Engineering Silkworms for Resistance to Baculovirus Through Multigene RNA Interference

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ABSTRACT *Bombyx mori* nucleopolyhedrovirus (BmNPV) that infects the silkworm, *B. mori*, accounts for >50% of silk cocoon crop losses globally. We speculated that simultaneous targeting of several BmNPV essential genes in transgenic silkworm would elicit a stable defense against the virus. We introduced into the silkworm germline the vectors carrying short sequences of four essential BmNPV genes in tandem, either in sense or antisense or in inverted-repeat arrangement. The transgenic silkworms carrying the inverted repeat-containing transgene showed stable protection against high doses of baculovirus infection. Further, the antiviral trait was incorporated to a commercially productive silkworm strain highly susceptible to BmNPV. This led to combining the high-yielding cocoon and silk traits of the parental commercial strain and a very high level of refractoriness (>75% survival rate as compared to <15% in nontransgenic lines) to baculovirus infection conferred by the transgene. We also observed impaired infectivity of the occlusion bodies derived from the transgenic lines as compared to the wild-type ones. Currently, large-scale exploitation of these transgenic lines is underway to bring about economic transformation of sericulture.

RNA interference (RNAi) is a mechanism by which cells silence the expression of foreign genes. This process often provides an adaptive innate immunity against viruses where double-stranded RNAs encoded by the viruses during infection act as pathogen trigger after they are taken up by the cellular RNAi machinery (Wang *et al.* 2006). Alternatively, this natural defense mechanism is exploited as an antiviral therapy via the artificial inhibition of the expression of essential viral genes (Leonard and Schaffer 2006). Multiple protocols of delivery of dsRNA or of constructs encoding dsRNAs in the organism are currently under assay to combat infections of various viruses. The efficiency of the assays is still challenged by the delicate setting of the proper dosage of the RNAi, the relative longevity of the effect, the occurrence of RNAi driven toxicity, and the virus-intrinsic susceptibility. A few attempts have been made in animal and plant models where the antiviral trait was installed by transgenesis to confer stable protection to the transformed individuals and to their progeny. Successes have been reached in plants (Bucher *et al.* 2006; Bonfim *et al.* 2007; Zhang 2010) but, to our knowledge, no case has yet been reported in animals showing a stable and robust protection against a virus after a RNAi-aided antiviral trait was introduced through germline transformation.

The baculovirus, *Bombyx mori* nucleopolyhedrovirus (BmNPV), is a major pathogen that affects silkworm rearings and hampers silk cocoon production in Asia. In India alone >50% of silk cocoon crop losses are attributed to baculovirus infection (Khurad *et al.* 2006). Effective treatment against the virus has been elusive due to its sturdy nature and the lack of control strategies. Interestingly, the

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biology of the virus is reasonably well studied, and its entire genome sequence and gene annotation are unraveled. Baculovirus infectious cycle is characterized by the occurrence of two forms of the virus: the so-called budded viruses (BV) that spread systemic infection within the host and the occlusion-derived viruses (ODV) that are encapsulated within polyhedral inclusion bodies (PIBs/OBs) and spread the baculovirus from host to host through *per os* infection.

During proliferation *in culturo*, baculoviruses express genes in a cascade form with early classes of proteins accumulating immediately upon infection, while late and very late classes of proteins appear subsequently (Huh and Weaver 1990a,b; Hoopes and Rohrmann 1991). Transient plasmid-based analyses have led to recognition of several essential genes. Among these, one codes for IE-1, a transregulator that activates a number of late expression factors (encoded by *lef* genes), another encodes LEF-1, a DNA primase of the replication complex (Mikhailov and Rohrmann 2002), and a third codes for LEF-3, which carries single-stranded DNA-binding capabilities (Evans *et al.* 1997). All these three genes are critical for the accomplishment of viral DNA replication. Another gene codes for the *per os* infectivity factor, P74, a conserved ODV structural protein, among baculoviridae, which, though not required for the making of budded viruses, is necessary for oral infectivity and the contamination from individual to individual (Kuzio *et al.* 1989; Peng *et al.* 2012).

The availability of a well-established method of transgenesis in *Bombyx mori* (Royer *et al.* 2005; Tomita 2011) has already led to the construction of transgenic silkworm lines harboring a dsRNA-encoding transgene targeting the essential viral gene *lef*-1 (Isobe *et al.* 2004) or *ie*-1 (Kanginakudru *et al.* 2007). Both attempts resulted in moderate but short-lasting effects, which were also reproduced in cultured BmN cells. It is thought that incomplete gene silencing may be the cause of the rapid recovery of viral proliferation.

Targeting simultaneously several viral genes from a single transgene construct is possible since only a few hundred-basepair long double-strand RNA sequence is sufficient to elicit RNA interference in the silkworm (Gandhe *et al.* 2007; Mrinal and Nagaraju 2010; Shukla and Nagaraju 2010; Terenius *et al.* 2011). With the aim of installing a strong antiviral trait, we assayed the properties of a transgene carrying four tandem sequences arising from four essential genes of the baculovirus (*ie1*, *lef1*, *lef3*, and *p74)*. The transgene was equipped with silkworm *cytoplasmic actin A3* gene promoter, to deliver dsRNA in all tissues and cells. The dsRNA-encoding transgene was shown to confer a high level of baculovirus resistance in transgenic silkworm. Further, the incorporation of the antiviral trait to a commercial productive silkworm strain highly susceptible to baculovirus led to combining the sericultural traits of interest and the transgene-based baculovirus resistance. The following presentation shows that this experimental strategy proved very efficacious to the point that it can elicit stable refractoriness over generations of silkworms to the infection of the virus. Further, our study also demonstrates that the baculovirus-infected transgenic silkworms produce OBs that are far less infective compared to wild-type OBs and, hence, are likely to be less potent in horizontal transmission.

Materials and Methods

Silkworm strains and virus stock

The *B. mori* strain, Nistari, a nondiapausing, polyvoltine, low-silk-yielding and moderately resistant to baculoviral infection (LD_{50} ~4000 OBs/larva), was used in the study. A diapausing, high-yielding silkworm strain, CSR2, highly susceptible to baculoviral infection (LD_{50} ~250 OBs/larva) was used as a recurrent parent for the introduction of the transgene from the transgenic Nistari strain. TAFib6, a transgenic Nistari line that expresses nonviral dsRNA, and nontransgenic Nistari and CSR2 strains were maintained as controls. The transgenic lines targeting the BmNPV *ie1* gene were used and are referred to as IE 126A, IE 126B, and IE 58E, as already described (Kanginakudru *et al.* 2007).

The baculovirus used for infection is the wild-type BmNPV that is usually prevalent in farmers' silkworm rearing house. The OBs were harvested from the infected hemolymph by brief centrifugation at 1000 rpm for 1 min and the pellet was washed in water and suspended in phosphate buffer saline (PBS). The silkworm larvae that were out of third molt were starved for 12 hr before infection. The OBs in 50 μl suspension were spread on to a 1-cm-diameter piece of mulberry leaf at 6000 OBs/leaf piece and fed to each larva. A total of 50 larvae were fed and only those larvae that consumed the entire leaf piece were maintained. The virus-fed larvae were subsequently reared on fresh mulberry leaves and observed for symptoms of viral infection. The dead larvae were removed immediately to prevent spreading of the secondary viral infection. Our initial *per os* infection experiments showed that 6000 OBs/larva ingested at third instar resulted in ~100% mortality of CSR2 strain and >50% mortality of Nistari strain (data not shown). We thus used 6000 OBs/larva to infect third instar larva.

Construction of sense, antisense, and inverted repeat vectors

The sense, antisense and inverted-repeat vectors were constructed using a *piggyBac* vector backbone. The constructs were made in such a way that dsRNA is generated for the four essential viral genes *ie1*, *lef1*, *lef3*, and *p74* in the host cells in sense, antisense orientation, or inverted-repeat arrangement under the control of the silkworm *cytoplasmic actin A3* gene promoter. The coding sequence of green fluorescent protein (*gfp*) was also introduced along with the stretches of viral gene sequences, in the same respective orientation. The constructs *pPiggyMG(+)3XP3–GFP* (sense), *pPiggyMG(−)3XP3–DsRed2* (antisense), and *pPiggyMG (+/−)3XP3–DsRed2* (inverted repeat) are represented in Figure 1. The targeted viral gene nucleotide sequence information is provided in Supporting Information, File S2.

