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Short hairpin RNA expression for enhancing the resistance of *Bombyx mori* (*Bm*) to nucleopolyhedrovirus *in vitro* and *in vivo*

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A new paradigm of RNAi technology has been studied for enhancing the resistance to virus in plants and animals. Previous studies have shown that the *Bombyx mori* (*Bm*) U6 promoter based shRNA is an effective tool for inducing RNAi in *Bombyx mori* cell line. However, widespread knockdown and induction of phenotypes in *Bm* larvae have not been fully demonstrated. In this study, we examined *Bm* U6 promoter based shRNA expression for suppressing *Bm* nucleopolyhedrovirus (NPV) in the *Bm* cell line and silkworm larvae. We measured the relative expression level of replication genes of *Bm*NPV in hemolymph of silkworm larvae and *Bm*N cells transfected with recombinant targeting shRNA by quantitative real time polymerase chain reaction (PCR). These results indicated that the recombinant shRNA expression system was a useful tool for resistance to *Bm*NPV *in vivo* and *in vitro*. The approach opens the door of RNAi technology as a wide range of strategies that offer a technically simpler, cheaper, and quicker gene-knockdown by recombinant shRNA for future genetics in silkworm *Bm* and other related species.

Key words: RNA interference (RNAi), Silkworm *Bombyx mori* (*Bm*) cell line, short hairpin RNA (shRNA), *Bm* nucleopolyhedrovirus (*Bm*NPV), quantitative real time polymerase chain reaction, *Bm* U6 promoter.

INTRODUCTION

RNA interference (RNAi) is the mechanism of introducing a small RNA into a cell to suppress the target gene's expression. RNAi technologies have been used as a highly useful genetic tool for therapeutic and specific knockdown of particular genes in mammals, invertebrates, and plants (Agrawal et al., 2003; Dawe, 2003; Fire et al., 1998). Characteristic feature of RNAi and antiviral role were first identified in plants. Successively, RNAi had been used as an antiviral curative in animal systems and a gene-therapeutic agent (Gitlin et al., 2002; Li et al., 2002; Sato et al., 2002). RNAi was induced in mammalian cells by the transient transfection of short dsRNA oligonucleotides (21- to 23- bp siRNA). Since the use of artificial siRNA does not lead to a long term effect, researchers developed plasmids encoding short hairpin RNA (shRNA), which are processed in the cell to generate siRNA (Arendt et al., 2003; Peng et al., 2007).

The *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV) is one among the most destructive diseases in silkworm. Although scientists have developed resistance of *Bm* to *Bm*NPV through breeding, it is not completely effective. Control of *Bm*NPV in silkworm is a major factor of silk industry. *BmNPV* is an insect virus which is in baculoviridae family. It has a circular double stranded DNA of 130 kbp. Studies have shown that specific viral genes such as *P*143 and *P*35 are required for multipli-cation of viruses. These two genes were first identified in *Autographa californica* multiple nucleopolyhedrovirus

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(AcMNPV) (Marcel et al., 1994). *P*143 gene is encoded 143 kDa polypeptide with motifs conserved among DNA helicases. The presence of this gene is necessary for *Bm*NPV DNA replication. *P*35 gene is stimulated by DNA replication in AcMNPV. Lack of *P*143 and *P*35 genes in cells infected with NPV could be effective in control *Bm*NPV infections in silkworms.

Isobe et al. (2004) successfully suppressed the multiplication of *Bm*NPV *in vivo* when the *lef1* dsRNA was infected in *Bm*N cells. Using transgenic silkworms to transcribe ie1 dsRNA of *Bm*NPV by ie1 promoter decreased the lethality of *Bm*NPV in the silkworm larvae.

