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RNA Interference to Prevent *Bombyx mori* Nuclear Polyhedrosis Virus Infection *in Vivo*

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RNA interference has been used as a powerful tool in preventing virus proliferation in many species. In this study, we injected the dsRNA *in vitro* transcripts into *Bombyx mori* to investigate the resistance to *B. mori* nuclear polyhedrosis virus (BmNPV). Through vivisectional observation and real-time quantities PCR analysis, we found that these dsRNA can prevent the BmNPV to a certain extent, and delay the viruses' proliferation.

Key words: *Bombyx mori* nuclear polyhedrosis vinus, DsRNA, RNAi, Real-time quantities PCR, Silkworm

Introduction

Baculoviridae is a family of enveloped, double-stranded DNA viruses that infect arthropods. *Bombyx mori* nuclear polyhedrosis virus (BmNPV) was the first virus discovered in previous studies of insect virology (Lu, 1998a). Although silkworm biology has been developed to improve silkworm productivity around the world, Silkworm nucleopolyhedorosis disease remains a highly infectious disease, which has resulted in great losses for the world's silkworm industry. How to control this disease has aroused focus from all over the world.

RNA silencing, originally recognized as post-transcriptional gene silencing in plants (PTGS), was first found in Petunia (Napoli *et al.*, 1990), and its mechanism as an evolutionary conserved gene-silencing mechanism was investigated and shown in the nematode *Caenorhabditis elegans* (Fire *et al.*, 1998; Reinhart *et al.*, 2000). Sequence-specific posttranscriptional gene silencing by double-stranded RNA is conserved throughout a wide range of organisms: plants, *Neurospora*, *Drosophila*, *C. elegans* and mammals (Williams and Rubin, 2002; Kamath *et al.*, 2003; Susi *et al.*, 2004; Kawasaki *et al.*, 2005; Nolan *et al.*, 2005). The use of RNAi as a tool for gene-specific therapeutics has also been studied more and more widely (Izquierdo, 2005; Karagiannis and El-Osta, 2005). Recent studies have provided a number of indirect and tantalising clues to support the participation of RNA silencing in viral infection of both vertebrates and plants (Mak, 2005; Wang and Metzlaff, 2005). In addition, dsRNA has been reported to be used for the investigation of virus resistance in insects *in vitro* and in vivo (Valdes *et al.*, 2003; Agrawal *et al.*, 2004; Flores-Jasso *et al.*, 2004).

The sequence-specific gene silencing effect of resistance of BmNPV by dsRNA has also been observed in the BmN cells (Isobe *et al.*, 2004; Xu *et al.*, 2004). However, no complete protection has been reported through *in vivo* investigation (Isobe *et al.*, 2004). Many genes of *B. mori* such as *ie-1, lef-1,lef-2, lef-3* and *dnapol,* are essential for the replication of viral DNA (Kool *et al.*, 1994; Lu and Miller, 1995; Lu, 1998b). Recent studies have shown that the N-terminal acidic domain of IE1 is required for transactivation (Yamada *et al.*, 2002). In this study, using dsRNA corresponding to the N-terminal acidic domain of IE1 for RNAi, we found partial antiviral effects in preventing BmNVP infection *in vivo*.

Materials and Methods

Reagents

T7 RiboMAX™ Express RNAi System was purchased from Promega corporation. SYBR GREEN PCR mix, PCR reagents, T4 DNA ligase and PM18-T vector were obtained from Takara Company (Dalian). DNA Gel Extraction Kit and Blood Cell Genomic DNA Mini-pre

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Kit were obtained from V-gene Biotechnology Limited. Other reagents were purchased from Shanghai Sangon Bio-technology Corporation. The primers used in this study were designed in the website (http://www.genefisher.de/), and synthesized by Shanghai Genebase Gen-Tech Ltd.