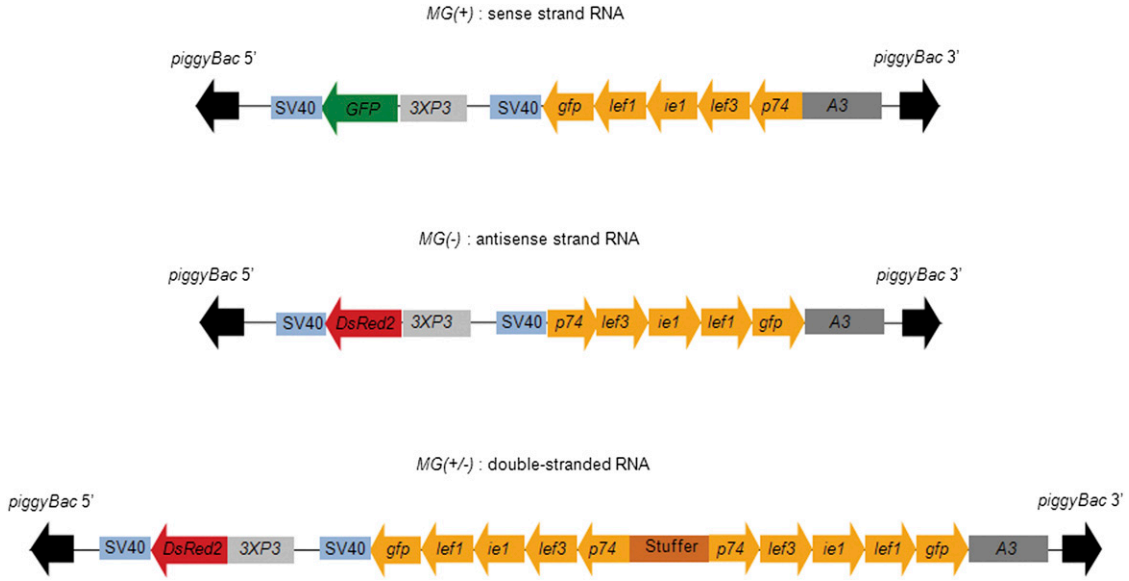

Figure 1 Representation of *piggybac*-inserted sequences in the various silkworm transgenic lines. The transgenes were carried in the vector constructs *pPiggyMG(+)3XP3–GFP* (9677 bp), *pPiggyMG(−)3XP3–DsRed2* (10097 bp), and *pPiggyMG(+/−)3XP3–DsRed2* (12422 bp). *PiggyBac* 5′ and *piggyBac* 3′ indicate the left and right terminal inverted repeats of the *piggyBac* element that flank the inserts. Each transgene comprised a segment of four BmNPV genes, namely *ie1* (310 bp), *lef1* (326 bp), *lef3* (316 bp), and *p74* (310 bp) and the entire coding sequence of green fluorescent protein (800 bp) in the same relative orientation. SV40 represents a 200-bp fragment carrying the SV40 polyadenylation site. The MultiGene(+) transgene, abbreviated MG(+), consisted of segments of the four targeted viral genes and *gfp* gene, transcribed as a sense-strand RNA under the control of the silkworm *cytoplasmic actin A3* promoter. The marker gene, *gfp*, under the control of the *3XP3* promoter is expressed in ommatidia, and embryonic nervous tissues facilitates identification of the transgenic individuals. The MG(−) transgene consists of segments of four viral target genes and *gfp* gene transcribed as an antisense strand RNA with the same *A3* gene promoter. The marker gene encoded red fluorescent protein (*DsRed*). The *gfp* gene in the MG(+) and the MG(−) transgenes helped to verify the RNAi-aided extinction of GFP accumulation in transgenic hybrid lines that carried both constructs. MG(+/−) transgene harbored a portion of each of the essential baculoviral genes in sense and antisense orientation separated by a 323-bp-long stuffer sequence and encoded a double-strand RNA of the baculovirus partial gene sequences.

The gene encoding GFP driven by a *3XP3* promoter in the MG(+) vector was used as an eye-color selection marker. Similarly, a DsRed coding sequence downstream of the *3XP3* promoter in the antisense and inverted repeat vectors enabled identification of transgenic individuals by their DsRed eye (Uchino 2006). The various steps involved in the construction of the vectors are schematically represented in Figure S1 and detailed in File S1.

Germline transformation of B. mori

Germline transgenesis was carried out essentially as described previously (Tamura *et al.* 2000; Royer *et al.* 2005) using Nistari strain. Freshly laid silkworm eggs were microinjected with 1 μg/μl of any of the three *piggyBac*-derived constructs and the helper plasmid *pHA3PIG* in equimolar ratio. Eggs were incubated at 25° in humidified chambers for 10 days until hatching. The transgenic lines obtained by using *pPiggy*MG(+)*3XP3GFP*, *pPiggy*MG(−)*3XP3–DsRed2*, and *pPiggy*MG(+/−)*3XP3–DsRed2* are hereafter referred to as MG(+) (sense), MG(−) (antisense), and MG(+/−) (inverted repeat), respectively.

Generation of G_0 and G_1 broods

The G_0 larvae from the injected eggs were reared on fresh mulberry leaves under standard rearing conditions. G_0 adults originating from injected eggs were mated among themselves and the G_1 progeny was screened under a fluorescence stereomicroscope (Leica MZFLIII) for the expression in ommatidia of either GFP or DsRed as described previously (Thomas *et al.* 2002). Data on the number of eggs injected and the final transgenics obtained with the three constructs were collected. G_1 adults were sib-mated to obtain G_2 offspring.

Generation of homozygous lines and F_1 hybrids

Homozygous silkworm lines carrying a single insertion were generated for each transgenic group by sib-mating for three generations. Based on the intensities of the selection markers, lines from each group were selected for making F_1 hybrids or for testing their antiviral property. Crosses were also made between MG(+) and MG(−) transgenic lines, which also express GFP in sense and antisense orientation, to bring together sense- and antisense-producing loci in the hybrid offspring.

Viral inoculation and mortality analyses in transgenic silkworms and their hybrids

For *per os* viral infection, OBs were spread onto fresh leaf pieces as described above. One hundred day 1 third-instar larvae of each of the Nistari transgenic lines, MG(+), MG(−), MG(+/−) and MG(+) × MG(−), along with control lines, were fed with OBs and only the larvae that consumed

all the leaf pieces were retained and reared on fresh mulberry leaves until the end of larval life. BmNPV infection was triggered *per os* using 6000 OBs/larva. Mortality of the silkworm larvae that ingested OBs was estimated by counting the number of dead ones until the time of eclosion of moths from the infected batches. Larvae of single viral gene target transgenic lines (IE 126A, 126B, 58E), nonviral target transgenic line, TAFib6, and nontransgenic strains, Nistari and CSR2, as well as their hybrids (Nistari × CSR2 and TAFib6 × CSR2), were used as controls.

Viral titer determination and viral DNA quantification in the infected transgenic and the nontransgenic lines

Transgenic lines as well as the control lines were infected with BmNPV as mentioned above. For each line, 100 larva of day 1 of third instar were infected individually with 6000 OBs/larva. The larvae that completely consumed the OBs were further reared until eclosion of the moths. Hemolymph was collected from each larva and the number of OBs were counted using Neubauer hemocytometer. The baculovirus accumulation was measured at day 4 of the fifth-instar larvae as the concentration of the OBs in the hemolymph of the infected larvae.

The virus accumulation level was also measured by viral DNA quantification. Briefly, the viral DNA was isolated from the hemolymph collected from five larvae each of the BmNPV-infected transgenic lines as well as their nontransgenic lines using DNeasy kit (Qiagen). The 25 μl qPCR reaction mixture included 20 ng DNA, 12.5 μl of 2× SYBER Green Master Mix (Applied Biosystems), 5 μM of viral *lef3* gene primers, and nuclease-free water to make up the total volume. The PCR was performed on a RT-7500 system (Applied Biosystems) with the initial denaturation for 2 min at 50° and 10 min at 95° followed by 40 cycles at 95° for 15 sec and 60° for 1 min. Each of the reactions was performed three times independently, in duplicate, and the results were normalized against 18S rRNA gene of *B. mori*. The *lef3* primers used for amplification of viral DNA do not overlap with the hairpin of *lef3* in the transgenic vector. The viral primer sequences used are provided in Table S1. The qPCR data were analyzed by using RT-7500 system software and plotted by using standard delta delta-Ct (ddCt) method.

Western blot analysis by using antibodies against GP64

Total pupal protein was isolated from IE, MG(+/−), MG(+), and MG(−) transgenic and control (Nistari, CSR2, TAFib6) lines infected with 6000 OBs/larva and western blotting was carried out as described previously (Kanginakudru *et al.* 2007). We incubated the membrane with primary antibody GP64 and secondary antibody conjugated to horseradish peroxidase. The primary antibody against GP64 coat protein of baculovirus was obtained from Novagen (Millipore, India). This antibody was found to cross-react with the GP64 of BmNPV. After addition of HRP substrate, the chemiluminescent signal was detected with X-ray film. The same membrane was stripped and reused for detection of α-tubulin as a loading control. The intensity of bands was quantified using QuantJ software. Each band density was first normalized by dividing it by the density of the α-tubulin band in the same lane and expressed as fold change.

Chromosomal localization of transgene in the transgenic lines

The site of insertion of the transgenes in the MG(+/−) lines was determined using transposable element display (TED) method following the modified protocol of van der Linden and Plasterk (2004). This method was used to target the left arm of the *piggyBac* transposon. The expected size of the amplicon after the second-round PCR was >120 bp, which comprises the transgene portion and the genomic DNA of unknown length. The PCR product from the second amplification step was used for sequencing. Formation of a single PCR amplification product after the second PCR reaction showed the presence of single-copy insertion of the transgene in the MG(+/−) lines (Figure S2).

We examined the genomic sequence at the insertion sites to verify whether the insertion of the transgenes was *piggyBac* transposon mediated. For all insertions, the *piggyBac* inverted terminal repeats were recovered and found to be bordered by the characteristic *piggyBac* target TTAA sequence (File S3). The location of insertion was further confirmed by the PCR product sequences, which were blasted against the complete genome sequence of *B. mori*, and the relative position of the transgene on the chromosome was determined for each of the transgenic lines (Figure S3 and Table S3).

Introduction of pPiggyMG(+/−)3XP3-DsRed2 to the high-yielding commercial strain, CSR2 through recurrent backcross strategy

The transgene from the Nistari genetic background was transferred to baculovirus-susceptible and high-yielding commercial strain, CSR2, by recurrent backcrosses followed by inbreeding (Figure 5). Males from three MG(+/−) Nistari transgenic lines were crossed to CSR2 nontransgenic line to raise F_1 hybrids. For each of the crosses, two replications were made (a replication refers to the whole silkworm population derived from eggs of a mated female moth). The F_1 offspring (males) were backcrossed with the recurrent parent, CSR2, female to generate BC_1 population. The performance of BC_1 generation was recorded under normal and BmNPV inoculated conditions. The backcross offspring that carried the transgene as indicated by DsRed eye color were selected as donors of the antiviral trait, for further breeding. BC_1 generation was carried forward to BC_4 on the basis of survival rate upon BmNPV infection as well as on the basis of DsRed eye and other cocoon characteristics of the recurrent parent. BC_4 males and females carrying the transgene were sib-mated and advanced to BC_4F_{13}.

Marker-assisted selection

DNA isolation from MG(+/−) transgenic moths was carried out as mentioned above. A total of ten simple sequence

repeats (SSRs) that were polymorphic between donor (Nistari) and recurrent (CSR2) parents were employed to screen the backcross offspring at each generation. Only those offspring that carried CSR2-specific alleles and DsRed eye were selected as parents of the next generation. DNA samples from BC_4F_6 were analyzed by PCR using primers specific for the 10 SSR markers and the number of CSR2-specific alleles across all the SSR loci was scored to estimate the extent of CSR2 genome in the offspring. The PCR conditions for each of the 10 SSR loci along with the flanking primer sequences are given in Table S6.

