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Transovarial Transmission of *Bombyx mori* Nuclear Polyhedrosis Virus in the Silkworm

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Whether *Bombyx mori* nuclear polyhedrosis virus (BmNPV) can be transmitted to offspring, has been a noticeable question for a long time. When fifth instar larvae of the silkworm were orally inoculated with BmNPV, dot hybridization and PCR amplification analysis demonstrated that BmNPV was not detected in the eggs laid by BmNPV productively infected female moths. The results indicated that BmNPV could not be transovarially transmitted.

Key words : *Bombyx mori*, Nuclear polyhedrosis virus, Dot hybridization, PCR, Transovarial transmission

Introduction

Silkworm has been domesticated for thousands of years because of its economic importance for the silk production. *Bombyx mori* nuclear polyhedrosis virus (BmNPV) is one of the most harmful pathogen to the silkworm, especially in relatively warm and moist sericultural areas. BmNPV can invade most silkworm tissues, such as the midgut, hemolymph, fat body, nerve, trachea and epidermis, etc. The reproductive system of BmNPV was clarified (Lü, 1991). Silkworm is also very useful as host for the production of heterologous proteins by baculovirus expression vectors (Maeda *et al.*, 1985; OReilly *et al.*, 1992). However, gene expression is transient, because the infected insects ultimately die from virus infection. *Autographa californica* nuclear polyhedrosis virus (AcNPV) can replicate and express foreign genes in the silkworm (Zhang *et al.*, 1993), which was also reported to be used as a vector for the transovarian transmission of foreign genes in the silkworm. Luciferase (Mori *et al.*, 1995) or green fluorescent protein (*gfp*) (Lee *et al.*, 2000) gene was introduced into the AcNPV genome, female fifth instar larvae or pupae were inoculated with the recombinant baculovirus, and foreign gene product activity was detected in the larvae of subsequent generation. The result indicated that the foreign gene had been vertically transmitted. AcNPV has been demonstrated to be a gene-targeting vector for transgenesis of silkworm (Yamao *et al.*, 1999). However, whether BmNPV can be transovarially transmitted has been a disputed question all along. It is generally considered that eggs laid by female moths infected with BmNPV can transmit the virus to the next generations via the contaminated surface of eggs, and this kind of infectious way can be cut off effectively by egg-surface sterilizing. As to whether BmNPV can infect transovarially, there are two different point of views: With the help of fluorescent antibody diagnosis technique, Epmakoba and Tapacebny found that BmNPV was present in the eggs laid by the infected female moths, and they proposed that BmNPV could be transoverially transmitted. However, it was failed to find the transoverially infected BmNPV in the offspring (Lü, 1982). In this study, we investigated the possibility of transovarial transmission of BmNPV by dot hybridization and PCR analysis.

Materials and Methods

Silkworm and virus inoculation

Silkworm (75Xin 7532) and BmNPV (ZJ-8 strain) were obtained from Sericultural Research Institute, Chinese Academy of Agricultural Sciences. Six hundred newly molted fifth instar larvae were orally inoculated with BmNPV (1×10^4 polyhedra per larva). Polyhedra were

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visualized in the hemolymph of BmNPV productively female moths. Six batches of eggs from these female moths were selected.

Sample preparation

DNA samples from the hemolymph of BmNPV-infected postoviposited female moths were prepared as described (Tu *et al.*, 1994). One-hundred μl hemolymph of BmNPV-infected silkworm female moth was transferred to a fresh Eppendorf tube, added with 100 μl 1 M NaOH. The solution was vortexed and incubated for 5 min at room temperature, followed by adding 20 μl 10 M ammonium acetate to each tube, mixed and incubated for 5 min at room temperature. The mixture was extracted with an equal volume of phenol/chloroform (1:1). One-hundred μl supernatant of each sample was taken out for the use of dot hybridization, the remainder was precipitated with 2 vol of 100% ethanol. After the centrifugation, the pellet was washed with 75% ethanol. The final pellet was resuspended in 20 μl TE buffer, 5 μl of which was taken as the PCR template.