In this paper, we generated the targeting of shRNAs against *Bm*NPV and analyzed the efficiency of *Bm*NPV knock-down by shRNAi plasmid, which is regulated by a *Bm* U6 promoter with a marker gene of green fluorescence protein (GFP), in *Bm* cells and silkworm larvae. We selected the most efficient shRNAs for resistance to *Bm*NPV. The results suggested that this technique is useful for suppressing the *Bm*NPV both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Cell lines

The *Bm* ovary derived cell line (*Bm*N cells) was conserved in our lab and cultured on TC-100 medium (GIBCO, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum followed by incubation at 27°C.

Virus

*Bm*NPV was purified from strain T3 and propagated in the *Bm*N cell lines at 27°C.

Experimental animals

A hybrid strain of silkworm (Commercial name: Baiyu x Qiufeng) was used in this experiment. The larvae were reared with fresh mulberry leaves at 25 to 27°C.

Chemistry reagents

Lipofectamine-2000 was purchased from Invitrogen, Shanghai, China First-strand of cDNA synthesis ReverTra Ace® qPCR RT Kit was purchased from TOYOBO, Japan MMLV first-strand cDNA synthesis kit was purchased from Sangon Company, Shanghai, China, and genomic DNA extraction kit was purchased from Sangong, China.

Construction of shRNA-expression plasmid

The shRNA were constructed according to knockout RNAi systems user manual. We constructed shRNA plasmids in PXL-BACII vector (*Piggy*Bac transposition vector). Briefly, recombinant plasmid were transformed in *Escherichia coli* strain using ampicillin containing LB plate for growing colonies at 37°C overnight. After growing colonies, we picked a colony and place them again on the fresh 0.5 µg/ml ampicillin LB agar for overnight shaking. We confirmed by sequencing and digested by *Bam*H1 and *Eco*RI endonuclease enzymes.

Already, the PXL-BACII-EGFP-BmU6-shRNAs were constructed in our lab. We constructed nine types of shRNA of *P*143 and *P*35 genes such as *P*143A, *P*143B, *P*143C, *P*143D, *P*143E, *P*35A, *P*35B, *P*35C, and *P*35D. Enhanced green fluorescence protein (EGFP) was used as reporter plasmid.

Transfection of *Bm*N cells line

*Bm*N cells (1×10^6/well) were cultured on a 6-well plate. Each well was transfected with 5 µg of PXL-BACII-EGFP-*Bm*U6-shRNA. plasmid DNA using by Lipofectamine-2000 according to the manufacturer's instructions.

After 6 h incubation, the TC-100 medium with 10% fetal bovine serum (FBS) was replaced. After 24 h, each well was inoculated with 15 µl of *Bm*NPV (at a MOI of 3) and incubated at 26°C. From 24 to 72 h post transfection of plasmid, the GFP was observed under a fluorescent microscope (Nikon ECLIPSE Ti). The genomic DNA was isolated by TIANamp genomic DNA Kit (Tiangen Biotech, Beijing co., LTD.) 72 h post transfection of plasmid, and genes were confirmed by PCR using primers *Bm*ie1-f (5'-tcgacaacggctattcagag-3') and *Bm*ie1-r(5'-ctgcagtctcgctgtcagat-3').

Recombinant shRNA injected in silkworm larvae

The 5th instar first day of silkworm larvae were reared in six different groups. Each group's larvae were 50 pieces. First groups were normal as a negative control. Second groups were microinjected with 15 µl *Bm*NPV polyhedra (1×10^6/each larva) as a positive control. Other four groups were microinjected with same concentration and quantity of recombinant shRNA plasmid (5 µg) with lipofectamine-2000. After 24 h post infection, the larvae were challenged with *Bm*NPV (1×10^6/each larva). Observations were conducted day by day. After 96 h post infection, the hemolymph was collected in an eppendorf tube, and then centrifuged at 1000 rpm for 10 min.