Cocoon and silk characters

The transgenic, backcross, and control lines were studied for the silk yield characters: (1) cocoon weight (in grams), (2) cocoon shell weight (in grams), and (3) cocoon shell ratio (the ratio of cocoon shell weight to the cocoon weight expressed in percentage). The silk technological characters, namely, silk filament length (meters) and raw silk (percentage), were obtained from the silk-reeling properties of cocoons measured using standard procedures.

Comparison of the infectivity of OBs generated in transgenic and nontransgenic lines

We chose the transgenic CSR2 (164C) line and the nontransgenic CSR2 and infected with the OBs derived from wild-type BmNPV. The OBs were then harvested separately from the infected transgenic and nontransgenic larvae at fifth instar before pupation of the larvae. The two types of OBs were administered *per os* separately to transgenic as well as nontransgenic CSR2 larvae. Eight doses of 1000, 2000, 4000, 6000, 8000, 10,000, 20,000, and 40,000 were made from a stock of 4×10^6 OBs/ml for infection studies. A 1-cm^2 piece of mulberry leaf was smeared with the suspension of OBs of specified dose at 10 μl per piece and infected *per os* to each larva in a paper boat on day 1 of the fourth instar. A total of 30 larvae were used for each dosage. The larval mortality was recorded from day 1 of postinfection until pupation. The percentage mortality values were converted to probit values by reading the corresponding probit units from the probit table. The probit values were plotted against log doses, and the LD_{50} values, standard error, and fiducial limits were calculated with the help of GraphPad Prism 6.

Statistical analyses

The transgenic lines were reared with appropriate controls from the year 2007 to 2011. The data presented is an average of two replications with 50–500 larvae per replicate. To improve homogeneity of variances, the data on the percentage mortality and viral proliferation count were subjected to arcsin or logarithmic transformations, respectively. A two-way analysis of variance (ANOVA) was used to estimate variation sources as well as their statistical significance between control and BmNPV inoculated groups using StatPlus 2007 Professional v. 4.9. The pairwise comparisons for various parameters among groups was performed after post-hoc analysis using Tukey's multiple comparison test of the corresponding treatment factor using main effects in a one-way ANOVA. The significance of each group was calculated as a *P*-value. The difference with $P < 0.05$ was considered significant.

Results

Establishment of transgenic silkworm lines with potent antiviral traits

We introduced transgenes that carried concatenated sequences from the four baculovirus genes *ie1*, *lef1*, *lef2*, and *p74*. MG(+) and MG(−) refer to transgenes expressing the sense and the antisense strands, while MG(+/−) encodes double-strand RNA of the viral gene sequences. Germline transgenesis was carried out as described earlier (Tamura *et al.* 2000; Royer *et al.* 2005) by co-injecting a *piggyBac* vector and helper plasmid to freshly laid eggs of the Nistari strain (Table S2). Based on the higher intensity of the eye color three MG(+) (2G, 58L, and 244E), two MG(−) (126C and 239A), and eight MG(+/−) (118A, 118B, 154C, 154D, 164B, 164C, 170A, and 170B) transgenic lines were retained. Accurate chromosomal insertion sites were mapped for the MG(+/−) lines (see *Materials and Methods*).

Crosses were also made between the three MG(+) and the two MG(−) transgenic lines to drive the expression of the sense and antisense strands in the same cells. A total of 18 homozygous, single insertion lines were constructed by sib-mating for three successive generations. Together with the four viral gene sequences, the transgenes carried an entire GFP coding sequence in the same orientation (Figure 1). This drove the MG(+) lines to express an intense GFP fluorescence in the intestine cells driven by the *cytoplasmic actin A3* gene promoter. This fluorescence was abolished in the MG(+) × MG(−) hybrids, reflecting the effectiveness of the RNAi process in the transgenic silkworm lines.

BmNPV challenges show reduced mortality in transgenics

We analyzed the baculovirus infectivity by monitoring the mortality of the infected larvae. Figure 2 shows the virus effects on the mortality according to the status, transgenic or not, of the tested lines. A two-way ANOVA revealed highly significant differences between control and BmNPV treatment ($P < 0.0001$) as well as between the different groups of transgenic and nontransgenic lines ($P < 0.0001$) (Table 1). Significant treatment × lines interaction demonstrated that the lines responded differently under BmNPV-inoculated conditions. We observed the highest mortality in control lines, Nistari (75%) and CSR2 (95%), while the MG (+/−) lines, on an average, showed only ∼20% mortality upon viral inoculation. The pairwise comparisons revealed that the mortality rate after viral infection in MG(+/−) lines was significantly less than IE ($P < 0.0001$), MG(+) ($P < 0.0001$), and MG(−) ($P < 0.0008$) lines (Table S4). We

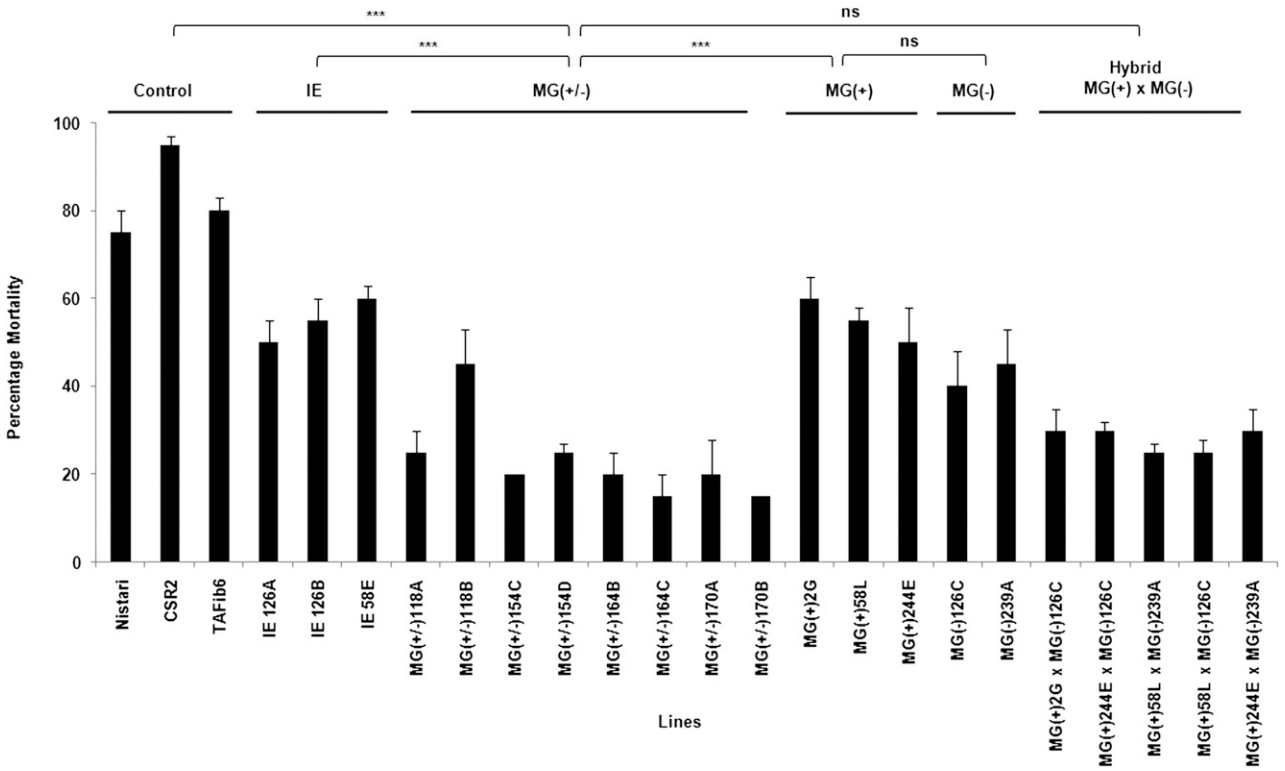

Figure 2 Transgenics show reduced mortality upon BmNPV infection. The histogram shows percentage mortality in the transgenic Nistari, nontransgenic, and control lines. TAFib6, transgenic line that expresses nontarget dsRNA; IE, transgenic line with single viral gene target; MG(+), transgenic line with segments of four viral target genes transcribed in sense orientation; MG(−), transgenic line with segments of four viral genes transcribed in antisense orientation; MG (+/−), transgenic line with four essential baculoviral target genes expressing as a stem-loop structure to induce RNAi; and MG (+) × MG(-), transgenic lines obtained by crossing MG(+) and MG(−) lines. Third-instar larvae were infected *per os* at a dose of 6000 OBs/larva. Mortality was scored as number of individuals succumbed to viral infection by counting the live moths eclosed from the infected larvae. Mortality was reduced significantly in lines having the multiviral genes. Bar indicates SD, $N = 48$. (***) $P < 0.001$; ns, not significant.

found no difference in the survivability upon viral infection between MG(+/−) and MG(+) × MG(-) lines ($P < 0.0848$). In comparison to transgenic lines targeting single viral gene previously generated by us (Kanginakudru *et al.* 2007), all the multigene transgenics recorded significantly ($P < 0.0001$) higher survivability against viral infection indicating potential benefit of multigene targeting.

Viral accumulation is reduced in multigene transgenic lines

The baculovirus accumulation was compared among the transgenic lines. Results of one-way ANOVA showed significant differences ($P < 0.0001$) in viral accumulation among BmNPV-inoculated transgenic lines (Table 2). Of all the transgenics, MG(+/−) lines showed lowest viral accumulation and differed significantly from IE ($P < 0.0001$), MG(+) ($P < 0.0001$) and MG(−) ($P < 0.0029$) lines (Figure 3A and Table S5). The viral accumulation in MG(+/−) lines was comparable to MG(+) × MG(−) ($P < 0.6732$) lines.

