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Targeting *ie-1* gene by RNAi induces baculoviral resistance in lepidopteran cell lines and in transgenic silkworms

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Abstract

RNA interference (RNAi)-mediated viral inhibition has been used in a few organisms for eliciting viral resistance. In the present study, we report the use of RNAi in preventing baculovirus infection in a lepidopteran. We targeted the baculoviral *immediate early-1* (*ie-1*) gene in both a transformed lepidopteran cell line and in the transgenic silkworm *Bombyx mori* L. Constitutive expression of double-stranded RNA was achieved by *piggyBac*-mediated transformation of Sf9 cell line with a transgene encoding double-stranded *ie-1* RNA (*dsie-1*). Strong viral repression was seen at early stages of infection but subsequent recovery of viral proliferation was observed. In contrast, the same transgene inserted into the chromosomes of transgenic silkworms induced long-term inhibition of *B. mori* nucleopolyhedrovirus infection, with nearly 40% protection compared with nontransgenic animals. Protection was efficient at larval stages after oral infection with occlusion bodies or hemocoel injection of budded viruses. Virus injected pupae also displayed resistance. These results show that heritable RNAi can be used to protect silkworm strains from baculovirus infection.

Keywords: *Bombyx mori*, RNAi, baculovirus, transgenic, silkworm.

Introduction

The virus BmNPV is a member of *Baculoviridae*, one of the 14 major families of invertebrate viruses that infect arthropods, primarily insects (Murphy *et al*., 1995; van Regenmortel *et al*., 2000). BmNPV, like most of the baculoviruses, is host-specific and highly infectious to *Bombyx mori* cultured cells, but does not replicate in the cells of the closely related lepidopteran *Spodoptera frugiperda* (Kondo & Maeda, 1991). Baculovirus infection ensues when the caterpillar eats environmentally resistant viral particles that are deposited on the leaves as occlusion bodies (OBs), which on entering the alkaline environment of the midgut, are released as free virions. Viral genes start transcribing in a temporal manner (Huh & Weaver, 1990a) with early genes such as *immediate early-1* (*ie-1*) and *-2* (*ie-2*) being transcribed from early promoters by host RNA polymerase II (Huh & Weaver, 1990b; Hoopes & Rohrmann, 1991; Glocker *et al*., 1993). This is followed by the expression of other late effector factors (lefs), while amanitin-resistant viral RNA polymerase initiates the transcription of very late genes (Huh & Weaver, 1990b). Finally, the virions encapsulated within the polyhedral envelope are released to the environment by cell lysis and dissolution of the infected larvae. In contrast to OBs, released free viruses (FV or budded viruses BV) are formed inside the cells without polyhedral cover, and spread infection into the organism. The larval tracheal system provides the major channel for the baculovirus to pass through basal laminae and reach other tissues (Engelhard *et al*., 1994).

The baculoviral *immediate early-1* (*ie-1*) gene is one of the five essential genes needed for viral replication (Kool *et al*., 1994; Lu & Miller, 1995). IE-1 has been shown to activate promoters of early genes including *ie-2*, *39K* and *p35*

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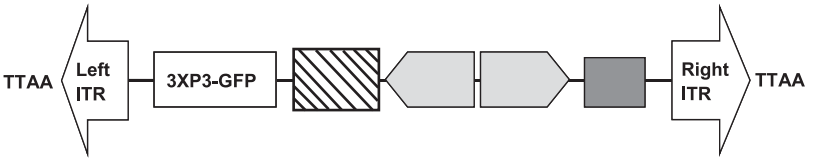


Figure 1. *PiggyBac*-based RNAi vector, *pPIG3XP3-GFP-FF* (11 kb) used for constructing transgenic silkworms with *3XP3-GFP* gene as a selection marker. Left ITR and Right ITR indicate left and right terminal inverted repeats, respectively. *ie-1*FF cassette (light grey boxes) comprises 630 bp of *ie-1* promoter (hatched box), 470 bp of *ie-1* gene fragment in 'flip-flop' orientation and 200 bp polyadenylation signal (dark grey box). The construct *pPIGA3GFP-FF* that was used for cell culture assays had the A3-GFP cassette instead of the 3XP3-GFP cassette.

(Guarino & Summers, 1986; Nissen & Friesen, 1989; Carson *et al.*, 1991) and is directly or indirectly involved in the expression of late genes (Passarelli & Miller, 1993). IE-1 oligomerizes within the cytosol (Olson *et al.*, 2002) and gets involved with the viral DNA replication machinery (Okano *et al.*, 1999). Thus, *ie-1* is an appropriate target for RNA interference (RNAi)-mediated gene silencing.

RNAi is a phenomenon of selective mRNA destruction leading to gene knockdown (Fire *et al.*, 1998). RNAi has been demonstrated in many insect species including the silkworm *B. mori* (Riu *et al.*, 2004). Double-stranded RNA injected into the haemolymph of *S. frugiperda* larvae has been shown to transiently suppress AcNPV proliferation (Valdes *et al.*, 2003), while *lef-1* targeting RNAi was partially effective to lower the BmNPV proliferation in transgenic *B. mori*, but not to reduce the mortality of virus-treated silkworms (Isobe *et al.*, 2004). In the present study, we report RNAi-mediated inhibition of baculovirus proliferation in cell culture and in transgenic silkworms by targeting *ie-1* gene.