RNA extraction and cDNA synthesis from *Bm*N cell and hemolymph

The total RNA was extracted from 96 h post infected cell samples using RNAiso Plus (TaKaRa Biotechnology Co., Ltd. China). We followed the same methodology for extraction of RNA from 96 h post infection of silkworm samples. The RNA was used as a template for first-strand of cDNA synthesis using ReverTra Ace® qPCR RT Kit (TOYOBO, Japan) under reaction conditions of 5 min at 65°C, 15 min at 37°C, 10 min at 98°C. For gene fragment amplification using actin A3 forward primer 5'-GCGCGGCTACTCGTTCACTACC-3' and reverse primer 5'-GGATGTCCACGTCGCACTTCA-3'. The PCR was conducted in a volume of 20 µl containing 7 µl double distilled water, 10 µl premix Ex Taq and 1 µl 10 mmol/1 primer F, 1 µl 10 mmol/1 primer R and 1 µl cDNA. The conditions of reaction were set as 30 s at 94°C, 30 s at 60°C, 1 min at 72°C and final extension for 10 min at 72°C.

Quantification of polyhedra of silkworm larvae

Hemolymph were harvested from single silkworm larva injected with *Bm*NPV as a positive control and simultaneously, hemolymph were harvested from larva injected with recombinant shRNA-plasmid at 48 and 96 h post infection, and centrifuged at 12000 rpm for 5 min. After which piled was collected and solubilized in 0.1% SDS in PBS buffer (pH 7.0) for 30 min at room temperature. The solubilized polyhedra were diluted 10 times with purified water. The larval hemolymph was checked for the presence of polyhedra under a hemocytometer at 48 and 96 h post infection. Each experiment was done in three replications.

Table 1. Synthesized shRNA sequences targeting the regions of the *p*143 and *p*35 of *Bm*NPV.

Construct	Position	Sequence
*p*143A	329-347	GGACTATTGTTGGTGCTCA
*p*143B	538-556	GGCAAACTTAACGCTGTCT
*p*143C	606-624	GTCATAATCGTCCACGTAC
*p*143D	1100-1118	TGCGCATGTAGAATCGAGT
*p*143E	1731-1749	GTTTAACGCGACTCGCATA
*p*35A	64-82	GACGAACAAACCAGAGAGT
*p*35B	117-135	GACAAAACCCGTTCTCATG
*p*35C	585-603	GTCTTAGCTTACGTGGACA
*p*35D	225-243	GATCAACTAGAACGCGAAT

Scanning electron microscopy (SEM)

The polyhedra were allowed to settle on the surface on to carbon-coated grids for 2 min. The grids were washed three times with distill water and stained with 2% uranyl acetate. The grids were examined using a Nikon Eclipse ME600 electron microscope (Nikon, Japan).

SYBR real-time quantitative PCR

PCR amplifications were performed using a 7500 fast real-time PCR System. The PCR was conducted in 25 µl system containing 6.8 µl double distilled water, SYBR premix Ex Taq 10 µl, 1 µl 10 mmol/1 primer F, 1 µl 10 mmol/1 primer R, 1 µl cDNA and ROX Reference Dye 0.4 µl (ROX) qPCR was completed according to protocol. Relative values of the genes expression data were calculated by Microsoft Excel.

RESULTS

Selection of shRNA and construction of recombinant plasmids

A total of 9 targeted sequences in the coding regions of *p*143 and *p*35 of *Bm*NPV were selected and served as a basis for the design of the complementary shRNA template oligonucleotides (Table 1).

The shRNAs were synthesized, annealed and inserted into the *Bam*HI and *Eco*RI sites of the siRNA expression vector pRNAi-Ready pSIREN-RetroQ-ZsGreen (Clontech). The ZsGreen and Neo resistant genes were amplified and sub-cloned into the vector PXL-BACII (*Piggy*Bac transposition vector. The *Bm*NPV targeting shRNAs were cut out from the above recombinant pSIREN with restriction sites *Bgl* II and *Eco*R I and the above constructed sub-cloned *piggy*Bac vector was designated as PXL-BACII-EGFP- *Bm*U6-shRNA.

Purification of polyhedra of silkworm

The single silkworm larva was injected with *Bm*NPV with 15 µl of *Bm*NPV (at a MOI of 3). After 96 h post infection, the hemolymph was harvested from larva and solubilized in 0.1% SDS in PBS buffer (pH 7.0). The polyhedra were identified under electronic microscope (Figure 1).

