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# Production of the Eggs with Abnormal Shape from the Domestic Silkworm, *Bombyx mori*, Infected with *Autographa californica* Nuclear Polyhedrosis Virus

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**The female pupae of the silkworm, *Bombyx mori*, were injected with recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV) expressing green fluorescent protein (GFP) by percutaneous inoculation. When the 4 day-old female pupae were injected with $1\times 10^7$ or $2\times 10^7$ plaque forming units (pfu) of the recombinant AcNPV, oviposited number and egg weight were significantly decreased. Furthermore, the shape of the eggs was obviously divided into normal and abnormal shapes. The percentage of the eggs with an abnormal shape was 7.8% and 57.1% at $1\times 10^7$ and $2\times 10^7$ pfu inoculation, respectively. PCR analysis of the genomic DNA extracted from the eggs revealed that *gfp* and AcNPV ecdysteroid UDP-glucosyltransferase genes were amplified from both types of eggs with normal and abnormal shapes. The results demonstrate that AcNPV DNA, and a *gfp* gene cloned into the AcNPV genome, injected in pupal stage were transmitted to eggs and remained stable through at least next generation.**

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

## Introduction

Silkworm, *Bombyx mori*, is 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; OReilly *et al.*, 1992). However, gene expression by baculovirus expression vectors is transient, because the infected insects and cells ultimately die from the virus replication. Because of economic importance for the silk production, above all, silkworm has been domesticated for thousands of years, and silkworm is throughly being studied for the development of transgenic insects. *Autographa californica* nuclear polyhedrosis virus (AcNPV) (Mori *et al.*, 1995; Jin *et al.*, 2000; Yamao *et al.*, 2000) or PiggyBac (Tamura *et al.*, 2000) has been utilized for a stable transformation of silkworms and constitutes 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 (Coates *et al.*, 1998; Handler *et al.*, 1998; Jasinskiene *et al.*, 1998; Zwiebel *et al.*, 1995).

Although AcNPV can replicates in silkworm, the AcNPV-infected silkworm larvae survive and grow (Jin *et al.*, 2000; 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) or green fluorescent protein (*gfp*) (Jin *et al.*, 2000) 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 gene had been vertically transmitted. AcNPV has also been demonstrated to be a gene targeting

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vector for transgenesis of silkworm (Yamao *et al.*, 2000). There are some reports of transovarian transmission of AcNPV in a non-target insect, silkworm (Jin *et al.*, 2000; Mori *et al.*, 1995; Yamao *et al.*, 2000). The previous reports introduced a recombinant AcNPV into the 5th instar larvae of silkworm. In this report we examined the transovarian transmission by the injection of recombinant AcNPV into the pupae and abnormal shape eggs production from the AcNPV-infected silkworms.

## Materials and Methods

### Virus and cell line

Recombinant AcNPV expressing GFP (Jin *et al.*, 2000) was propagated and titered in Sf9 cells (Vaughn *et al.*, 1977). The titer was expressed as a plaque forming units (pfu) per ml (O'Reilly *et al.*, 1992). Sf9 cells were maintained at 27°C in TC100 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA) as described by standard methods (O'Reilly *et al.*, 1992).

### Injection of pupae with AcNPV

The silkworm eggs (Jam 123×Jam 124) were obtained from Department of Sericulture & Entomology, National Institute of Agricultural Science & Technology, Korea and the silkworms were reared on mulberry leaves. The 2-, 4-, 6- and 8 day-old female pupae were injected with 100 μl or 200 μl containing $1\times10^7$ or $2\times10^7$ pfu of the recombinant AcNPV expressing GFP by percutaneous inoculation. Eggs from the AcNPV-infected silkworm were visualized by light microscopy (Nikon, Japan). The egg number and egg weight were counted from individual silkworms, and the percentage of eggs with abnormal shape was calculated from the egg mass per silkworm.

### Genomic DNA extraction

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

### PCR analysis

Genomic DNA extracted from the eggs 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 visualized under 1% agarose gel. The *gfp* gene from the genomic DNA of the eggs 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 *gfp* gene (Chalfie *et al.*, 2000). The AcNPV ecdysteroid UDP-glucosyltransferase (*egt*) gene from the genomic DNA of the eggs was amplified using a synthetic primer set, 5'-CAGTACAGTTATTCGGGTTGAAGC-3' for the 5' promoter region and 5'-CTCACCAACCAGAGCAGTCAT-3' for the 3' untranslational region of AcNPV *egt* gene (O'Reilly and Miller, 1990; O'Reilly *et al.*, 1991).

## Results and Discussion

The female pupae of the silkworm were injected with $1\times10^7$ or $2\times10^7$ pfu of the recombinant AcNPV by percutaneous inoculation. The egg numbers and egg weights were counted from the silkworm infected with AcNPV (Table 1). The numbers of eggs oviposited by the AcNPV-infected silkworms were significantly decreased than those of control. When the 2 day-old female pupae were injected with AcNPV, all silkworms did not emerge normally, and ovary development was arrested. When the 4 day-old pupae were injected with AcNPV, egg numbers and egg weights were dramatically decreased. Although egg numbers were decreased, still most of the 6- or 8 day-old pupae injected with AcNPV emerged, but AcNPV did not transmit to ovary (data not shown). These results indicate that when AcNPV was injected into the pupae, virus replication occurred and caused abnormal development, such as premature pupation, death during the pupal stage, or interference of normal embryogenesis, as reported previously in the larvae (Shikata *et al.*, 1998).