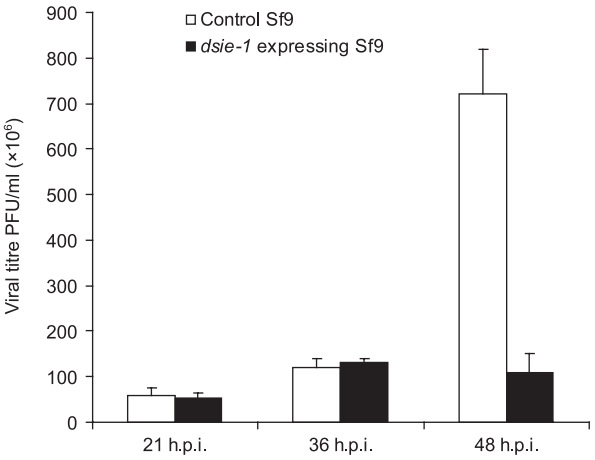


Figure 2. Plaque assay on polyclonal transformed Sf9 cells. Cells were infected with 5 MOI of free AcNPV. Plaque assay was carried out from the media collected at the scheduled time postinfection. The viral titre is expressed as plaque-forming unit (PFU) per ml of the culture media. Significant reduction of plaque formation was noticed in transformed cells at 48 h.p.i.

Results

AcNPV resistance in transformed Sf9 cell line

Obtaining transformed Sf9 cells having pPIGA3GFP-FF. To trigger heritable RNAi-induced baculovirus inhibition, we generated a *piggyBac*-derived vector carrying the *ie-1* promoter and two 470 bp fragments of the 5′ sequence of the *ie-1* gene placed in opposite orientation (*pPIGA3GFP-FF*) (Fig. 1). The vector also carried a GFP expressing selection gene. Sf9 cells were transformed by transfection of the gene vector with a helper construct providing the *piggyBac* transposase. Following stabilization of GFP expression after six passages, the transformed Sf9 cells were FACS selected. The polyclonal population of GFP-positive Sf9 cells was amplified and used for viral challenge.

Plaque assay. Transformed Sf9 cells carrying the *pPIGA3GFP-FF* were tested for viral resistance with cells harbouring *pPIGA3GFP* as a negative control. For viral challenge, cells were infected at a multiplicity of infection (MOI) of five of wild-type AcNPV and assayed after different hours postinfection (h.p.i.). The culture medium containing BVs was collected at scheduled times and viruses were titrated by plaque assays. The results are shown in Fig. 2. The transformed cells showed a sevenfold decrease of budded viruses compared with control cells at 36–48 h.p.i.

Microscopic observations. GFP-positive and GFP-negative Sf9 cells of polyclonal population were observed after infection with wild-type AcNPV. As shown in Fig. 3, the cells expressing GFP displayed reduced OBs compared with the nonfluorescing cells. However, GFP-positive cells gradually accumulated OBs and at the fifth day after infection, all the surviving cells were laden with OBs and started lysing.

BmNPV resistance in dsie-1 transgenic silkworms

Construction of transgenic silkworm lines with pPIG3XP3GFP-FF. For germ-line transgenesis, *pPIG3XP3GFP-FF* construct was used and the results of microinjection are summarized in Table 1. From the five broods six independent transgenic silkworm founders were selected and after sib mating, we established six independent homozygous lines for the transgene. *Per os* infection, and intra-hemocoel injection of baculovirus particles were tested on three of these lines (126A, 126B and 58E) and on control lines NM and TAFib6 to evaluate the protection against baculovirus imparted by the transgene.

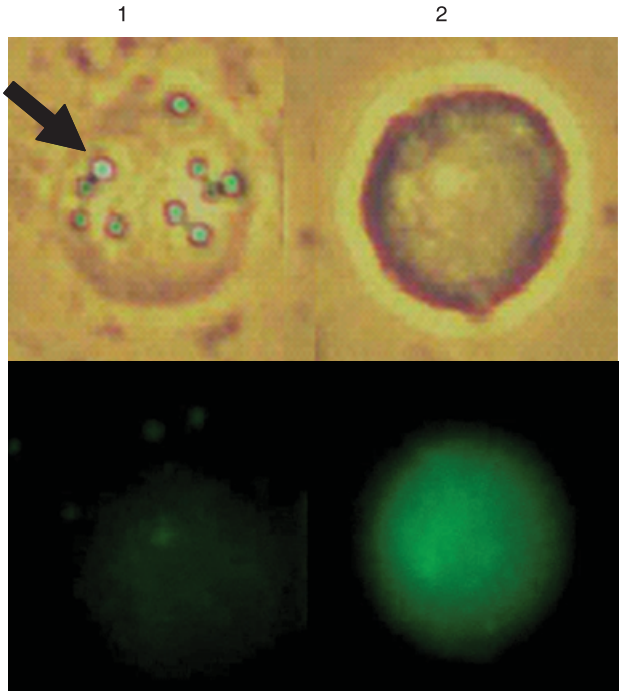


Figure 3. Microscopic observation of the infected polyclonal transformed Sf9 cells. Occlusion bodies (arrow) could be seen predominantly in nonfluorescing cells (1) compared with in fluorescing cells (2).

Table 1. Summary of microinjection for generating transgenic silkworm lines

Injected embryos	Hatched larvae*	Fertile adults†	G_1 broods‡	Broods with GFP positive larvae§
510	345	251	143	5

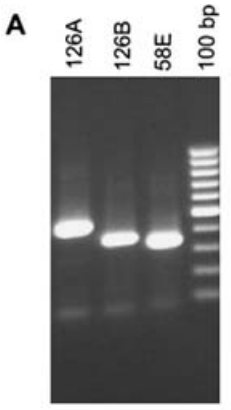
*Hatched larvae (G_0) were allowed to develop to moths. Hatching % = 67.
†The moths were intercrossed or backcrossed.
‡Neonate larvae from G_1 broods were screened for GFP fluorescence.
§GFP-positive larvae were reared and survived moths were crossed with wild-type silkworms to establish G_2 transgenic lines.