Viral challenge and investigation of interference against BmNPV *in vitro*

We investigated whether shRNA expression plasmid constructed by our method can induce gene expression in *Bm* cell line and larvae. The recombinant shRNA expression plasmid together with lipofectamine-2000 was co-transfected into the *Bm*N Cell and silkworm larvae.

After transfection of shRNA expression plasmid DNA (5 µg) containing lipofectamine-2000 in *Bm*N cell, all cells were clearly visualized for EGFP after 24 h when the cells were transfected with recombinant shRNA plasmid, and challenged with *Bm*NPV polyhedra (1×10^6). Observation suggested that almost 90% cells were transfected and EGFP was visualized. The *Bm*NPV challenge experiment showed that different shRNA has different suppression effect against *Bm*NPV in cells. Among them shRNA targeting *p*143E was most effective (Figure 2).

After challenge with *Bm*NPV, transfected shRNA with lipofectamine-2000 in cell was observed. The different suppression rates depend on different shRNA sequences.

Silkworm larvae challenged by *Bm*NPV

We chose best sequences for further experiments in the silkworm larvae. We selected four shRNAs (*p*143E, *p*35A, *p*35B, *p*35C) which were more suppressed from Figure 2. Four days after *Bm*NPV was infected; silkworm were observed to die (Figure 3B).

However, *P*143E shRNA infected silkworm was able to survive up to 6 days in comparison to infected positive control (Figure 3F). It was interesting to find that ShRNA infected silkworms were more active than *Bm*NPV infected silkworm and could be easily distinguished with

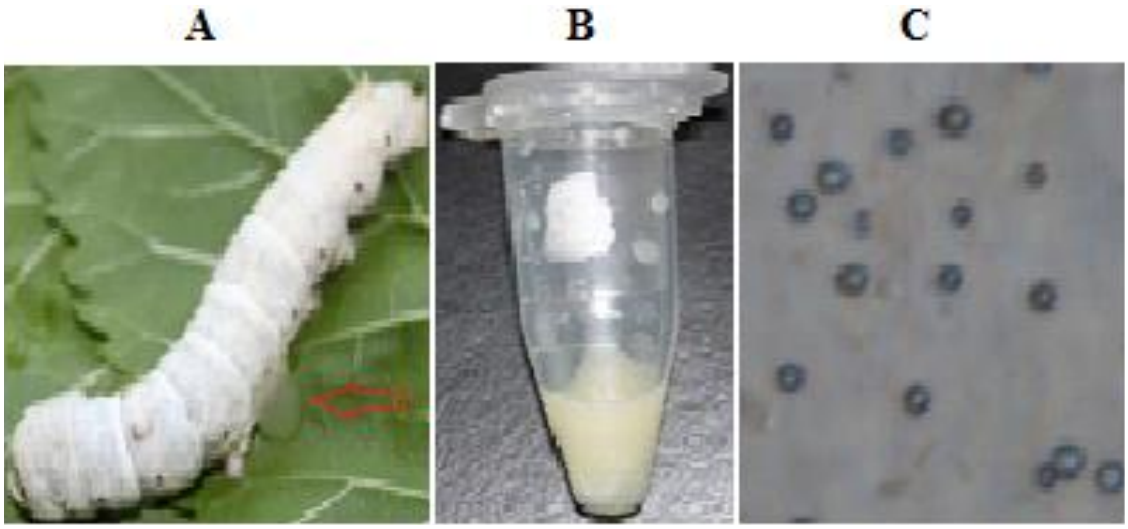

Figure 1. Purification of polyhedra of silkworm. A) Collect hemolymph from the silkworm. B) Purification of the hemolymph with 0.1% SDS in PBS (pH 7.0). C) Polyhedra of the *Bm*NPV under electronic microscope.