The viral titer was determined by quantifying the viral DNA levels using the *lef3* gene of BmNPV. The viral load, as determined by qPCR, showed significant ($P < 0.001$) reduction in the Nistari transgenic lines (118A, 170B, and 164C) as compared to Nistari nontransgenic lines. Overall, there was >2.5-fold decrease in viral load in the Nistari transgenics as compared to the nontransgenic line (Figure 3B).

Table 1 A two-way ANOVA for the percentage mortality of the transgenic and control lines under normal and BmNPV infected conditions

Source of variation	Sum of squares	d.f.	Mean squares	*F* ratio	*P*
Lines	4738.72	5	947.74	41.25***	0.0001
Treatments	14120.53	1	14120.53	614.67***	0.0001
Lines × treatments	3285.97	5	657.19	28.60***	0.0001
Within	1929.68	84	22.97		
Total	24074.92	95			

A two-way analysis of variance for the percentage mortality of the IE, MG(+), MG(−), MG(±), MG(+) × MG(−) and control lines under normal and BmNPV infected conditions. Degrees of freedom (d.f.) for each source of variation, treatment, lines, and interactions between these is shown with sum of squares. The average numbers of three experiments, $N = 96$. ***$P < 0.001$.

Table 2 A one-way ANOVA for the rate of viral proliferation of the transgenic and control lines under BmNPV-infected conditions

Source of variation	Sum of squares	d.f.	Mean squares	*F* ratio	*P*
Between lines	5.61	5	1.12	43.81***	0.0001
Within lines	1.08	42	0.03		

A one-way analysis of variance for the rate of viral proliferation of the IE, MG(+), MG(−), MG(±), MG(+) × MG(−) and control lines under BmNPV-infected conditions. Degrees of freedom (d.f.) and sum of squares for each source of variation is shown. $N = 48$. ***$P < 0.001$.

Reduction in viral proliferation was further confirmed by Western blot experiments using antibody against GP64, an envelope fusion protein of budded viruses required for cell-to-cell transmission of infection (Monsma *et al.* 1996). Western blot experiments demonstrated that the accumulation of GP64 was lowest in the MG(+/−) lines as compared to all other transgenic or nontransgenic lines (Figure 3C). Taken together, our results confirm that the viral accumulation is significantly reduced in the transgenic lines in comparison to control lines and that the MG(+/−) lines are the most robust lines that could resist baculoviral infection.

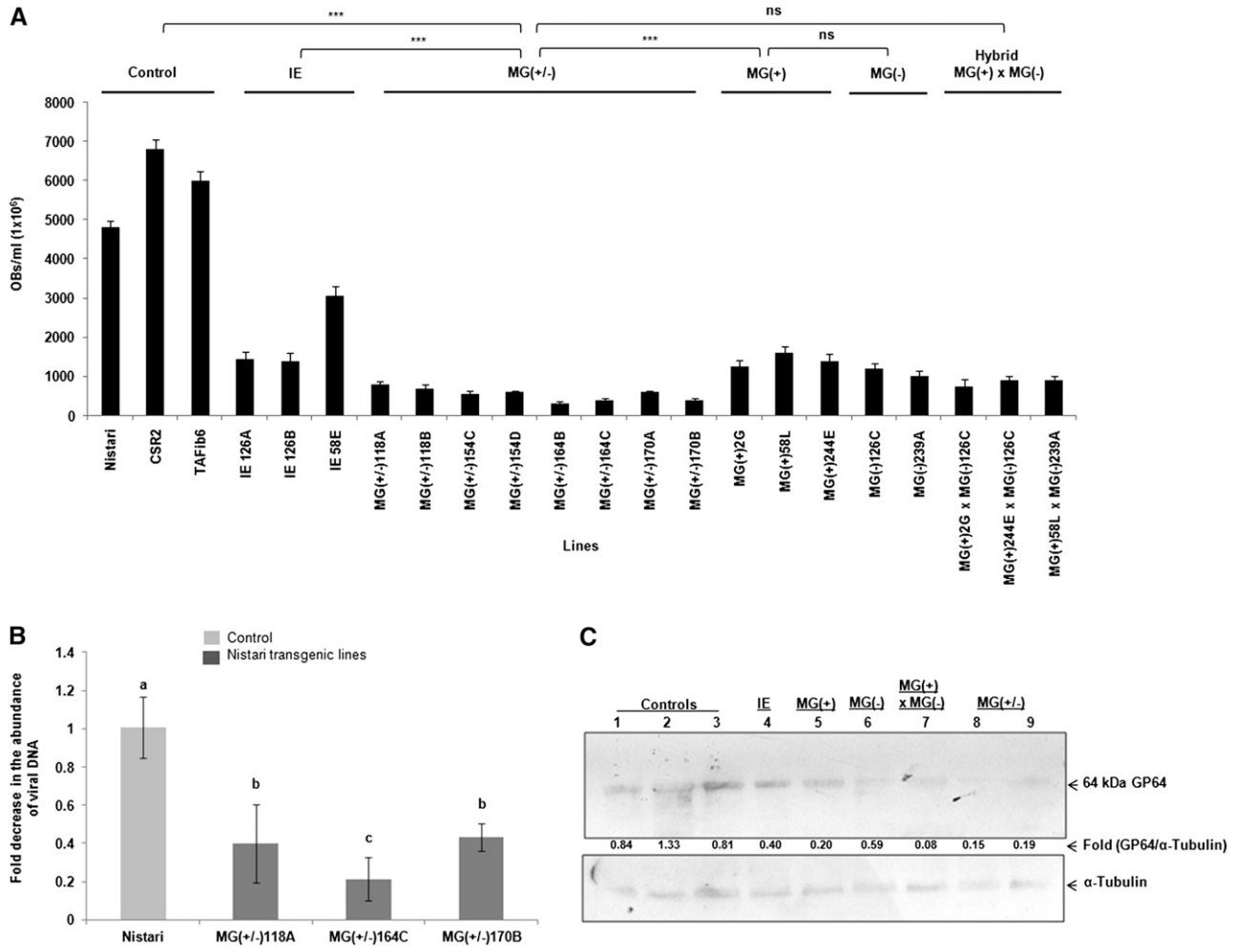

Figure 3 Viral accumulation as measured by OBs titer in transgenic and nontransgenic lines. (A) Virus load was determined by scoring the OBs from hemolymph of BmNPV-infected larvae. The number of OBs is much lower in transgenic lines in comparison to nontransgenic control lines. Bar indicates SD, $N = 36$. (***) $P < 0.001$; ns, not significant. (B) Quantitative PCR analysis of *lef3* gene of BmNPV to determine the viral load in the hemolymph of infected transgenic and nontransgenic larvae. Three independent experiments were carried out in duplicates, with a set of five larvae and the results were normalized against endogenous 18S rRNA gene. The bars denoted by different letters differ significantly ($P < 0.001$). (C) Western blot showing reduced expression of viral coat protein in transgenics compared to nontransgenic moths. Western analysis was performed using anti-GP64 antibody. Lane 1, control CSR2 line; lane 2, TAFib, a transgenic line that expresses nontarget dsRNA; lane 3, control Nistari line; lane 4, transgenic line (IE 126A) with single viral gene target; lane 5, transgenic line MG(+)58L with segments of four viral target genes transcribed in sense orientation; lane 6, transgenic line MG(−)126C with segments of four viral genes transcribed in antisense orientation; lane 7, transgenic hybrid line MG(+)58L × MG (−)126C obtained by crossing MG (+) and MG (−) lines; lanes 8 and 9, transgenic lines MG(+/−) (164C and 170A) lines with four essential baculoviral target genes expressing in sense and antisense orientation.

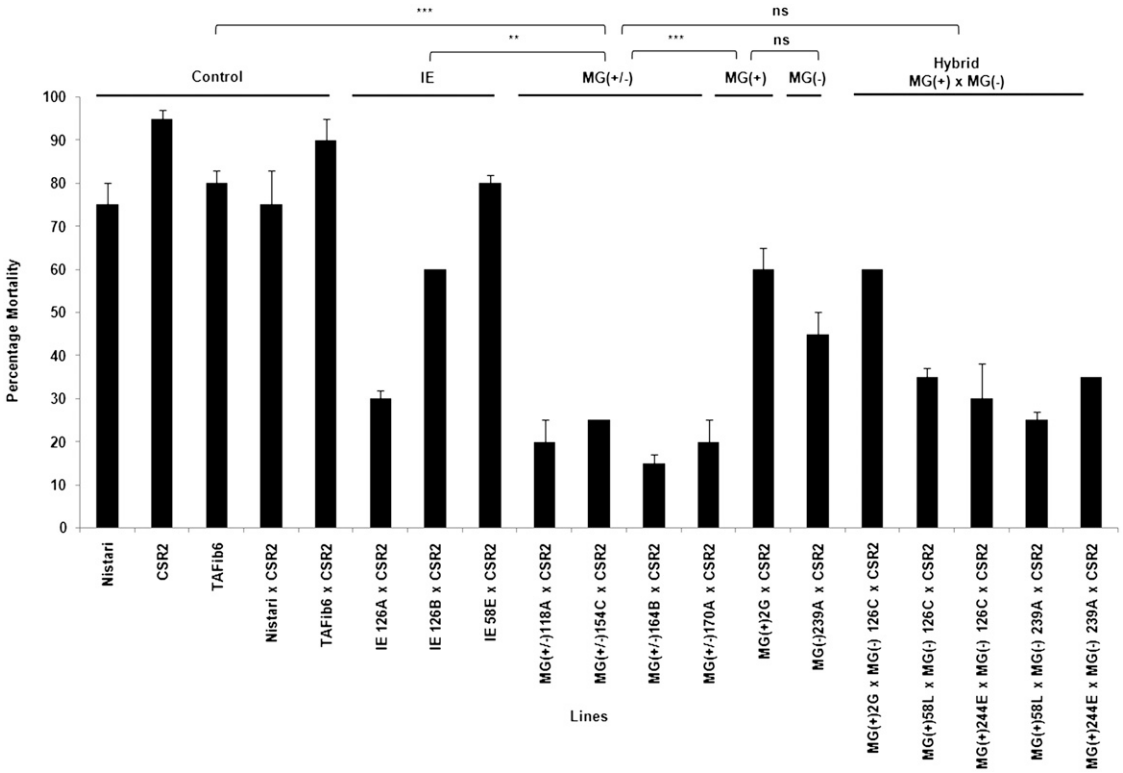

Figure 4 Histogram showing percentage mortality in the hybrids of transgenic Nistari and nontransgenic CSR2 lines. TAFib6, transgenic line that expresses nontarget dsRNA; IE, transgenic line with single viral gene target; MG(+), transgenic lines with segments of four viral target genes transcribed in sense orientation; MG(−), transgenic lines with segments of four viral genes transcribed in antisense orientation; MG(+/−), transgenic line with four essential baculoviral target genes expressing as a stem-loop structure to induce RNAi; and MG(+) × MG (−) transgenic lines obtained by crossing MG(+) and MG(−) lines. Third-instar larvae were infected *per os* at a dose of 6000 OBs/larva. Mortality was scored as number of moths succumbed to viral infection by counting the live moths eclosed from the infected larvae. Mortality was reduced significantly in lines having the multiviral genes. Bar indicates SD, $N = 48$. (**) $P < 0.01$; (***) $P < 0.001$; ns, not significant.