Eighty silkworm eggs from each batch were treated in 0.1 M HCl to discard the possibly contaminated viruses on the egg surface. They were washed twice with TE buffer and then ground in 100 μl TE buffer. The supernatant was transferred to a fresh tube, and the following steps were performed as the above described.

Dot hybridization

The 1.6 kb *Sal* I fragment of BmNPV polyhedrin gene (Wang *et al.*, 1985) was used as the probe, which was labeled with Random Primer labeling system (Amersham). Dot hybridization was performed as described (Sambrook *et al.*, 1989).

PCR analysis

For these studies, two target DNA sequences were used to design PCR primers. They were from the promoters of immediate early gene (*ie-1*) and DNA helicase gene (*dnahel*) of BmNPV, respectively. The above two genes have been demonstrated to be essential for baculovirus DNA replication (Lu and Miller, 1995). The *ie-1* gene promoter was amplified using a synthetic primer set, 5'-TAGAATTCATCCCAACGGCGCAGTGTAC-3' for the 5' coding region and 5'-ATGGATCCAATAGTCGTTTGGTTGTTCACG-3' for the 3' coding region (Ayres *et al.*, 1994). The *dnahel* gene promoter was amplified using a synthetic primer set, 5'-TAGTCTAGACATTTTGGTTGTCCAAGTCCA-3' for the 5' region and 5'-GTTGGATCCAATTTTGGCTATCGTGTTTATATTT-3' for the 3' region (Gomi *et al.*, 1999).

DNAs extracted from the hemolymph of BmNPV-infected female moths and their eggs were used as templates. The amplification was performed with PTC-100^{TC} (MJ Research Inc., USA) by using a single denaturation step (5 min at 95°C), followed by a 35-cycle program, with each cycle consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, a final extension step (72°C for 5 min) was also used. After the amplification, 10 μl PCR product was analyzed by 1% agarose gel electrophoresis.

Results and Discussion

When the fifth instar larvae of the silkworm were fed with 1×10^4 polyhedra of BmNPV per larva by oral inoculation, many of which were dead before the emergence and ovary development was arrested. A positive result was obtained from the hemolymph of BmNPV-infected female moths using the polyhedrin gene probe by dot hybridization, while negative signal in DNAs extracted from their eggs (Fig. 1). The result showed that there were BmNPVs in the haemolymph of BmNPV-infected female moths, whereas no detectable polyhedrin gene template in the eggs laid by these female moths.

To further inquire into the transovarial transmission of BmNPV, PCR analysis of DNAs extracted from the hemolymph of BmNPV-infected female moths and their eggs was carried out using the primer sets derived from BmNPV *ie-1* and *dnahel* gene promoters. The PCR products of the expected size, about 360 bp fragment for *ie-1* gene promoter and 510 bp fragment for *dnahel* gene promoter were amplified from the hemolymph of BmNPV-infected female moths, respectively, while absent in DNAs extracted from these eggs (Fig. 2). When the PCR template was prepared from DNAs extracted from the healthy eggs where 5×10^5 pfu of BmNPV virions were

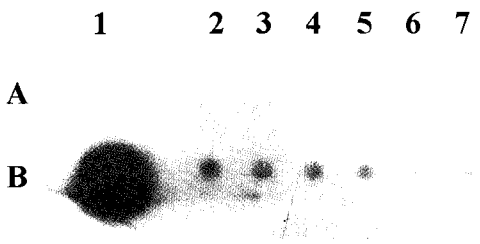

Fig. 1. The results of dot hybridization with BmNPV polyhedrin gene. (A) DNA was extracted from the silkworm eggs. Lane 1, the healthy eggs; Lanes 2-7, the eggs from BmNPV-infected female moths. (B) DNA was extracted from the hemolymph of silkworm female moths. Lane 1, the positive control (the healthy eggs added with 5×10^6 pfu BmNPV virions); Lanes 2-7, the hemolymph from BmNPV productively infected female moths.