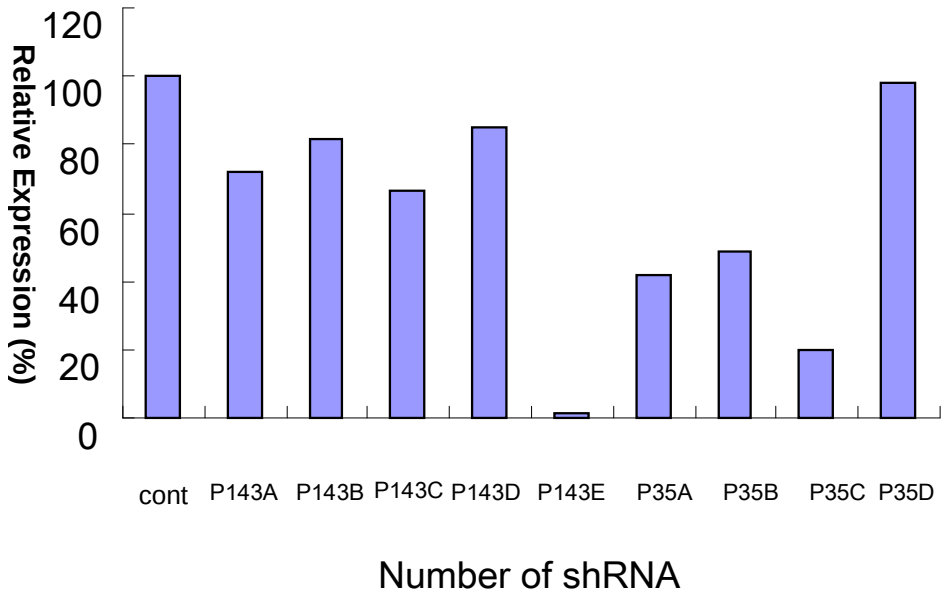

Figure 2. The target sequence-dependent suppression by shRNAs with U6 promoter in *Bm*N cells analysis by quantitative real- time PCR.

phenotypic characters. Some of the phenotypic traits were blackening of the silkworm body, loss of appetite, growth retardation, early maturing, swelling of the body and hemolymph becoming whitish (Figure 3).

Further, we examined the density of *Bm*NPV polyhedra in the infected hemolymph in each larva and then compared by hemacytometer under a microscope (Figure 4). We observed that *P*35C shRNA has less polyhedra (than positive control *Bm*NPV infected silkworm. We observed two different times at 48 and 96 hpi, the results were not same and varied with time period. The results indicated that *P*35C is less polyhedral than other infection of shRNA and also showed that the polyhedra were less than *Bm*NPV infected silkworm (Table 2).

Gene expression analysis

Figure 5 indicates that *p*35C is the most effective suppression of all. Though *p*143E shows the least suppression rate here, their suppression rate shows better in *Bm*N cell lines. Our findings demonstrate that at 5 µg