Viral resistance in the hybrids of transgenic and nontransgenic lines

Crossbreeding is extensively used as a means of exploiting heterosis in the silkworm, and only hybrids are reared for large-scale silk production (Nagaraju *et al.* 1996). To test whether the protective effect of transgenic lines could be observed in the hybrids of transgenic and nontransgenic lines, we infected hybrids with BmNPV. Results of two-way ANOVA revealed significant difference ($P < 0.0001$) between infected and control lines as well as between transgenic and nontransgenic F_1 hybrids. The ability of the transgene to suppress mortality was higher in hybrids made with MG(+/−) lines (Figure 4). The pairwise comparisons revealed that the CSR2 hybrids with MG(+/−) showed higher survivability and differed significantly from IE ($P < 0.0017$), MG(+) ($P < 0.0001$), and MG(−) ($P < 0.0018$) lines (Table S4). There was no difference in the survivability upon viral infection between CSR2 hybrids of MG(+/−) and MG(+) × MG(−) lines ($P < 0.0758$). Our data support that the RNAi-mediated suppression of baculoviral infection could be achieved in the hybrids involving transgenic lines MG(+/−) or MG(+) × MG(−) as one of the parental lines.

Transfer of transgenes from Nistari genetic background to nontransgenic CSR2 strain

We aimed at combining the RNAi-aided antiviral trait with high-yield characteristics of strains used in sericulture. We selected the strain CSR2, which is currently being exploited in Indian sericulture for its high silk productive values, but highly susceptible to baculovirus infection. We introduced the multigene inverted-repeat transgene from the Nistari genetic background into the CSR2 line by recurrent back-cross strategy followed by inbreeding as shown in Figure 5. A total of four backcrosses were made followed by 13 generations of sib-mating. The CSR2 line carrying the transgene showed significantly lower mortality (<15%) as compared to >75% mortality observed in nontransgenic CSR2, when administered with OBs per orally (Figure 6A). Consistent with this result, the viral DNA, isolated from the larvae infected with BmNPV and quantified by qPCR using primers for viral *lef3* gene, showed significant ($P < 0.001$) reduction in the CSR2 transgenic lines (118A, 170B, and 164C) as compared to the nontransgenic lines. Overall, there was more than threefold reduction in viral load in the CSR2 transgenic lines as compared with the nontransgenic lines (Figure 6B).

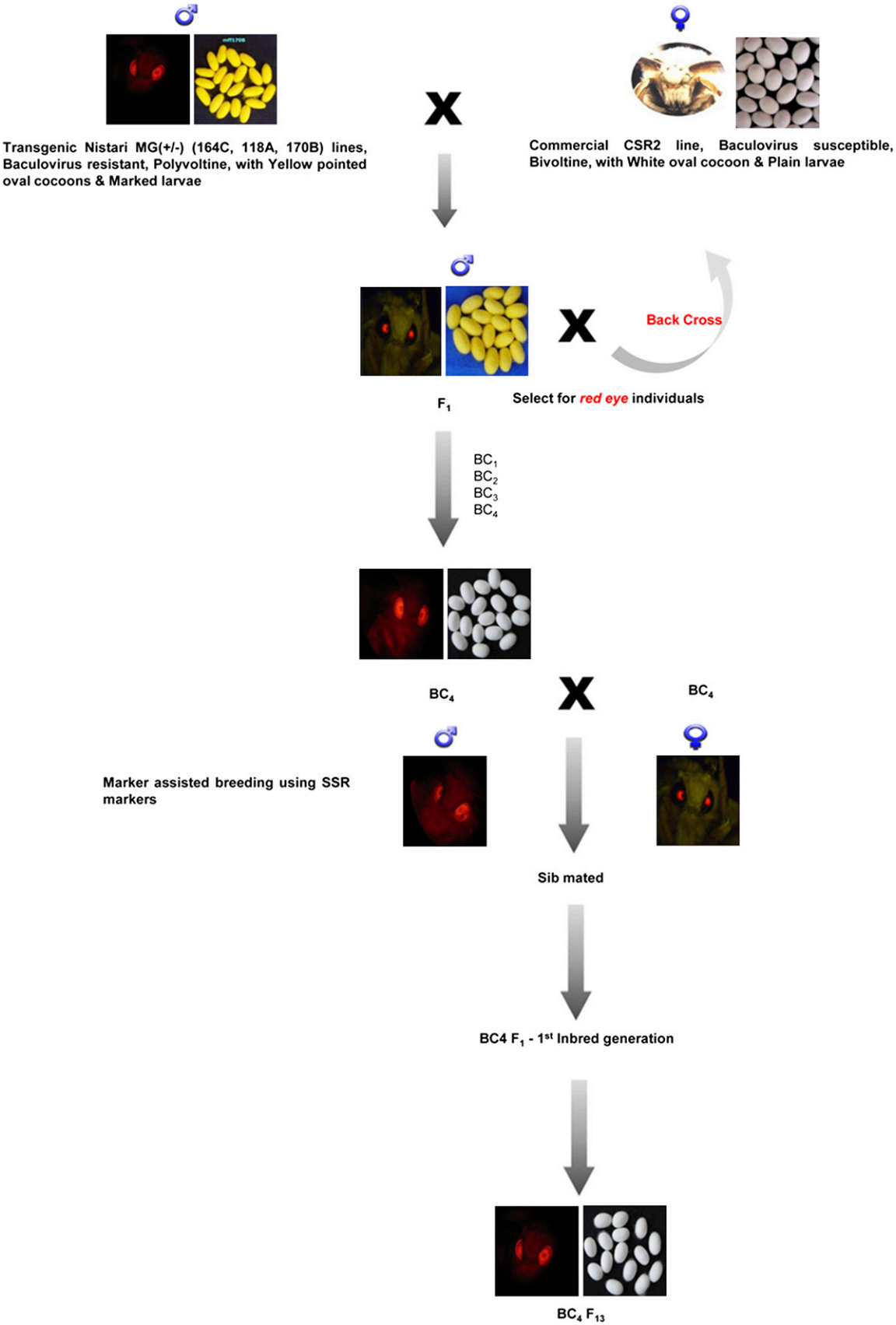

Figure 5 Schematic showing introduction of transgene (dsRNA for multiple essential baculoviral genes) from the transgenic Nistari lines to high-yielding CSR2 strain.

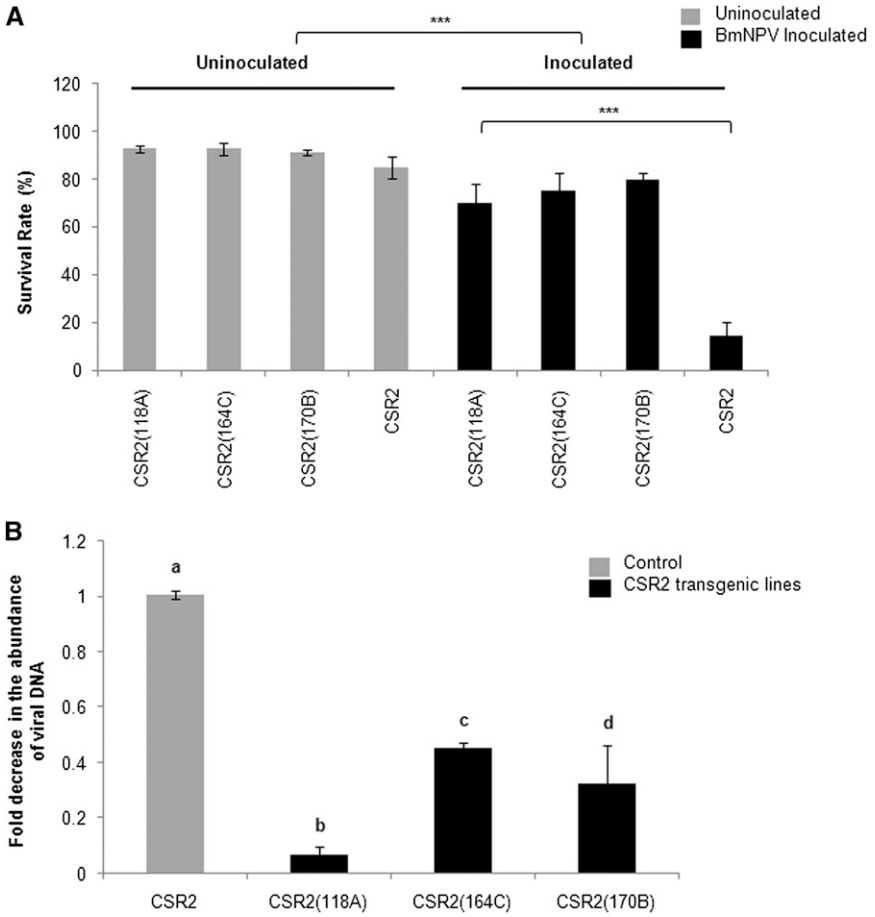

Figure 6 Performance of CSR2 transgenic MG (+/−) (118A, 164C, and 170B) lines obtained from recurrent backcross population (BC_4F_{13} batch). (A) Survival rate as compared to BmNPV-infected lines. Statistical significance (*P*) was calculated by Student's *t*-test. Bar indicates SD, $N = 24$. (***) $P < 0.001$. (B) Quantitative PCR analysis of BmNPV using *lef3* as a target gene to determine the viral load in the CSR2 transgenic and nontransgenic lines infected with 6000 OBs/larva. Three independent experiments were carried out in duplicates with a set of five larvae each, and the results were normalized against endogenous 18S rRNA gene. The bars denoted by different letters differ significantly ($P < 0.001$).