Silkworm and virus

The silkworm (*Bombyx mori*) was inbred in our lab. Highly susceptible silkworm strain 306 was used for this study. All larvae were raised with mulberry leaf. BmNPV T3 was purified by centrifugation at 10,000 × g for 5 min in 4°C centrifuge and the pellet was washed three times with double-distilled water. The precipitate was then resuspended in double-distilled water. 0.1 M Na_2CO_3 was administed to the virus for 30 min in room temperature and resuspended in 0.01 M PBS to prepare occlusion-derived virus (ODV) for hemolymph injection. BmNPV DNA template for dsRNA Synthesis was prepared following the description of Wang *et al.* (1999).

DsRNA synthesis and injection of *Bombyx mori*

Double-stranded RNA was synthesized *in vitro* using the T7 RiboMAX™ Express RNAi System (Promega corporation), following the manufacturer's instructions. Double PCR strategy was used, and nucleotides 60-675 (ATG is in position 1) of BmNPV IE1 gene corresponding to the N-terminal domain from the IE1 protein (Yamada *et al.*, 2002) were amplified by PCR from the entire virus genome. Two pairs of primers were synthesized, and the second pair of primers all contained a 5' T7 RNA polymerase binding site (TAATACGACTCACTATAGG) followed by sequences of the first one pair (Forward: 5' GTTCGACAACGGCTATTCAG; Reverse: 5'ATAACCAGTCTCGGAC ATATGG). The purified PCR products were used as templates by T7 RiboMAX™ Express RNAi System. The dsRNA products were precipitated by adding 0.1 volume of 3 M Sodium Acetate (pH 5.2) and one volume of isopropanol, and resuspended in nuclease-free water. Finally, the dsRNA were inspected with Gene spec III (Naka Instruments Co., Ltd.) and analyzed by 1.5% agarose gel electrophoresis. The dsRNA were stored at −70°C for further use. When *B. mori* larvae were raised to second day of fifth instar, 1 μg of the dsRNAs nude or distilled water (control) were injected into the hemolymph. 24 hrs later, every *B. mori* larvae was injected into the hemolymph with BmNPV ODV (20 ng/larvae).

Real-time quantities PCR analysis

Hemolymph of *B. mori* was collected for virus titer analysis. Genome DNA was extracted by blood Cell Genomic DNA Mini-pre Kit, following the manufacturer's instructions, and every 30 *B. mori* per sample were used. Real-time PCR was performed with an MX3000PTM Real-time PCR System (Stratagene). The primers of BmNPV *dnapol* for real-time PCR analysis were: forward primer 5' AACCGATGACCGATTACAGC and reverse primer 5' TTGCCGGGCACAAAATCC. Plasmids containing BmNPV *dnapol* sequence were constructed and diluted to 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 copies/μl, respectively. Standard curve was made by using 1 μl of plasmids as standard samples. The 20 μl PCR mixture contained 1 × SYBR GREEN PCR mix (Takara), 300 nM of each primer, according to the manufacturer's instructions. After an initial denaturation at 95°C for 5 min, 40 cycles of 95°C 10 sec, 64°C 40 sec and 72°C 15 sec were carried out. And the fluorescence signal was detected at each annealing step at 64°C. Melting curve analysis was applied after the cycling protocol. The copies of BmNPV in hemolymph were calculated according to standard curve.

Results

Infection survival data

To determine the infection survival data, we designed two sets of control groups; the first set of controls were injected with distilled water and then BmNPV ODV. The second set was injected with distilled water in both stages. After electrophoresis on a 1.5% agarose gel, the dsRNA was injected followed by BmNPV ODV in the experimental group. Ninety-six hrs after the second injection, we examined the hemolymph of every *B. mori* under the microscope to determine whether *B. mori* was infected by BmNPV. In the second control group injected only with distilled water, all of *B. mori* survived. However, both in the experimental group as well as the first control group injected with distilled water and BmNPV ODV, different grades of infection existed. We plotted out four grades of infection which are depicted in Fig. 1, and have designated them as serious infection, middling infection, light infection, and survival, respectively.