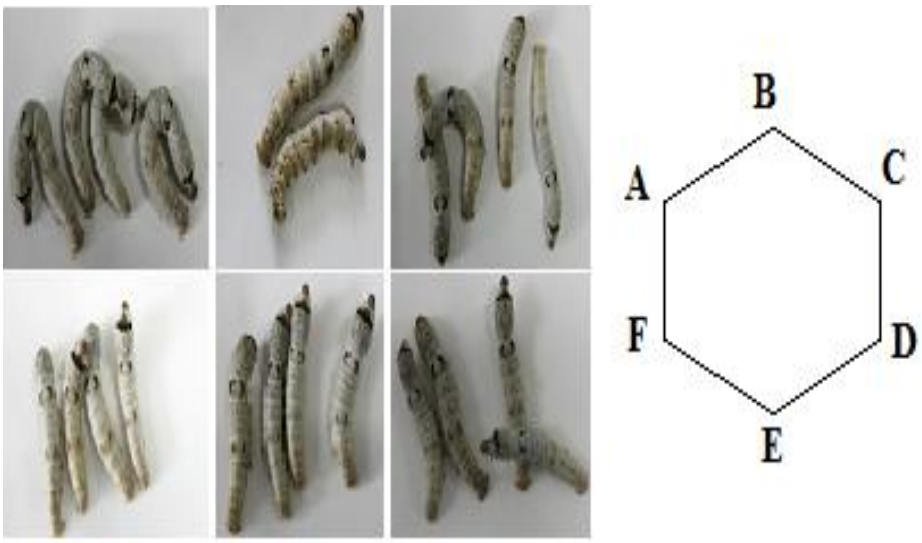

Figure 3. *Bm*NPV challenged with different recombinant shRNA *in vivo* after 96 hpi. A) Normal silkworm larvae (negative control), B) *Bm* larvae infected with *Bm*NPV, C) *Bm*NPV challenged with *P*35A shRNA, D) *Bm*NPV challenged with *P*35B shRNA, E) *Bm*NPV challenged with *P*35C shRNA, F) *Bm*NPV challenged with *P*143E shRNA. The silkworm was injected with *Bm*NPV polyhedra (1×10^5/each larva).

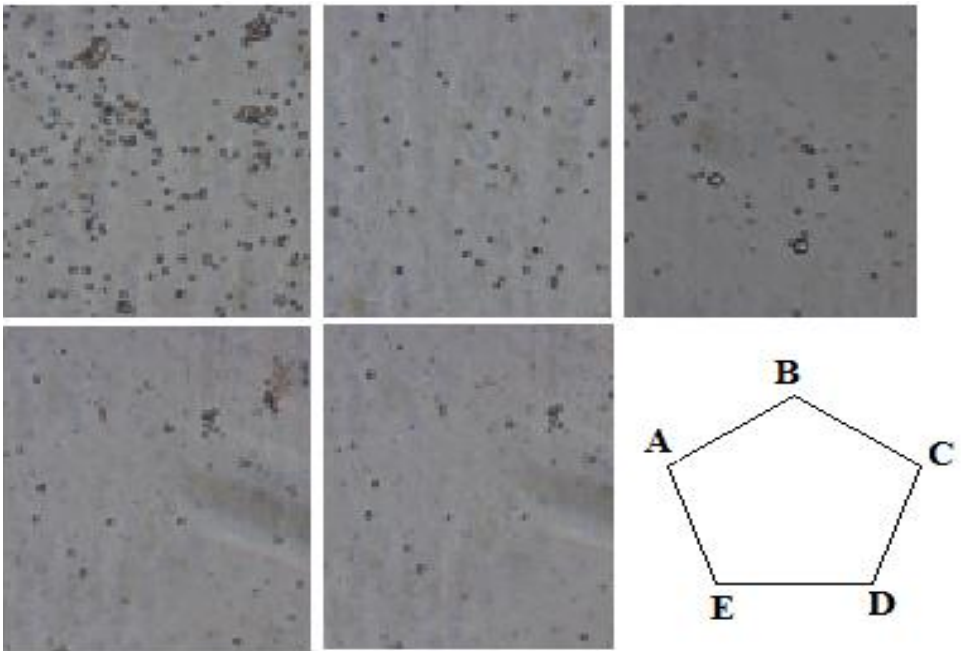

Figure 4. Density of polyhedra different shRNA treated silkworm hemolymph. A) *Bm*NPV infected silkworm hemolymph, B) *Bm* NPV challenge with *P*35A shRNA, C) *Bm* NPV challenge with *P*143B, D) *Bm* NPV challenge with *p*143E shRNA, E) *Bm* NPV challenge with *p*35C shRNA.

shRNA doses of injection to all silkworm larvae and *Bm*N cell lines, the level of expression varies, which indicates the variation mechanism involved *in vivo* and *in vitro* conditions.

*P*143 ShRNA is less suppressing than others. The result shows that in the *Bm* cell line *P*143E shRNA was

Table 2. Observation of *Bm*NPV polyhedron in hemolymph of silkworms at different times under hemocytometer microscope.