Chromosomal location of the transgenes. Copy number and chromosomal location of the inserts were characterized by the method of transposon element display (TED). Formation of a single polymerase chain reaction (PCR) amplification product after the second PCR reaction showed the presence of a single copy insertion of the transgene in all three transgenic lines (Fig. 4A). TED was used to target the left arm of the *piggyBac* transposon. The presence of TTAA sequence near the genomic integration site further confirmed the *piggyBac*-based transgenesis (Fig. 4B).

To confirm the location of insertion, the PCR products were sequenced and blasted against the complete genome sequence of *B. mori.* Lines 126A and 126B, which carry a distinct insertion showed a high homology to RAMEN contig102893 (85% similarity) and contig16157 (98%) respectively. The insertion site of line 58E could not be ascertained, as it showed no significant homology to any of the yet available contigs of the sequenced silkworm genome.

BmNPV feeding experiments showed reduced mortality in transgenics. Per os BmNPV infection was carried out with a viral dose of 6000 and 12 000 OBs per larva at III and IV instar stages, respectively. The results are shown in Fig. 5. The larval mortality was registered until the eclosion of the moths. The percentage of mortality of the control lines NM, TAFib6, and the transgenic lines 126A, 126B and 58E was 92, 80 29, 65 and 90 respectively at IV instar stage (Fig. 5). The mortality rate at III instar also followed similar trend but efficient suppression of viral proliferation was very much evident at IV instar stage (Fig. 5). Thus, the three lines tested presented different degrees of resistance to baculoviral infection, 126A appearing as the most resistant followed by 126B, and 58E showing negligible protection against the pathogen. In 126A, the survival of individuals was enhanced by threefold compared with the parental line. Correlatively, the quantification of OBs in haemolymph showed reduction of the presence of the virus in 126A and 126B, which carried 45 000 and 2000 times less viral particles than the infected parental or control lines. No beneficial effect was observed for the transgene carried by the line 58E.

We also assayed the properties of the transgenic silkworms after a viral challenge that consisted of direct injection of budded viruses into the haemolymph. In these experiments, we used the recombinant virus *BmNPV-P10GFP.* When a



B

126A

TGTTTTTTTTTTCAGAAGTCCTTCTTCAGGCTTAAACATGTAATAACTGTGGC
TTATTAACCGTTTAGAATCTGTGAAAGTGGAGGAAAATAAAGCAAAATCACT
GGCCATAGCCTCTCGAGTTCTTCCAAAAAGTCCAGTGAAGTCTTA**TTAA**CC
CTAGAAAGATAGTCTGCGTAAAATTGACGCATGCATTCTTGAAATATTGCTCTC

126B

AAATAATTTACTAAAATAACTACTAAATAATTAATAATATTTATCACTACATAGTA
TAAAACAAAGTCGCTTTCTCTGTCCCTATATCTGTCTGTCCCTATGTATGCTTCA
ATCTTTAAAACTACGCAACGGATTTTGATGCAGTTTTAT**TTAA**CCCTAGAAAGA
TAGTCTGCGTAAAATTGACGCATGCATTCTTGAAATATTGCTCTC

58E

GAAAACGTCACACCACGCCACGTGACGTCACCGTCTTATTTGCGAACAGTCTG
CCTTTACCGTTATAAATAAATATTTACACGATTGTAAATCTCTAGACTAAGTCAA
TTAGCGGAAGGAAAACTCTAAACAAATTATGTCAGTCATTTA**TTAA**CCCTAGAA
AGATAGTCTGCGTAAAATTGACGCATGCATTCTTGAAATATTGCTCTC

Figure 4. (A) Transposable element display shows the single insertion of transgene in the three transgenic lines. PCR amplification using LIR specific primer gave a single amplicon in all the three transgenic lines suggesting the integration of one copy of the transgene. These products were sequenced to identify the chromosomal location of the integrated transgene. (B) Sequence information of the integrated site showing the flanking region of the left inverted repeat. The characteristic TTAA repeat is highlighted.

Table 2. Percentage of mortality after intrahemocoelic injection of *BmNPV*-P10GFP in fourth instar larvae. The intensity of fluorescence is indicated by '+' symbol

Lines tested	Number of larvae inoculated	Mortality (%)
NM	25	100.0 + 0.0 (++++)
126A	25	56.0 + 5.6 (++)
126B	25	60.0 + 5.6 (+++)
58E	25	90.0 + 2.8 (++++)

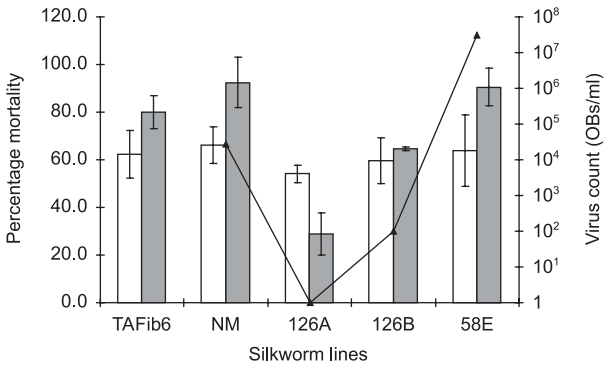


Figure 5. BmNPV resistance in four silkworm homozygous transgenic lines and in the control parental nontransgenic line *Nistari* (NM). TAFib6 control line contains a transgene for producing nontarget unrelated dsRNA. Third and fourth instar larvae were fed with *BmNPV-P10GFP* at a dose of 6000 and 12 000 OBs/larva, respectively. The white and grey bars indicate mortality in different silkworm lines infected at third and fourth larval instars, respectively. The black line indicates the number of OBs/ml of haemolymph present in the larvae that were infected at the fourth instar stage. Bar indicates standard deviation.

viral load of 1000 BV/larva was injected to fourth instar larvae, we confirmed that mortality was lower compared with controls (Table 2) and, consistently, the appearance of OBs was less pronounced with the corresponding reduction in severity of symptoms in transgenics, as indicated by the intensity of virus-derived fluorescence (Table 2). The relative lower effect of the transgene when the virus bypasses the intestinal barrier is accounted for by the intense activity of the *ie-1* promoter in the intestinal cells.