We determined the median lethal dose (LD50) of 306 strain was 8.516 ng ODV/larvae 96 hrs after injection at room temperature (15°C – 25°C). The survival datas of experimental group A and the first control group B are presented in Table 1. From the statistical data, the survival rate of experimental group A was 20%, while the first control group B was 0. In addition, we found that the degree of infection of the experimental group was lighter than the first control group, and both percentages of serious and middling infection are comparative smaller in the experimental group. Although not completely, these results suggest that the dsRNA got some effects, and the proliferation

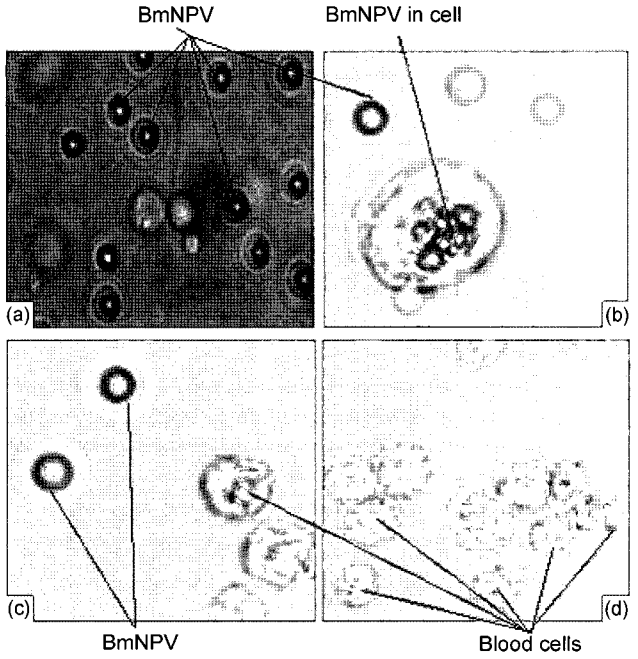

Fig. 1. Hemolymph of *B. mori* infected by BmNPV ODV after 96 hrs. a: Serious infected hemolymph, the NPV titer is so high and nearly no blood cells could be observed; b: Middling infected hemolymph, many BmNPVs exited, a few blood cells can be observed and most of these cells were invaded and occupied by NPV; c: Light infected hemolymph, a few BmNPV exited, many intact blood cells can be observed and a few cells were invaded and occupied by NPV; d: Survival *B. mori* hemolymph, no BmNPV can be found in the hemolymph and the blood cells were observed clearly.

Table 1. Infection survival data

	Serious infection	Midding infection	Light infection	Survival
A	7.1%(5/70)	40.0%(28/70)	30%(21/70)	20%(14/70)
B	31.4%(22/70)	52.9%(37/70)	15.7%(11/70)	0

A: the experimental group, injected with dsRNA and BmNPV ODV; one *B. mori* was died of bacteria infection; B: the first control group, injected with distilled water and BmNPV ODV.

of virus was delayed after interference by dsRNA.

Virus titer determined by real-time quantities PCR

Hemolymph of A and B groups of *B. mori* were collected in three different time intervals (6 hrs, 24 hrs and 48 hrs), Real-time quantitative PCR was used to detect virus copies (Fig. 2). The data showed that the number of virus particles in the first control group was much higher than the experimental group at 48 hrs, more by 580 times. Early on after injection with BmNPV ODV, at 6 hrs, the copies of virus were all so little and nearly the same in quantity. The

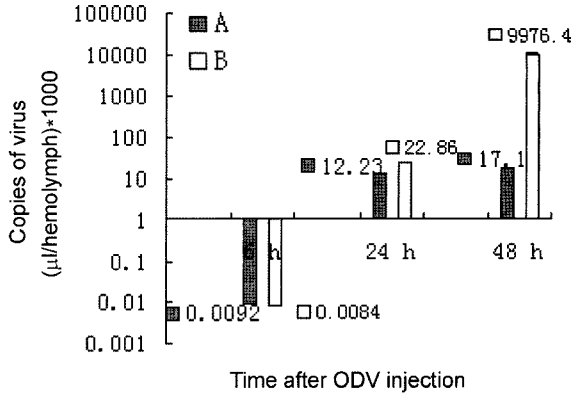

Fig. 2. Copies of virus determined by real-time quantities PCR. A: the copies of virus in experimental group injected with dsRNA and followed by BmNPV ODV; B: the copies of virus in the first control group injected with distilled water and followed by BmNPV ODV. As the differences of copies of virus in different samples are so great, the logarithmic scale was used in Y-axis.