ShRNA name	Polyhedron observed (No.) (48 h post-injection)	Polyhedron observed (No.) (96 h post-injection)
Hemolymph of silkworm	0	0
NPV with silkworm	80	100
Bm NPV challenge with *P*35A	49	68
Bm NPV challenge with *P*35B	59	80
Bm NPV challenge with *P*143E	57	70
Bm NPV challenge with *P*35C	40	50

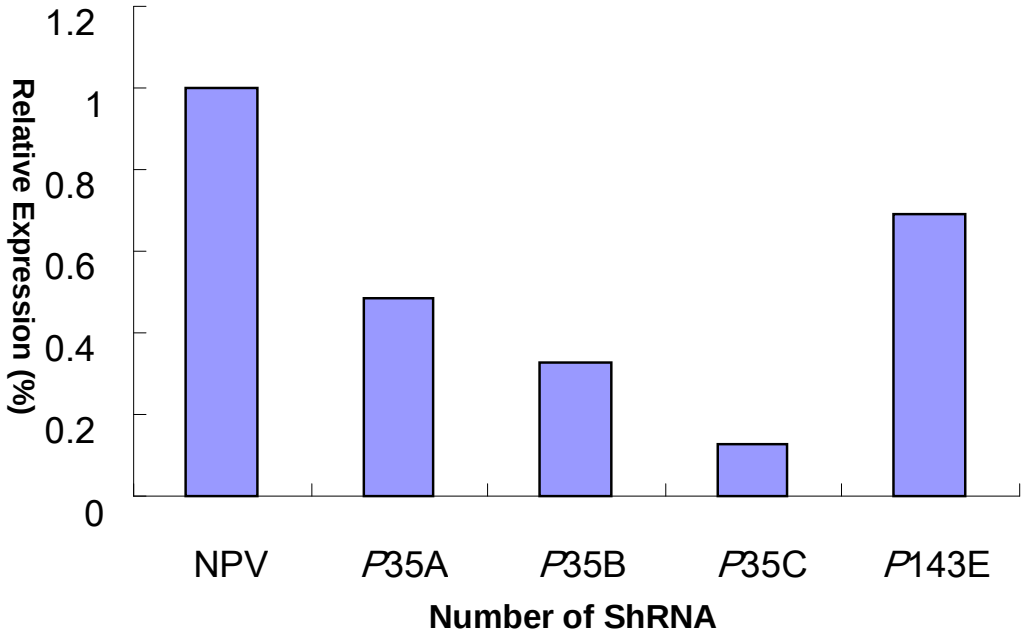

Figure 5. Relative expression analysis of shRNA in silkworm hemolymph post 96 h transfection. Indicated are: *Bm*NPV = Control larvae; *p*35A, *p*35B, *p*35C and *p*143E = Challenged *Bm*NPV.

more suppressive but in the organism, *P35C* is the most suppressive shRNA. Our findings demonstrate that with same amount of shRNA the suppression of the gene also differs in the organism. This behavior may be due to differences *in vivo* and *in vitro* mechanisms involved. The results showed almost similar suppression rate *in vivo* and *in vitro* conditions.

The *p*143 and *p*35 genes were identified by PCR. The samples were collected from infected larvae. The results showed that lane 2 was a negative control and does not have a band; lane 3 was a positive control and has a band; lanes 4 to 6, the *p*35A, *p*35B, *p*35C challenged with *Bm*NPV, also have a band of 430 bp; lane 7 was a positive control and has a band and lane 8 *p*143E challenged with *Bm*NPV and has a band of 275 bp. We confirmed from this picture that the *p*143 and *p*35 genes were presented in infected silkworm larvae (Figure 6).