As a whole, these virus challenge results suggest that resistance to baculovirus could be conferred by the transgene, but the relative ability to inhibit the infection varies according to the transgenic line examined.

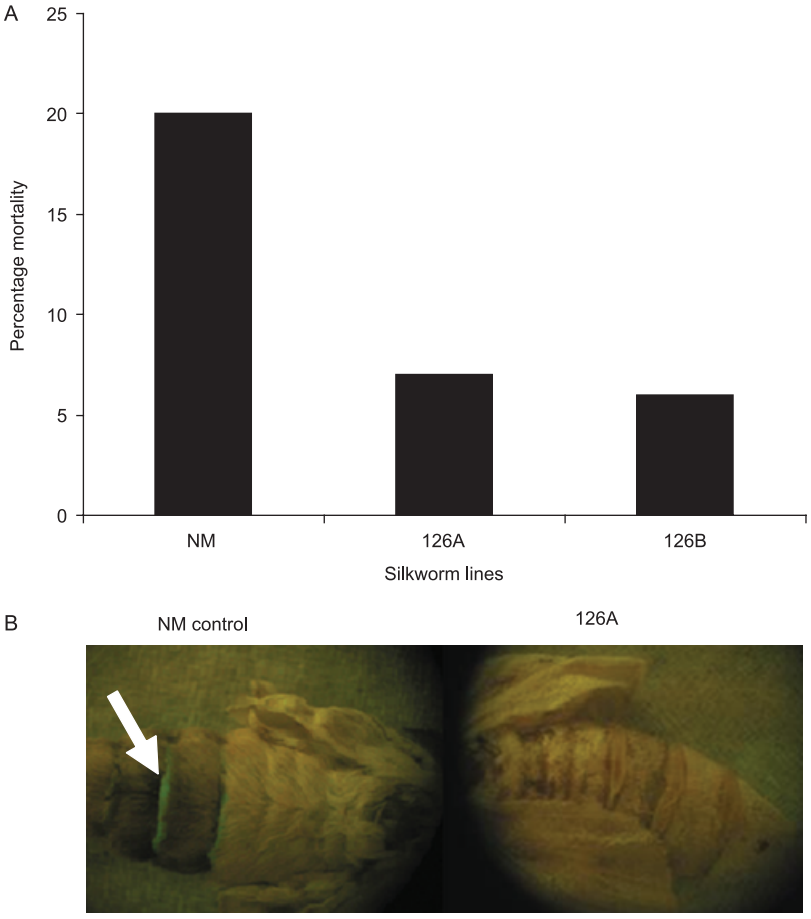


Figure 6. (A) BmNPV resistance in transgenic pupae injected with budded virus. Pupae from two transgenic lines and the control NM line were injected with free virus at a dose of 60 BV/pupa. The result indicates threefold reduced viral induced mortality in transgenic line 126A and 126B, confirming antiviral property conferred by the transgene. (B) Stereomicroscope micrograph of intact nontransgenic and a transgenic moths emerged from the pupae injected with a recombinant GFP expressing BmNPV, *BmNPV-P10GFP*. The white arrow points to GFP expressed from BmNPV, which is found predominantly in the nontransgenic NM control line compared with the transgenic 126A line.

Virus injection in transgenic pupae also leads to reduced mortality. The best performing line, 126A was further investigated to estimate the effect of the transgene in pupae injected with budded viruses. Fifteen pupae from 126A, 126B and the NM parental line were injected with a viral dose of either 60 BV/pupa or water (control). The percentage of survival of these virus-injected pupae was calculated based on the number of moths that emerged. All control water-injected pupae emerged without any mortality. The mortality rate in the transgenic line was three times lower than that of the parental strain (Fig. 6A). These results confirmed the capacity of the transgene to confer protection against baculovirus infection. When using the recombinant baculovirus, the amplification of which can be visualized by the production of GFP under the *P10* promoter, we could observe the proliferation of the virus in nontransgenic animals but not in moths emerged from pupae carrying the transgene (126A in Fig. 6B). When the viral dose was increased to 1000 BV/pupa we still observed a similar trend with transgenic line of 126A showing a relatively lower mortality (70% ± 12%) when compared with 100% mortality in NM line.

Western blot of infected pupa shows reduced GP64 levels. Total protein extracts were prepared from moths of the 126A, 126B and NM lines that emerged from pupae injected with *BmNPV-P10GFP* viruses. The results indicated that GP64 production was strongly reduced in transgenic silkworm lines as compared with the nontransgenic (Fig. 7). Upon quantitation of the GP64-specific band by QuantityOne software, we observed a very low level of GP64 in transgenic 126A (6% of the control NM) and 126B (42% of NM) lines, confirming the suppression of BmNPV infection in the transgenic lines.

Real-time PCR analyses. The relative abundance of *ie-1* transcripts was estimated by real-time PCR analyses performed on the total RNA extracted from the moths emerged from the transgenic and nontransgenic larvae infected at fourth instar stage. In transgenic lines 126A and 126B, the accumulation of the target gene, *ie-1* transcripts was lower by 60% and 40% respectively compared with the control line NM. In the line 58E the *ie-1* transcript level was similar to that of control line (Fig. 8).