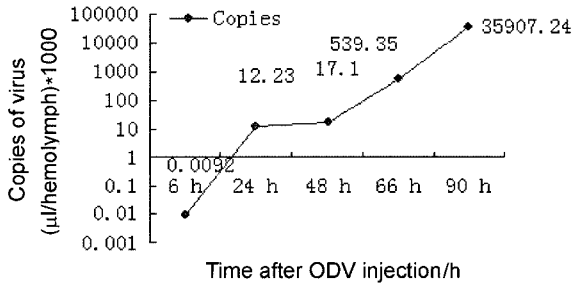

Fig. 3. Viruses' proliferation dynamic analysis after interfered with dsRNA by real-time quantity PCR. the logarithmic scale was used in Y-axis.

results demonstrate that *B. mori* injected with dsRNA exhibited some degree of resistance to BmNPV ODV.

To investigate the proliferation of *B. mori* nuclear polyhedrosis virus in hemolymph interfered with dsRNA in vivo, hemolymph was collected to perform dynamic analysis by real-time quantity PCR. Hemolymph was collected at different times after BmNPV ODV injection (6 hrs, 24 hrs, 48 hrs, 66 hrs and 90 hrs, respectively). In the early stage from 6 hrs to 24 hrs, the virus in both experimental group and control group B increased rapidly. The data showed that the virus proliferated comparatively slowly at the early 48 hrs, and broke out after 66 hrs (Fig. 3). This result accorded with the vivisectional observation under the microscope.

Discussion

Many studies have reported siRNA mediated inhibition of

replication of various viruses of mammals and it is clear that RNA interference appears to be a powerful tool in controlling virus replication (Tan and Yi, 2004; Gadkari, 2005; Wu *et al.*, 2005). To examine the prevention of viruses in invertebrates, dsRNA generally was used (Valdes *et al.*, 2003; Isobe *et al.*, 2004; Bonvin *et al.*, 2005; Robalino *et al.*, 2005). RNA silencing pathways convert the sequence information in long RNA, typically double-stranded RNA, into approximately 21-nt RNA signaling molecules known as small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Cerutti, 2003; Tomari and Zamore, 2005). While double-stranded RNA has been used to prevent the baculovirus infection in Sf21 cells and in *Tenebrio mollitor* (mealworm beetle) *in vivo* successfully (Valdes *et al.*, 2003), no study about dsRNA application in resistance of BmNPV *in vivo* has been reported at present, with the exception of in BmN cells (Isobe *et al.*, 2004; Xu *et al.*, 2004).

In this study, we used the nucleotides of the BmNPV IE1 gene corresponding to the N-terminal domain from the IE1 protein as a template to synthesize dsRNA *in vitro*, and investigated the effect of this dsRNA in prevention of BmNPV in *B. mori in vivo*. Although we were unable to prevent BmNPV infection completely or at a high level from this study, the result suggests that dsRNA could delay the multiplication of the virus *in vivo*. In our study, we also found that the virus in both experimental group and control group B increased comparatively quickly in the early stage from 6 hrs to 24 hrs (Fig. 3). This may be due to the low transfection efficiency of dsRNA *in vivo*, and accordingly the restrain effect was not palpable in the experimental group while the abundance of virus and siRNA produced from dsRNA were all low. It may also show that this dsRNA has a function in resistance to virus in the early 3 days after the dsRNA injection, and then may decrease or miss its function. This may provide some information for future study.

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