of the *gfp* gene (lane 1). The *gfp* gene under the control of AcNPV polyhedrin gene promoter was amplified with primers of AcNPV polyhedrin gene promoter region and 3-coding region of the *gfp* gene (lane 2). PCR of genomic DNA extracted from control silkworm as a negative control was carried out with the specific primers of 5'- and 3'-coding regions of the *gfp* gene. The molecular size of amplified genes is indicated by arrow.

infection (Huang *et al.*, 1997; Mori *et al.*, 1995), the larvae finally died during the spinning stage. The result indicates that when AcNPV was injected into the silkworm larvae, virus replication occurred and caused abnormal development, resulting in premature pupation or death during the pupal stage, as reported in the cases of the silkworm larvae (Shikata *et al.*, 1998). We also tested the expression and transmission of the *gfp* gene in the next generation of Daiseungjam (Fig. 3 and 4). The expression of the GFP in the fatbody of the newly hatched larvae was observed by fluorescent microscope (Fig. 3). As expected, GFP expression from the fat body of the control silkworm was not detected. To determine transovarian transmission of the *gfp* gene and AcNPV DNA, therefore, we extracted the genomic DNA from the newly hatched larvae, and carried out PCR with the specific primers for the *gfp* gene and polyhedrin gene promoter region (Fig. 4). The PCR products of the expected size were detected and confirmed by sequencing, indicating that the *gfp* gene was transmitted to the following generation and apparently contained the polyhedrin gene promoter of AcNPV. However, the mechanism of the transmission and expression of the *gfp* gene in the subsequent generations remains unclear.

In this study the transovarian transmission by injection of recombinant AcNPV into the silkworm larvae was consistent with the previous results (Mori *et al.*, 1995; Yamao *et al.*, 2000). However, the *gfp* gene expression under the transcriptional control of the AcNPV polyhedrin gene promoter in the silkworm larvae has not been previously reported. AcNPV, many of whose genes showing an extensive homology with the genes of *B. mori* NPV, exhibits a relatively wide host range, and has been shown to infect successfully more than 30 species of lepidoptrean insects (O'Reilly *et al.*, 1992). In the cell culture, AcNPV accumulated viral structural polypeptides and budded virions in the BmN cells derived from the silkworm, *B. mori*, although polyhedra did not produce (Aoki *et al.*, 1998). The GFP as a visible marker is very sensitive, and it is a stable protein which emits green light on excitation with ultraviolet or blue light at 395 or 470 nm (Chalfie *et al.*, 1994). In this study, the *gfp* gene expression under the transcriptional control of the AcNPV polyhedrin gene promoter was clearly detected in the silkworm larvae, as shown in Fig. 1, demonstrating that AcNPV polyhedrin gene promoter is functional in the silkworm larvae. It was also revealed that this result in the silkworm with recombinant AcNPV was due to the great sensitivity and ease monitoring of the GFP compared with the other reporter gene products, luciferase or β-galactosidase.

Our findings demonstrate that the viral replication was detected in the silkworm treated with the recombinant AcNPV, and the *gfp* gene was expressed under the transcriptional control of the polyhedrin gene promoter. Furthermore, the *gfp* gene was transmitted to the next generation. It is suggested that this system can be applied for the further development of visible transgenic silkworms.

Acknowledgments

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