When the 4 day-old pupae were infected with AcNPV, interestingly, the shape of the eggs from the silkworms was divided into two types, normal and abnormal shapes

**Table 1.** Egg number and egg weight from the silkworm infected with AcNPV

| Virus | Treatment stage | Virus titer (pfu/pupa) | Egg number (number/ silkworm) | Egg weight (mg/egg) |
|---|---|---|---|---|
| Control* | - | - | 445 | 0.55 |
| AcNPV | 2 day-old pupa | $1\times10^7$ | - | - |
| | | $2\times10^7$ | - | - |
| | 4 day-old pupa | $1\times10^7$ | 167 | 0.43 |
| | | $2\times10^7$ | 56 | 0.43 |
| | 6 day-old pupa | $1\times10^7$ | 297 | 0.54 |
| | | $2\times10^7$ | 265 | 0.49 |
| | 8 day-old pupa | $1\times10^7$ | 326 | 0.60 |
| | | $2\times10^7$ | 280 | 0.63 |

*Control represents mock infection.

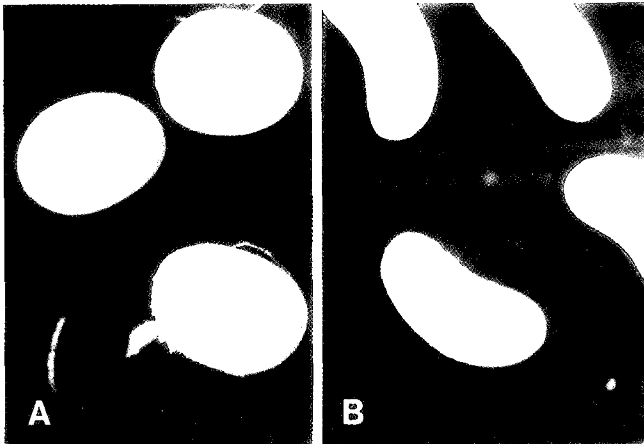


**Fig. 1.** Micrographs of the eggs with abnormal shape from the silkworm infected with AcNPV in the 4 day-old pupal stage. Eggs from the control (A) and AcNPV-infected silkworm (B) were observed by light microscopy (×70).

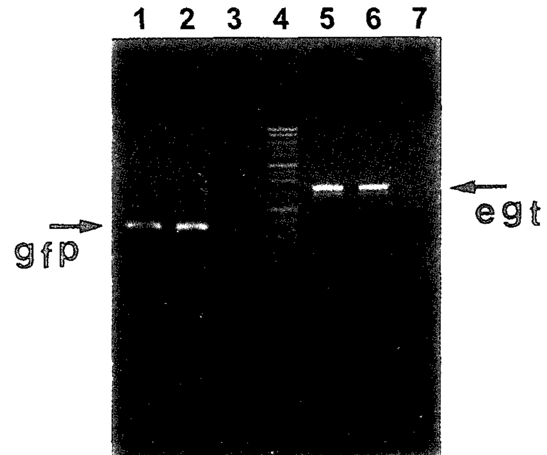


**Fig. 2.** PCR of genomic DNA of the eggs from the silkworm infected with AcNPV in the 4th day-old pupal stage. Genomic DNA was extracted from the control silkworm eggs (lanes 3 and 7), and normal (lanes 1 and 5) and abnormal (lanes 2 and 6) shape eggs of the AcNPV-infected silkworm. The amplified *gfp* and *egt* genes are indicated by arrow. Molecular size markers are represented at the middle lane (lane 4).

**Table 2.** The percentage of the eggs with abnormal shape from the silkworm infected with AcNPV in the 4 day-old pupal stage

| Virus titer (pfu/pupa) | Percentage of the eggs with abnormal shape (%) |
|---|---|
| $1\times10^7$ | 7.8 |
| $2\times10^7$ | 57.1 |

(Fig. 1). The eggs with abnormal shape were lengthy oval. In case of the 6- or 8 day-old pupae injected with AcNPV, however, the silkworm did not produce the eggs with abnormal shape. The percentage of the eggs with abnormal shape from the AcNPV-infected silkworms was 7.8% and 57.1% at $1\times10^7$ and $2\times10^7$ pfu inoculation, respectively (Table 2). The percentage of the eggs with abnormal shape from the silkworm injected with $1\times10^7$ was approximately 7.3-fold greater than that with $2\times10^7$. These results show a dose dependence between the pupal development and the amount of virus injected.

To determine transovarian transmission of the *gfp* gene and AcNPV DNA, PCR analysis of the genomic DNA extracted from the eggs was carried out using the primer set derived from the *gfp* and AcNPV *egt* genes (Fig. 2). The result revealed that *gfp* and *egt* genes were amplified both types of eggs, normal and abnormal shapes, demonstrating that the recombinant AcNPV was transmitted to ovary. As expected, *gfp* and *egt* genes from the control silkworm were not amplified. In the case of the eggs derived from the silkworm infected with AcNPV, PCR successfully amplified the products with expected sizes. PCR products of the *gfp* and *egt* genes were confirmed subsequently DNA sequencing (data not shown). When the 4 day-old pupae were injected with recombinant AcNPV, the *gfp* and *egt* genes were detected in eggs. This result suggests that the genes from AcNPV remained stable until at least the next generation.

In this study the transovarian transmission by injection of AcNPV in the pupal stage was consistent with the results in the larval stage (Jin *et al.*, 2000; Mori *et al.*, 1995; Yamao *et al.*, 2000). In conclusion, we have shown that AcNPV DNA, and a foreign gene cloned into the AcNPV genome, injected in pupal stage can be transmitted to eggs and remain stable through at least next generation. The results would be a useful tool for the development of transgenic silkworm by injection of baculovirus in the pupal stage as well as in the larval stage.

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