RT–PCR analyses. Semiquantitative RT–PCR analyses were carried out on RNA extracted from pupae inoculated with 60 BV/pupa to measure the impact of the transgene on virus amplification. We assayed the virus *lef-3* mRNA along

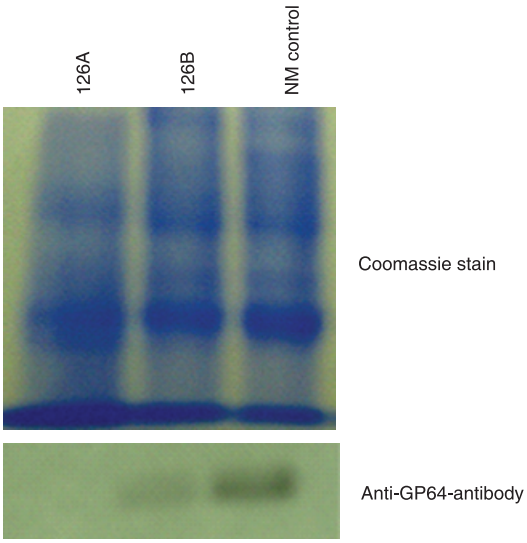


Figure 7. Western blot analyses of extracts from moths emerged from pupa injected with *BmNPV*-P10GFP. Total protein extract was analysed using anti-GP64 antibody. Total protein loading (Coomassie stain) was used as a loading control. Result confirms the low levels of viral coat protein production in transgenic lines indicating a reduction in viral titre.

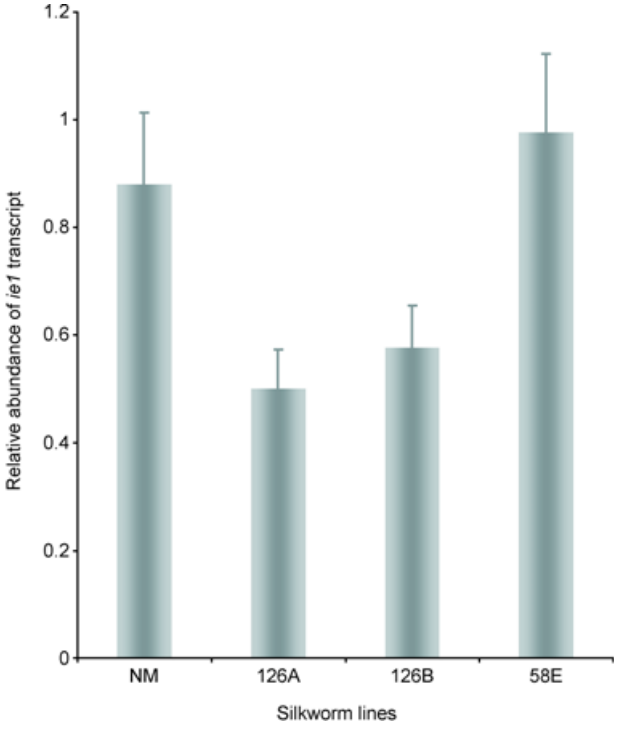


Figure 8. Real-time PCR analyses of total RNA from the moths eclosed from the BMNPV infected fourth instar larvae (12 000 OBs/larva) show the relative abundance of *ie-1* transcripts in the three transgenic lines, 126A, 126B and 58E and the nontransgenic control line, NM. The *ie-1* expression levels were normalized against the housekeeping cytoplasmic β-actin expression levels. The abrogation of viral proliferation was the most efficient in the line 126A followed by 126B. The line 58E was similar to the control line. The vertical lines are error bars.

with β-actin mRNA as control. The ratio of the *lef-3*-derived fragment to that of β-actin band indicates the decreased expression of *lef-3* in transgenic lines as compared with that of control (Fig. S1). The result once again confirmed the reduced viral transcription in transgenic lines.

Discussion

In this study we have developed a procedure to trigger RNAi-mediated baculoviral repression in the silkworm *B. mori*. For this purpose, we first validated the efficacy of baculovirus inhibition of infection in Sf9 cultured cells by targeting the baculoviral gene *ie-1*, one of the essential genes of the BmNPV. We developed a *piggyBac*-transformed Sf9 line carrying a transgene for *dsie*-1 production and GFP under *B. mori* cytoplasmic-actin (A3) promoter as selection marker. The transformed Sf9 line displayed an initial inhibition of viral proliferation as shown by plaque assay and microscopic observation, and also by a recombinant AcNPV expressing luciferase gene (data not shown). In this study, however, the observed suppression of baculoviral proliferation was followed by the recovery of virus replication leading to the lysis of the infected cells after 120 h.p.i. We interpret the limited viral blockage as a result of the polyclonal nature of the cell population with various degrees of resistance of individual cells likely conferred by the different chromosomal insertion sites of the transgene. To test the efficacy of BmNPV suppression by RNAi in the whole organism, we also constructed transgenic silkworms expressing *dsie-1*. Our *in vivo* results indicate that irrespective of the route of infection, at least two of the transgenic lines (126A and 126B) displayed a marked resistance to BmNPV infection when compared with nontransgenic/nontarget RNAi transgenic lines. We confirmed the complete degradation of the targeted *ie-1* gene transcript in an *in vitro* assay that consisted of incubating the *ie-1* RNA with lysates prepared from the transgenic line 126A and the control NM line (supplementary Fig. S2). We also observed that when the virus was injected into the haemolymph, the mortality was higher than through *per os* infection. This likely reflects the inhibition of dissemination of the virus in the intestinal cells where the *ie-1* promoter is very active. Western blot analyses, semiquantitative RT–PCR and real-time PCR assays and microscopic observations confirm that the virus proliferation was reduced in 126A and 126B transgenic lines, as compared with nontransgenics. We also observed a similar trend when the viral dose was low or when the infection was carried out at third instar stage though the effect was less pronounced as compared with the fourth instar stage. We did observe an increased mortality in transgenics (almost 90%) when the viral dose reached 20 000 OBs/larva or more at fifth instar. This corresponds to at least five times the dose to which silkworms are exposed in contaminated rearing facilities, suggesting that there is a limit beyond