In addition to the transgene marker (DsRed eye color), 10 SSR markers located on different chromosomes polymorphic between Nistari transgenic line and CSR2 line were employed at each generation to select backcross offspring that carried CSR2-specific allele. Results show that in the BC_4F_6 progeny, 86.7, 86.9, and 81.2% of CSR2 genome was found to be incorporated in MG(+/−)118A, MG(+/−)164C, and MG (+/−)170B lines, respectively, as estimated by CSR2-specific SSR alleles at 10 loci (Figure S4 and Table S6).

Cocoon and silk properties in the transgene incorporated CSR2 lines

The cocoon and silk characters such as cocoon weight, cocoon shell weight, cocoon shell ratio, filament length, and raw silk percentage were measured in the CSR2 transgenic lines obtained through recurrent backcrosses coupled with selection for the transgene and compared with that of the recurrent parent, nontransgenic CSR2. The silk characters of the progeny at BC_4F_{13} generation are given in Table 3. These results suggest that the CSR2 line incorporated with the transgene has commercial silk traits similar to the nontransgenic CSR2 line.

Per os infectivity of the OBs derived from the transgenic and nontransgenic lines

To test whether the knockdown of P74 by the dsRNA in the transgenic silkworms resulted in less virulent virus particles, we carried out infection experiments with the OBs derived from the infected transgenic and nontransgenic CSR2 lines and compared the LD50 values in these two lines. LD50 value for the OBs derived from the transgenic CSR2 larvae when fed to nontransgenic larvae of the same line was found to be >4500 (Table 4) as compared to the LD50 value of 1600 for OBs obtained from the infected nontransgenic CSR2 larvae administered *per os* to nontransgenic CSR2 line. Our results indicate >2.5-fold decrease in the infectivity of the OBs obtained from the transgenic line as compared to that of OBs from the nontransgenic line. When the OBs derived from the infected transgenic and nontransgenic CSR2 lines were used for infection *per os* of transgenic CSR2 line, there was 4-fold decrease in the infectivity of the OBs obtained from the transgenic line. The comparative mortality in the two groups was determined by subjecting the mortality data to probit analysis, and the infectivity of OBs was assessed from the LD50 values as presented in Table 4. The LD_{50} values between the two types of OBs differered significantly ($P < 0.05$) inferring a considerable difference in the infectivity of the OBs from the transgenic and nontransgenic lines.

Discussion

BmNPV are arthropod viruses that kill their hosts in a biphasic infection cycle with a systemic primary phase

Table 3 Cocoon and silk characteristics of transgenic CSR2 and nontransgenic CSR2 (Control) lines

Lines BC4-F13	Cocoon weight (g)	Shell weight (g)	Shell ratio(%)	Filament length (m)	Raw silk (%)
CSR2 118A	1.3124 ± 0.0233	0.2390 ± 0.0088	18.14 ± 0.5094	816.87 ± 27.73	14.08 ± 0.7957
CSR2 164C	1.3102 ± 0.0720	0.2407 ± 0.0226	18.48 ± 0.8143	823.50 ± 59.97	14.59 ± 1.4637
CSR2 170B	1.4312 ± 0.1411	0.2587 ± 0.0096	18.02 ± 1.1307	773.75 ± 24.75	13.59 ± 0.2970
CSR2(Control)	1.4405 ± 0.0100	0.2795 ± 0.0100	19.37 ± 0.0100	924.50 ± 87.38	17.11 ± 2.8072

Cocoon weight (grams), Shell weight (grams), Shell ratio (percentage), Filament length (meters) and Raw silk (percentage) are given as mean ± SD.

involved in multiplication of so-called BVs that establish infection within the host and a second phase where ODVs propagate the infection from host to host. Silkworm rearing suffers heavily from BmNPV infection, which accounts for more than half of the loss in cocoon production. Although a long-lasting question, no enduring therapy exists to combat nucleopolyhedrosis.

Recently, Jiang *et al.* (2012) revealed an anti-BmNPV activity in the gut juice of transgenic silkworm that could be enhanced by 33% by overexpressing the *Bmlipase-1* which is shown to have antiviral activity. Modifying the host gene could help developing control strategies, but, at the moment, the effects are measurable only at sublethal doses of virus.

Recent developments suggest that RNA interference-based anti-pathogen strategies are efficient in blocking the activity of genes crucial to the accomplishment of the viral cycle, when there is a lack of other effective methods (Tan and Yin 2004; Mueller *et al.* 2010; Davidson and McCray 2011). The first report demonstrating the use of RNAi to inhibit *Autographa californica* NPV proliferation showed that delivering dsRNA derived from *gp64* or *ie1*, two genes essential for baculovirus propagation, resulted in transitory inhibition of viral infection *in vitro* (*Spodoptera frugiperda* Sf21 cells) and *in vivo* (injection into *Tenebrio molitor* larvae) (Valdes *et al.* 2003). Combining RNAi and *B. mori* germline transformation (Tamura *et al.* 2000), Isobe *et al.* (2004) generated silkworms expressing BmNPV *lef1* dsRNA and observed a moderate inhibition of viral replication that, however, did not alleviate baculovirus-rendered larval mortality substantially. Also, we targeted the baculoviral *ie1* gene in both cultured cells and in the transgenic silkworm (Kanginakudru *et al.* 2007). While we noted a strong viral repression at early stages of infection, the viral proliferation recovered subsequently. All these attempts demonstrated the validity of the RNAi approach, but targeting a single gene to block baculovirus proliferation resulted in limited success. Hence, in the present study, we assayed the efficiency of the simultaneous inhibition of several key viral genes. To our knowledge, this is the first report where multiple genes of the same pathogen are being simultaneously used for effective RNAi mediated suppression of virus replication/infectivity in insects.

Indeed, we selected three baculoviral genes *ie1*, *lef1*, and *lef3* that are essential for DNA replication and transcription and another gene *p74* necessary for *per os* infectivity. We constructed *piggyBac*-derived vectors based on the concatenation of partial sequences from the four genes in the sense, the antisense strand, or inverted repeat arrangement. Transgenic lines were generated with the three types of transgenes. We also combined transgenes encoding the sense and the antisense strands from two distinct loci by crossing the corresponding parental lines. We assayed the capacity of the transgenes to inhibit the completion of the viral cycle by infecting the third-instar larvae with high doses of the OBs per orally, the natural mode of contamination that occurs in farmers' silkworm rearing facilities. With this mode of infection, mortality mainly results from the dissemination of the BV in the organism impairing vital functions of host cells and tissues.

We observed that the various transgenic lines displayed different levels of resistance against BmNPV infection, accounted for by the type of transgenes and likely, within one group of lines, the chromosomal environment of the inserted sequences. The less efficient protection was noted in the lines expressing either the sense or the antisense strand alone. Significantly, the combination of both conferred a high level of protection against the virus, which suggested that the two single-strand RNAs form duplexes that are further processed by the RNAi machinery. The transgenic lines that showed the lowest mortality rates upon infection were those expressed the long stem-loop double-

Table 4 Comparative infectivity of OBs obtained from transgenic CSR2 and nontransgenic CSR2 larvae

			95% fiducial limit				
Type of OBs used	Test strain	LD_{50}	Upper	Lower	1/slope	Regression equation	Standard error
OBs from transgenics	Nontransgenics	4786	3801	6025	1.804	$Y = 0.5542X + 2.962$	0.1013
OBs from nontransgenics	Nontransgenics	1698	1258	2398	1.146	$Y = 0.8728X + 2.176$	0.1320
OBs from transgenics	Transgenics	21877	18197	25703	1.948	$Y = 0.5134X + 2.773$	0.0747
OBs from nontransgenic	Transgenics	2570	2238	2951	1.822	$Y = 0.5488X + 3.127$	0.0573

Day 1 fourth instar transgenic and nontransgenic CSR2 lines were fed *per os* with doses of OBs ranging from 1000 to 40,000 per larva. Thirty larvae per dose per line were used. The mortality was recorded from day 1 postinfection until pupation. The probit values were plotted against log doses for calculation of LD_{50} using GraphPad Prism 6 software. LD_{50} values of the transgenic and nontransgenic lines infected either with transgenic- or nontransgenic-derived OBs differed significantly ($P < 0.05$).

strand RNA. The resistance that we measured was mostly the consequence of the coincident inhibition of the IE1, LEF1, and LEF3 accumulation, which affected viral replication. This is demonstrated by the almost disappearance of the GP64 envelop protein in the MG(+/−) lines, which recorded the highest level of resistance against baculoviral infection.

The drop in the OBs titer in hemolymph of the infected transgenic larvae further demonstrated that the constraints on DNA replication strongly impaired the formation of occluded viruses. Furthermore, we speculated that the inhibition of the *per os* infectivity factor P74 generates flawed viruses unable to propagate the infection in the population. Indeed, in the present study, we observed a significant decrease in the infectivity of the OBs derived from the transgenic lines as compared to that of the OBs derived from the nontransgenic lines. These results support the earlier findings that deletion of P74, a virion envelop protein involved in *per os* infectivity in most of the baculoviruses, and its deletion results in significant loss of infectivity in *Autographa californica* nucleopolyhedrovirus (Faulkner *et al.* 1997). Haas-Stapleton *et al.* (2004) showed that P74 of AcMNPV is critical for oral infection of *Trichoplusia ni* larvae. The study also provided evidence that P74 facilitates binding of AcMNPV ODV to a specific receptor within the larval midgut epithelia of another host species, *Heliothis virescens*. P74-deficient ODV failed to compete effectively with wild-type ODV binding, and as a result the overall binding level of the mutant ODV was one-third of the wild-type ODV. Wang *et al.* (2009) have shown that P74 of AcMNPV has a role in virion occlusion, as individual gene deletion of either *p26* or *p10* could not abolish virion occlusion, but the deletion of *p74* along with these two genes resulted in few or no virions in OBs. Peng *et al.* (2011) reported threefold reduction in host-cell-binding efficiency of the P74 mutant virus as compared to the wild-type ones. Taken together, our studies demonstrate that abrogation of P74 by the corresponding transgene-derived dsRNA resulted in more than twofold reduction in BmNPV infectivity. This is the first evidence to show the role of P74 of BmNPV in *per os* infectivity of OBs.