DISCUSSION

RNAi is a promising tool for studying gene silencing in all eukaryotes. DsRNA duplex can suppress the expression of target gene through either mRNA degradation or blocking mRNA translation (Mcmamus and Sharp, 2002). shRNAs can be generated by an oligonucleotide DNA sequence. The shRNA constructs can trigger siRNA molecules to introduce the gene-specific silencing. Each shRNA vector system has the ability to silence specific gene. It has been demonstrated that *piggy*Bac2 vector can be successfully used for shRNA expression. This vector can also be linearized and as such; be ready for ligation and direct use in transient transfection experiments. Sequence encryption shRNA is a 19 to 21 bp of homology to the targeted gene and are synthesized as 60 to 75 bp double stranded DNA oligonucleotides

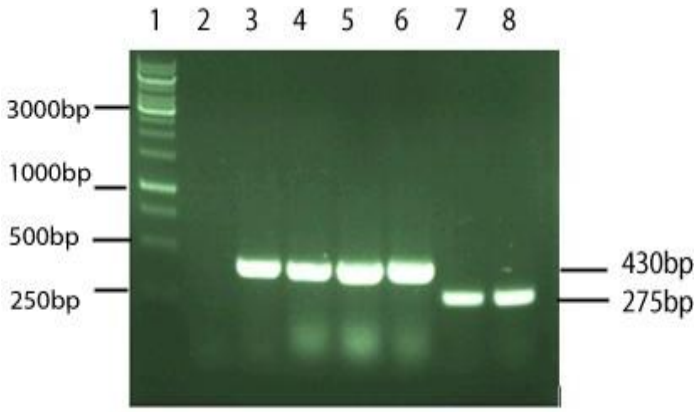

Figure 6. *P35* gene and *p*143 gene detection by PCR after injected recombinant plasmid challenged with *Bm*NPV in silkworm larvae. The genomic DNA as a template from hemolymph after 96 hr. infection. Lane1: DNA marker Lane 2: Control silkworm Lane 3: *Bm*NPV infected silkworm. Lane 4 to 6: p35 gene challenged *Bm*NPV. Lane7: *Bm*NPV infected silkworm Lane 8: *p*143 gene challenged with *Bm*NPV

(Patrick et al., 2002).

Recently, several works have been done on improving the pol lll expression system which successfully knock-down the gene expression (Isobe et al., 2004). However, there is need to improve this system for control of *Bm*NPV both *in vivo* and *in vitro* condition. Recently, dsRNA has been used for resistance *Bm*NPV in the silkworm by pol ll promotor (Ohtsuka et al., 2008). *Bm*NPV gene was silenced by small RNA using poll lll promoter; although, it is necessary to improve this technology for successful sequence-specific gene silencing in the *in vivo* and *in vitro* condition.

In this work, the selected *Bm*U6 promoter was used as a best tool for suppression of *Bm*NPV in the silkworm. We targeted five and four different positions of the genes *p*143 and *p*35, respectively (*p*143A, *p*143B, *p*143C, *p*143D, *p*143E and *p*35A, *p*35B, *p*35C, *p*35D) (Table.1). The shRNA presents a 7 to 9 nucleotide hairpin loop (5'-TTCAAGAGA-3') and the 19 base pair antisense sequence of the target site. The knock down efficiency of shRNA expression was different in *Bm* cell. The most effective suppression was observed in the plasmid targeting *p*143E gene (Figure 1, *p*143E) which suppressed the expression to about 90%, and *p*35C gene which suppressed the expression to about 70% (Figure 1, *p*35C). This result suggests that gene suppressing differs on the suitable sequence.

Further, we examined the density of *Bm*NPV polyhedra in the infected hemolymph in each larva transfected with both of *BM*NPV and shRNA plasmids. The results showed that *P*35C is less polyhedral than other infection of shRNA and also showed that the polyhedra were less than *Bm*NPV infected silkworm (Figure 4 and Table 2).

We examined relative expression level of the replicated *Bm*NPV genes in silkworm larvae, hemolymph and *Bm*N cells. Some of the shRNA worked successfully and showed less multiplication of *Bm*NPV. This RNAi system will be useful for suppression of *Bm*NPV in silkworm. It might eradicate *Bm*NPV in silkworm body in the future which will be valuable for silk industry.

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