which the RNAi machinery is not efficient enough to protect the organism. This may suggest inhibition in RNAi signal amplification system in the silkworm, while signal amplification is known to enhance the efficacy of RNAi in some organisms such as *Caenorhabditis elegans* and plants, but is not evident in the fruit fly and vertebrates (Novina & Sharp, 2004). We did not observe phenotypic loss of virus resistance over 14 generations, suggesting that the basal level of *dsie-1* production is not harmful to the silkworm. We hypothesize that differences in sensitivity of the three tested transgenic lines are accounted for by different expression levels of the transgene in relation to their distinct chromosomal insertion sites. The quantitative differences in *ie-1* RNA levels in the three transgenic lines (Fig. 8) and their correlation with the baculovirus resistance corroborate our contention.

Successful and transient suppression of AcNPV proliferation in *Spodoptera frugiperda* by RNAi-mediated targeting of IE-1 and GP64 was previously reported (Valdes *et al*., 2003). As they had directly injected the *in vitro* synthetized dsRNAs into the haemolymph the effect of dsRNA-mediated suppression was transitory and short lived. Isobe *et al*. (2004) targeted the gene *lef-1* and reported similar observations of breakdown of baculovirus infection in transgenic silkworms. In contrast to our study, they did not observe reduction of mortality in transgenic lines after infection. The difference may lie in the selection of the targeted gene, the strain of the silkworm used (it is generally known that the nondiapause strain *Nistari* that we used in the present study is sturdy in nature and hence could have contributed better resistance), or the selection of transgenic lines generated and tested for viral resistance.

In conclusion, we have shown here that the heritable RNAi-mediated baculoviral resistance is possible in the domesticated silkworm *B. mori*. Transgenic, virus-tolerant or resistant silkworm strains would be very useful in sericulture industry considering the high incidence of baculovirus-mediated mortality of larvae in rearing facilities, especially under subtropical regimes. As a further improvement, we are in the process of developing a multigene targeting strategy whereby different essential baculoviral genes are targeted simultaneously. Also, the use of better universal promoters, particularly ones activated by the baculovirus, need to be explored as drivers of the double-stranded target RNA.

Experimental procedures

Cell lines, virus stock and silkworm strain

Sf9 cells were maintained in Grace media (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 3.33 g/l yeast extract, 3.33 g/l lactalbumin hydrolysate, 10% FBS and 1 × antimycotic-antibiotic mix (Invitrogen). The cells were maintained at 26 ± 1 °C in a BOD incubator.

The recombinant BmNPV virus (*BmNPV-P10GFP*) was a generous gift of K.P. Gopinathan, Indian Institute of Science, Bangalore, India. The wild and recombinant baculoviruses were amplified in BmN cells and maintained as free viruses. *BmNPV-P10GFP* OBs were obtained by injection of BV to CSR2 silkworm strain and then OBs were harvested from haemolymph.

The nondiapausing Nistari strain of *B. mori*, moderately resistant to BmNPV infection with a LD_{50} value of ≈ 4000 OBs/larva, was used for germ-line transgenesis. Larvae were reared on mulberry leaves under standard conditions. We also used a transgenic *B. mori* line, TAFib6 that has a nonspecific, nonviral dsRNA expressing transgene.

pPIGA3GFP-FF *vector construction*

For generation of *pPIGA3GFP-FF* plasmid, sequential cloning strategy was adopted. The DNA of interest was PCR amplified using following primers and cloned into shuttle or intermediary vectors:

ie-1 promoter
ie-1 Prom F: GCCGGCCGATTTGCAGTTCGGGA
ie-1 Prom R: TTGTTCACGATCGTGTCCCGCC
ie-1 gene
ie-1 Gene F: CCAAACGACTATGACGCAAATT
ie-1 Gene R: TTGTTAAATTGGCCCACCAC
A3 cytoplasmic actin poly(A) signal
Poly(A) F: ATCGATAAACGAACGGAATGTT
Poly(A) R: GGCCGAGGCGGCCACAC

A typical PCR reaction consisted of 10 μl final volume with 5 pmol each of forward and reverse primers. The PCR amplification was performed in 10 mM Tris–HCl, (pH 8.3 containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 0.01% Triton X-100), 1 mM dNTPs and 0.5 U of IMMOLASE™ DNA Polymerase (Bioline, Luckenwalde, Germany) per reaction. Thermal cycling was carried out in a thermal cycler (PE9700, Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation of 3 min at 94 °C; 35 cycles of 30 s at 94 °C; 30 s at 52 °C (48 °C for poly(A) signal) and 2 min at 72 °C, and final extension of 10 min at 72 °C. The PCR products were quantified on 1.0% agarose gel, purified using Qiagen PCR purification columns, according to the manufacturer's protocol (Qiagen, Hilden, Germany). For *ie-1* promoter and gene fragment, *pBMNPVIEG*, a kind gift of R. Huybrechts (Katholieke Universiteit Leuven, Belgium), was used as the template, whereas for the polyadenylation signal, *pPIGA3-GFP* was used as a template. The vector *pPIGA3GFP-FF* was confirmed by restriction digestion and DNA sequencing. For DNA sequencing, 250 ng of plasmid was used in a sequencing reaction that contained 8 μl of Ready reaction mix (big dye terminator, BDTv 3.0, Applied Biosystems) and 5 pmol of M13 primers. The cycling conditions used were as follows: 25 cycles of 96 °C for 10 s, 50 °C 5 s, 60 °C 4 min. Samples were ethanol precipitated, washed with 70% ethanol and resuspended in Hi-Di™ formamide (Applied Biosystems). The sequencing was carried out in ABI Prism 3100 Genetic Analyzer (Applied Biosystems) and the final construct *pPIGA3GFP-FF* was obtained.