As a whole, the systemic infection as well as the horizontal (host-to-host) transmission is considerably impaired in transgenic silkworms to the point of conferring high level of refractoriness to the virus in the best lines. BmNPV belongs to the large virus family of baculoviridae for which 57 genome sequences are known (Miele *et al.* 2011). The comparative sequence analysis shows that the core genes *lef1* and *p74* that we targeted are widely conserved, suggesting that the protection may extend to other baculovirus genus or isolates for which *Bombyx* may be sensitive.

To commercially exploit the benefit of transgene-mediated baculoviral suppression, we introduced the RNAi-producing transgenes to the high-yielding, baculovirus-susceptible commercial strain, CSR2 which is currently in use in sericulture, through recurrent backcross breeding coupled with transgene marker selection (eye color), cocoon phenotype, and SSR marker-aided selection of the recurrent parent. In the BC_4F_6 progeny >85% CSR2 genome was found to be incorporated as screened by SSR markers. The resultant backcross lines (BC_4F_{13}) were evaluated for survival rate and other silk cocoon quality traits. Almost all the nontransgenic CSR2 lines succumbed to infection before reaching cocoon stage whereas >75% of the backcross-derived transgenic CSR2 lines survived and reproduced successfully. The cocoons, silk yield, and all other properties were similar to the control CSR2 line (Table 3). Although we deal with genetically transformed silkworms, this is reminiscent of the first in-field technology transfer developed by Hunter *et al.* (2010) whereby feeding bees (*Apis mellifera*) with dsRNA of Israeli acute paralysis virus protects them from colony collapse disorder.

In conclusion, we succeeded in obtaining high-yielding silkworm lines protected from the baculovirus infection that could be harnessed for the benefit of sericulture industry. The contained multilocational trials of the baculovirus-resistant transgenic silkworms are currently underway.

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Literature Cited

Bonfim, K., J. C. Faria, E. O. Nogueira, E. A. Mendes, and F. J. Aragao, 2007 RNAi-mediated resistance to Bean golden mosaic virus in genetically engineered common bean (*Phaseolus vulgaris*). Mol. Plant Microbe Interact. 20: 717–726.

Bucher, E., D. Lohuis, P. M. van Poppel, C. Geerts-Dimitriadou, R. Goldbach *et al.*, 2006 Multiple virus resistance at a high frequency using a single transgene construct. J. Gen. Virol. 87: 3697–3701.

Davidson, B. L., and P. B. McCray Jr.,. 2011 Current prospects for RNA interference-based therapies. Nat. Rev. Genet. 12: 329–340.

Evans, J. T., D. J. Leisy, and G. F. Rohrmann, 1997 Characterization of the interaction between the baculovirus replication factors LEF-1 and LEF-2. J. Virol. 71: 3114–3119.

Faulkner, P., J. Kuzio, G. V. Williams, and J. A. Wilson, 1997 Analysis of p74, a PDV envelope protein of *Autographa californica* nucleo-

polyhedrovirus required for occlusion body infectivity in vivo. J. Gen. Virol. 78: 3091–3100.

Gandhe, A. S., S. H. John, and J. Nagaraju, 2007 Noduler, a novel immune up-regulated protein mediates nodulation response in insects. J. Immunol. 179: 6943–6951.

Haas-Stapleton, E. J., J. O. Washburn, and L. E. Volkman, 2004 P74 mediates specific binding of *Autographa californica* M nucleopolyhedrovirus occlusion-derived virus to primary cellular targets in the midgut epithelia of *Heliothis virescens* larvae. J. Virol. 78: 6786–6791.

Hoopes, R. R. Jr., and G. F. Rohrmann, 1991 In vitro transcription of baculovirus immediate early genes: accurate mRNA initiation by nuclear extracts from both insect and human cells. Proc. Natl. Acad. Sci. USA 88: 4513–4517.

Huh, N. E., and R. F. Weaver, 1990a Categorizing some early and late transcripts directed by the *Autographa californica* nuclear polyhedrosis virus. J. Gen. Virol. 71: 2195–2200.

Huh, N. E., and R. F. Weaver, 1990b Identifying the RNA polymerases that synthesize specific transcripts of the *Autographa californica* nuclear polyhedrosis virus. J. Gen. Virol. 71: 195–201.

Hunter, W., J. Ellis, D. Vanengelsdorp, J. Hayes, D. Westervelt *et al.*, 2010 Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (*Apis mellifera*, Hymenoptera: Apidae). PLoS Pathog. 6: e1001160.

Isobe, R., K. Kojima, T. Matsuyama, G. X. Quan, T. Kanda *et al.*, 2004 Use of RNAi technology to confer enhanced resistance to BmNPV on transgenic silkworms. Arch. Virol. 149: 1931–1940.

Jiang, L., G. Wang, T. Cheng, Q. Yang, S. Jin *et al.*, 2012 Resistance to *Bombyx mori* nucleopolyhedrovirus via overexpression of an endogenous antiviral gene in transgenic silkworms. Arch. Virol. 157: 1323–1328.

Kanginakudru, S., C. Royer, S. V. Edupalli, A. Jalabert, B. Mauchamp *et al.*, 2007 Targeting ie-1 gene by RNAi induces baculoviral resistance in lepidopteran cell lines and in transgenic silkworms. Insect Mol. Biol. 16: 635–644.

Khurad, A. M., S. Kanginakudru, S. O. Qureshi, M. K. Rathod, M. M. Rai *et al.*, 2006 A new *Bombyx mori* larval ovarian cell line highly susceptible to nucleopolyhedrovirus. J. Invertebr. Pathol. 92: 59–65.

Kuzio, J., R. Jaques, and P. Faulkner, 1989 Identification of p74, a gene essential for virulence of baculovirus occlusion bodies. Virology 173: 759–763.

Leonard, J. N., and D. V. Schaffer, 2006 Antiviral RNAi therapy: emerging approaches for hitting a moving target. Gene Ther. 13: 532–540.

Miele, S. A., M. J. Garavaglia, M. N. Belaich, and P. D. Ghiringhelli, 2011 Baculovirus: molecular insights on their diversity and conservation. Int. J. Evol. Biol. 2011: 379424.

Mikhailov, V. S., and G. F. Rohrmann, 2002 Baculovirus replication factor LEF-1 is a DNA primase. J. Virol. 76: 2287–2297.

Monsma, S. A., A. G. Oomens, and G. W. Blissard, 1996 The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. J. Virol. 70: 4607–4616.

Mrinal, N., and J. Nagaraju, 2010 Dynamic repositioning of dorsal to two different kappaB motifs controls its autoregulation during immune response in Drosophila. J. Biol. Chem. 285: 24206–24216.

Mueller, S., V. Gausson, N. Vodovar, S. Deddouche, L. Troxler *et al.*, 2010 RNAi-mediated immunity provides strong protection against the negative-strand RNA vesicular stomatitis virus in Drosophila. Proc. Natl. Acad. Sci. USA 107: 19390–19395.

Nagaraju, J., U. Raje, and R. K. Datta, 1996 Crossbreeding and heterosis in the silkworm, Bombyx mori: a review. Sericologia 36: 1–20.

Peng, K., J. W. van Lent, J. M. Vlak, Z. Hu, and M. M. van Oers, 2011 In situ cleavage of baculovirus occlusion-derived virus receptor binding protein P74 in the peroral infectivity complex. J. Virol. 85: 10710–10718.

Peng, K., J. W. van Lent, S. Boeren, M. Fang, D. A. Theilmann *et al.*, 2012 Characterization of novel components of the baculovirus per os infectivity factor complex. J. Virol. 86: 4981–4988.

Royer, C., A. Jalabert, M. Da Rocha, A. M. Grenier, B. Mauchamp *et al.*, 2005 Biosynthesis and cocoon-export of a recombinant globular protein in transgenic silkworms. Transgenic Res. 14: 463–472.

Shukla, J. N., and J. Nagaraju, 2010 Two female-specific DSX proteins are encoded by the sex-specific transcripts of dsx, and are required for female sexual differentiation in two wild silkmoth species, *Antheraea assama* and *Antheraea mylitta* (Lepidoptera, Saturniidae). Insect Biochem. Mol. Biol. 40: 672–682.

Tamura, T., C. Thibert, C. Royer, T. Kanda, E. Abraham *et al.*, 2000 Germline transformation of the silkworm *Bombyx mori* L. using a piggyBac transposon-derived vector. Nat. Biotechnol. 18: 81–84.

Tan, F. L., and J. Q. Yin, 2004 RNAi, a new therapeutic strategy against viral infection. Cell Res. 14: 460–466.

Terenius, O., A. Papanicolaou, J. S. Garbutt, I. Eleftherianos, H. Huvenne *et al.*, 2011 RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. J. Insect Physiol. 57: 231–245.

Thomas, J. L., M. Da Rocha, A. Besse, B. Mauchamp, and G. Chavancy, 2002 3xP3-EGFP marker facilitates screening for transgenic silkworm *Bombyx mori* L. from the embryonic stage onwards. Insect Biochem. Mol. Biol. 32: 247–253.

Tomita, M., 2011 Transgenic silkworms that weave recombinant proteins into silk cocoons. Biotechnol. Lett. 33: 645–654.

Uchino, K., M. Imamura, H. Sezutsu, I. Kobayashi, K. Kojima *et al.*, 2006 Evaluating promoter sequences for trapping an enhancer activity in the silkworm *Bombyx mori*. J. Insect Biotechnol. Sericology 75: 89–97.