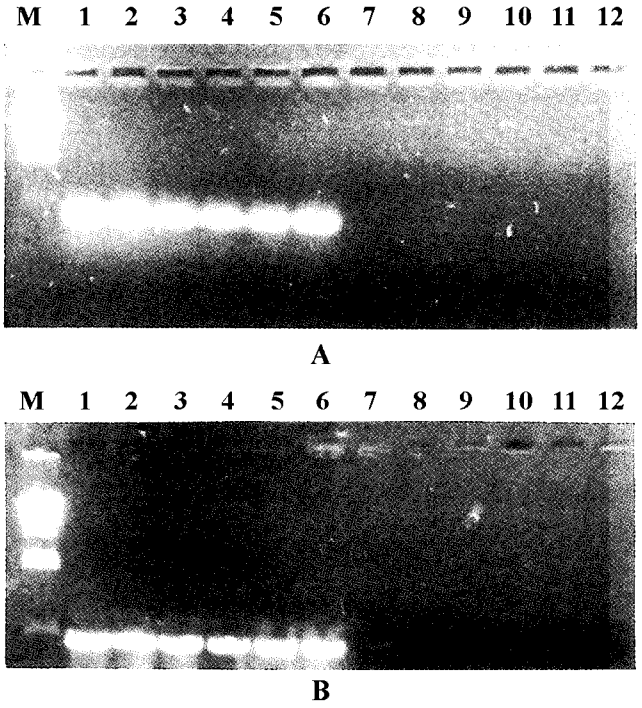

Fig. 2. PCR analysis of DNA extracted from BmNPV-infected female moths and their eggs. (A) The amplified *ie-1* gene promoter. (B) The amplified *dnahel* gene promoter. Lanes 1-6, the hemolymph of BmNPV-infected female moths; Lanes 7-12, the eggs from the corresponding female moths. M: λDNA/*Hin*d.

added, specific fragments could be amplified (data not shown). The evidence suggested that there were not BmNPVs in the eggs laid by BmNPV productively infected female moths. Thus we considered from our results that BmNPV could not be transovarially transmitted by productive infection.

It is very important for the control of silkworm diseases that whether BmNPV can be transovarially transmitted. When the newly molted silkworm fifth instar larvae were micro-infected with BmNPV, the development of partial individuals could be continued, such as pupation, emergence, or oviposition, in spite of BmNPVs present in their hemolymph. It has been confirmed that BmNPV could be transmitted by the silkworm egg-surface. The previous studies reported that BmNPV could be transovarially transmitted, but which was failed to be supported by the silkworm feeding experiment of the next generation (Lü, 1982). It was also reported that AcNPV, injected in silkworm larval or pupal stage could be transmitted to ovary and remained stable through the following generation (Jin *et al.*, 2000; Lee *et al.*, 2000; Yamao *et al.*, 1999). In the cell culture, AcNPV could accumulate 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). Furthermore, AcNPV has been demonstrated to get into mammalian cells well (Merrihew *et al.*, 2001). In this study, using both dot hybridization and PCR technique, we did not detect any template of BmNPV from the eggs laid by BmNPV productively infected silkworm female moths. Injection of BmNPV can cause the death of the silkworm during larval stage due to its strong pathogenicity. When silkworm larvae were orally micro-infected with BmNPV, abnormal development occurred, and premature pupation and death during the larval or pupal stage were caused. We only obtained limited valuable samples at last. A part of infected silkworms could continue their life; however, there were not BmNPVs in the eggs from infected female moths. In conclusion, our findings revealed that BmNPV could not be transmitted to ovary, thus we can prevent the vertical transmission of BmNPV by silkworm egg-surface disinfecting, even though BmNPV has infected the female moth.

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