In the vector *p3XP3GFP-FF* for germ-line transgenesis, the A3-GFP cassette of *pPIGA3GFP-FF* was replaced by 3XP3-GFP cassette of Thomas *et al.* (2002).

The representation of the vector used is shown in Fig. 1.

In culturo *RNAi-mediated viral resistance*

Transformation of Sf9 cell line with pPIGA3GFP-FF. The exponentially growing Sf9 cells were transfected with the *pPIGA3GFP-FF* and helper plasmids (1 : 1 molar ratio) by using DAC-30 as the transfection reagent. Briefly, the two plasmids in the DAC-30

mixture were prepared in Grace media devoid of serum and antibiotics and layered on to cells at 50% confluence. After 4 h, the transfection medium was replaced by fresh medium containing 10% serum and antibiotics and GFP expression was monitored from the second day onwards.

After the proportion of GFP-positive cells in the population had stabilized, the cells were sorted using a FACSVantage (Becton Dickinson, San Jose, CA, U.S.A.). Briefly, 20 000 transgenic cells were sorted aseptically using GFP-specific FL1H filters. The sorted cells were harvested by centrifugation at 900 ***g***, then cultured in conditioned Grace's medium containing 15% serum and 1 × antibiotic mix (Invitrogen).

AcNPV resistance in transformed Sf9 cell line

Viral infection and plaque assay. One million exponentially growing Sf9 cells were seeded in each well of six-well plates. Sf9 cells transformed with *pPIGA3-GFP* were used as a control. The transformed cells were infected at 5 MOI (5×10^6 BV per million cells) of wild-type AcNPV. The media from the infected cell lines were collected at different time points (2–48 h.p.i.). For plaque assay, 0.5 ml of the supernatant was diluted in two different ranges of 10^{-1}–10^{-4} (for 2–21 h.p.i.) or 10^{-5}–10^{-8} (beyond 24 h.p.i.). Each diluted media was layered in triplicate on to a million Sf9 cells in six well plates. After 1 h, the media was removed and cells were layered with *Seeplaque* agarose (FMC) dissolved in Grace's medium. The plates were incubated at 26 ± 1 °C in a BOD incubator. The number of plaques was counted manually.

Microscopic observation of infection in a polyclonal population. Transformed cells were infected with wild-type AcNPV at a MOI of 5, in a chamber slide and left in contact for 3–4 days. After the specific duration of incubation, the chambers were removed and the attached cells were observed under fluorescence microscope (Nikon Eclipse E600).

Germline transgenesis of B. mori *using P3 × P3GFP-FF.* Germline transgenesis was carried out essentially according to (Tamura *et al*., 2000; Royer *et al*., 2005) on the Indian nondiapausing strain *Nistari*. The DNA vector and helper plasmids were injected into preblastoderm eggs at a concentration of 0.5 μg/μl for each plasmid. Then the eggs were incubated at 25 °C in humidified chambers for 10 days until hatching. The larvae were reared on an artificial diet (Nihon Nosan Kogyo KK, Japan) or on fresh mulberry leaves under standard conditions. G_0 adults originating from injected eggs were mated and the G_1 progeny was screened for eGFP-positive expression in ommatidia as described previously (Thomas *et al*., 2002). A provisional Indian patent (no. 1125/CHE/2006) has been filed for the transgenic RNAi silkworm lines.

Copy number and site of insertion. Transposable element display (TED) method was used for identifying the site of insertion and copy number of the insertion according to the modified protocol of van der Linden & Plasterk (2004). Briefly, genomic DNA from the transgenic lines 126A, 126B and 58E was extracted according to Nagaraja & Nagaraju (1995) and digested with *Sau*3A. Resultant digested DNA was ligated to the vector cassette that carried *Sau*3A over hangs (Oligo 503 and 504). Two rounds of PCRs were carried out using transposon-specific LIR outer (5′-CTCACGCG-GTCGTTATAGTT) and LIR inner (5′-GGCGACTGAGATGTCCTAAA) primers designed within *piggyBac* transgene near the left inverted repeat (LIR of Fig. 1) and adaptor specific 505 and 337NEW primers of van der Linden & Plasterk (2004). The first round of PCR was carried out using LIR outer and 505 primers and the second round of PCR was performed using LIR inner and 337NEW primers using MBI taq. The expected size of the amplicon after the second round of PCR was more than 120 bp that comprises of the transgene portion (120 bp) and the genomic DNA of unknown length. PCR product from the second amplification step was used for sequencing, as mentioned earlier.

Viral inoculation and mortality analyses in transgenic silkworms

Oral inoculation. We carried out *per os* BmNPV infection of the transgenic lines 126A, 126B, 58E and control nontransgenic (NM) silkworms with 12 000 OBs per larva at the IV instar, as this viral dose is more than the LD_{50} for the *Nistari* strain ($LD_{50} \approx 4000$ OBs/larva at third instar). TAFib6, a transgenic line that expresses nontarget dsRNA was also used as a control. For third instar larvae, a viral dose of 6000 OBs per larva was used. The OBs were spread on to fresh leaf pieces of 1 cm diameter. One hundred larvae of each line were fed with OBs and only those larvae that consumed all the leaf pieces were retained and reared on fresh mulberry leaves till the end of larval life. The haemolymph of five *BmNPV-P10GFP*-infected larvae (IV instar) were pooled and the number of OBs were counted, expressed as OBs/ml of haemolymph.

Intra-hemocoel injection of BmNPV-P10GFP *to IV instar larvae.* For intra-hemocoelic injections, free budded viruses were prepared from cell culture media. Larvae at day 1 of IV instar were injected with 1000 BV in 25 μl, and mortality was recorded until moth stage.

Pupal injection of virus. Pupae of transgenic lines and control nontransgenic line were harvested on the fourth day of cocoon formation and the following day *BmNPV-P10GFP* were injected at a viral dose of 60 BV in a volume of 25 μl per pupa. Mortality was recorded until eclosion of moths. The injections were also carried out using a viral dose of 1000 BV/pupa in 20 pupae each in duplicates.

Western blot by using antibodies against GP64. The moths emerged from the infected pupae of two transgenic lines (126A and 126 B) and control NM were ground in lysis buffer (50 mM Tris–Cl, pH 8.0, 120 mM NaCl, 0.5% (v/v) NP-40, 0.2 mM sodium orthovanadate, 100 mM NaF) containing protease inhibitor cocktail (Sigma, St. Louis, MO, U.S.A.). Western blot was carried out using anti-GP64 antibody. Antibody against GP64 coat protein of baculovirus (Novagen, Darmstadt, Germany) was used for the detection of *BmNPV-P10GFP* GP64 protein. Equal quantities of proteins were denatured in SDS-loading dye, separated by 10% SDS–PAGE and blotted on to Hybond P+ membrane according to Sambrook & Russell (2001). Western blot was revealed with mouse anti-GP64 antibody and the primary antibody was detected using appropriate secondary antibody conjugated to horseradish peroxidase and the protein bands were visualized using ECL detection kit (Amersham Biosciences, Freiburg, Germany). The bands were quantified densitometrically by using QuantityOne software of the BioRad gel documentation system (BioRad Laboratories, Hercules, CA, U.S.A.).

Real-time PCR of ie-1 *transcripts.* Single-strand cDNA was synthesized from total RNA (125 ng) obtained from the moths eclosed from the larvae fed with 12 000 OBs of BmNPV at fourth instar stage using MMLV reverse transcriptase (Amersham Biosciences) and oligo dT sequences. In order to avoid genomic DNA contamination, the RNA samples were treated with RNase-free DNase I (1 U/μl). To estimate the relative abundance of *ie-1* transcripts in

the BmNPV-challenged silkworms, real-time PCR was performed using Eppendorf S gradient MasterCycler Detection System with SYBR Green. RT–PCR for *ie-1* (100 bp) was carried out using AAGTGAACCGAAGAGAGAGCAC forward and AAACGTTATAGCGTCGGACAAC reverse primers. Cytoplasmic A3-β-actin, a housekeeping transcript (200 bp) was used as a loading control for the normalization of *ie-1* transcripts. All the samples were run in triplicate in a 20 μl reaction volume containing 2 × SYBR green PCR master mix (Applied Biosystems), primers at a final concentration of 0.25 μM each, water to a 19 μl final volume, and 1 μl of the first strand cDNA solution. PCR was carried out according the thermal profile: 50 °C for 2 min, 95 °C for 15 min and 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 1 min.

The *ie-1* transcript levels were measured with SYBR green fluorescence using the standard curve method. The data were calculated on the comparative CT method where we observed the slope value to be –3.317520 and R^2 to be 0.984572. The standard curve was generated for cytoplasmic A3-β-actin control and *ie-1* was normalized for NM infected (nontransgenic control) and transgenic lines (126A, 126B and 58E).

RT–PCR analyses. Total RNA was extracted from the pupae injected with the *BmNPV-P10GFP* virus (5 days postinfection) by using Trizol® reagent (Invitrogen) according to standard protocol. RT–PCR was carried out from 1 μg of total RNA according to Sambrook & Russell (2001). First strand was synthesized using the superscript II enzyme (Invitrogen). Subsequently PCR was carried out using baculoviral *lef-3*-specific primers (F: 5′-GCGAATTCGGTTGTTTTTGAAGTCGCGTACA-3′ and R: 5′-GGGGATCCCCGGTCTTGCGTTTGTGCAATTTT-3′) and control cytoplasmic A3-β-actin-specific primers (F: 5′-CACTGAGGCTCCCCTGAAC-3′ and R: 5′-GGAGTGCGTATCCCTCGTA-3′). The densitometric ratio of *lef-3* band to that of β-actin was used for measuring viral proliferation and plotted as a graph.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1. Semiquantitative RT–PCR analyses of total RNA from pupa injected with *BmNPV-P10GFP* (60 BV/pupa). The *y*-axis indicates the ratio of the intensity of the viral-encoded *lef-3*-related fragment to that of the cellular β-actin fragment. The corresponding gel image is shown below the graph. The result shows decreased transcription of the *lef-3* transcript in transgenic lines as compared with the control line.

Figure S2. Details of *in vitro* RNAi using lysate extracted from transgenic and nontransgenic lines. Results indicate faster degradation of *in vitro* transcribed *ie-1* mRNA by lysate derived from the transgenic line than from the nontransgenic line.

This material is *available* as part of the online article from http://www.blackwellsynergy.com.