Valdes, V. J., A. Sampieri, J. Sepulveda, and L. Vaca, 2003 Using double-stranded RNA to prevent in vitro and in vivo viral infections by recombinant baculovirus. J. Biol. Chem. 278: 19317–19324.

van der Linden, A. M., and R. H. Plasterk, 2004 Shotgun cloning of transposon insertions in the genome of *Caenorhabditis elegans*. Comp. Funct. Genomics 5: 225–229.

Wang, L., T. Z. Salem, D. J. Campbell, C. M. Turney, C. M. Kumar *et al.*, 2009 Characterization of a virion occlusion-defective *Autographa californica* multiple nucleopolyhedrovirus mutant lacking the p26, p10 and p74 genes. J. Gen. Virol. 90: 1641–1648.

Wang, X. H., R. Aliyari, W. X. Li, H. W. Li, K. Kim *et al.*, 2006 RNA interference directs innate immunity against viruses in adult Drosophila. Science 312: 452–454.

Zhang, Z. Y.,F. L. Fu, L. Gou, H. G. Wang, and W. C. Li, 2010 RNA interference-based transgenic maize resistant to maize dwarf mosaic virus. J. Plant Biol. 53: 297–305.

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GENETICS

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Engineering Silkworms for Resistance to Baculovirus Through Multigene RNA Interference

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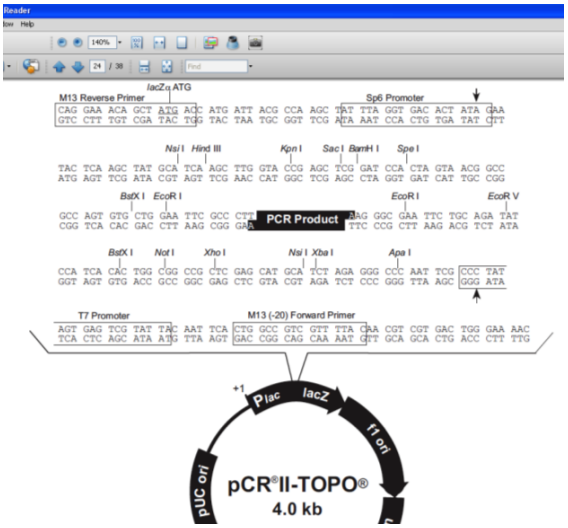
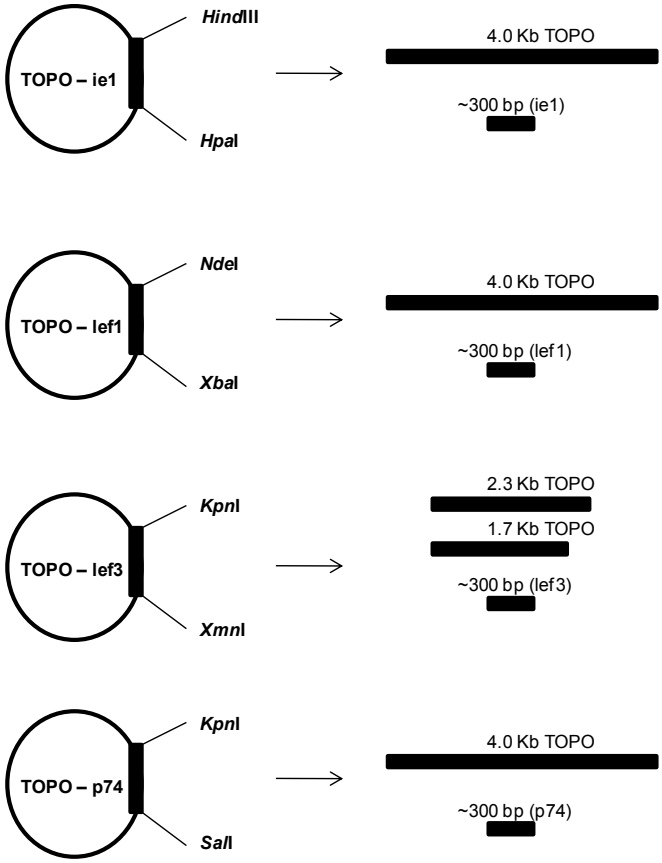
DOI: 10.1534/genetics.112.144402

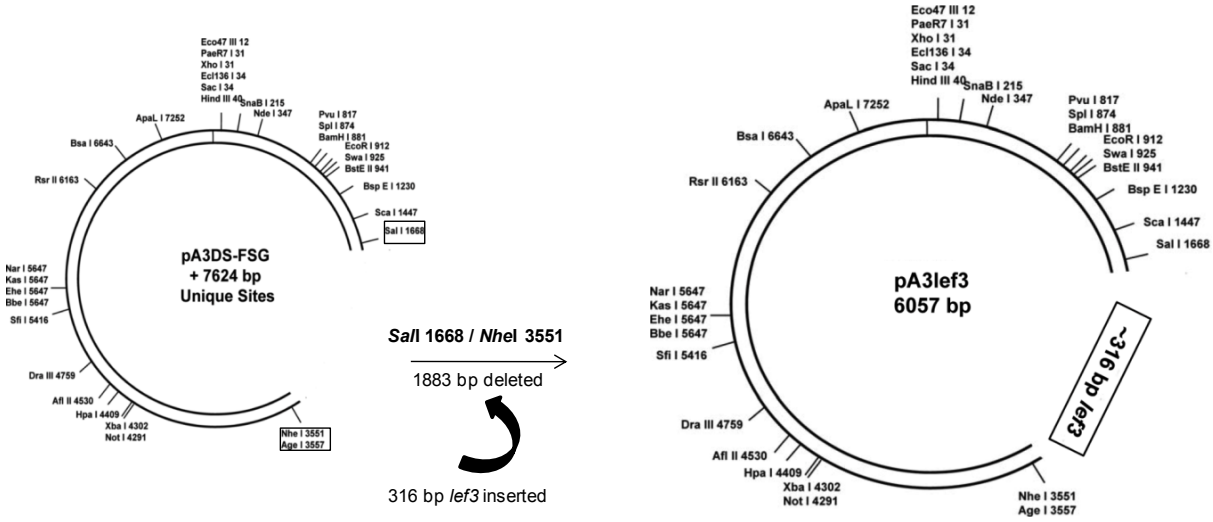
Cloning of baculoviral genes (*ie1*, *lef1*, *lef3* and *p74)* into TOPO vector

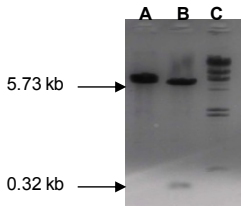




A 316 bp fragment of *lef3* was cloned into pA3DS-FSG vector backbone using *Sal*I and *Nhe*I sites to generate 1-gene construct, pA3Δlef3

Step 1



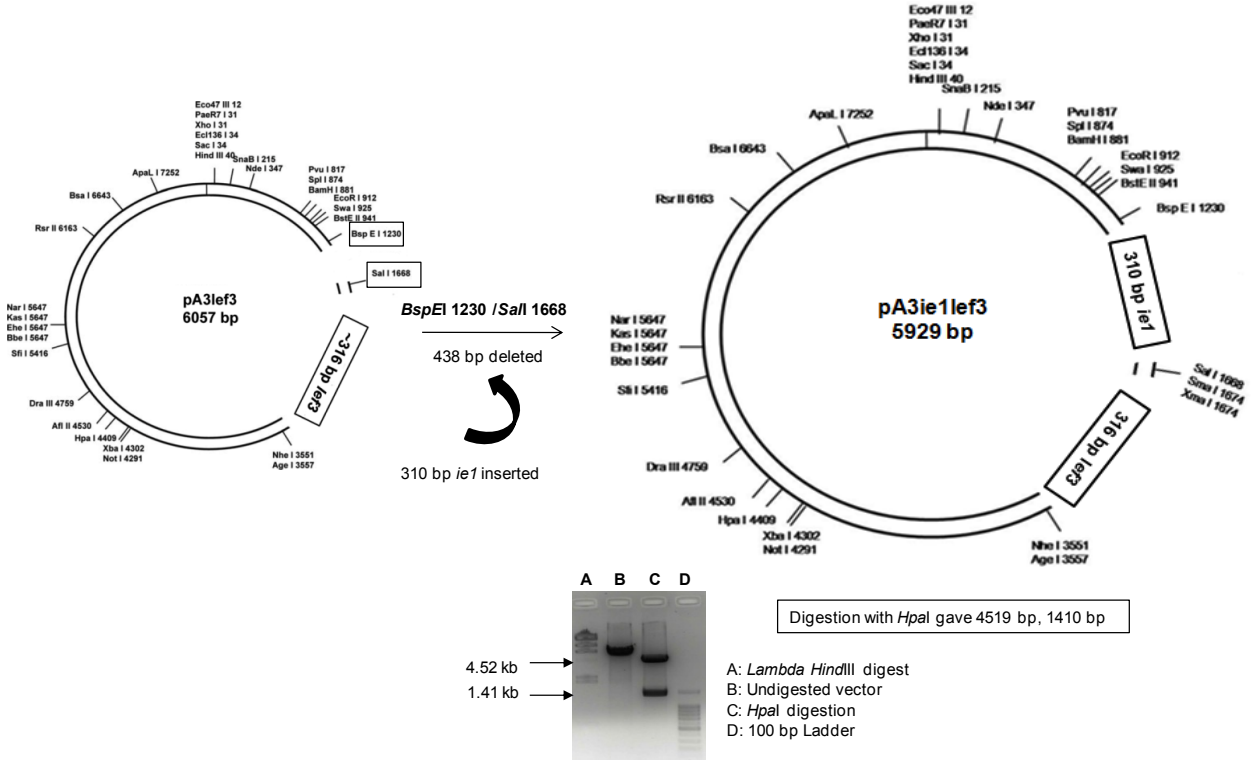


Digestion with *Xmn*I and *Sca*I gave 5734 bp and 323 bp fragments

A: *Xmn*I digestion
B: *Xmn*I and *Sca*I digestion
C: *Lambda Hind*III digest

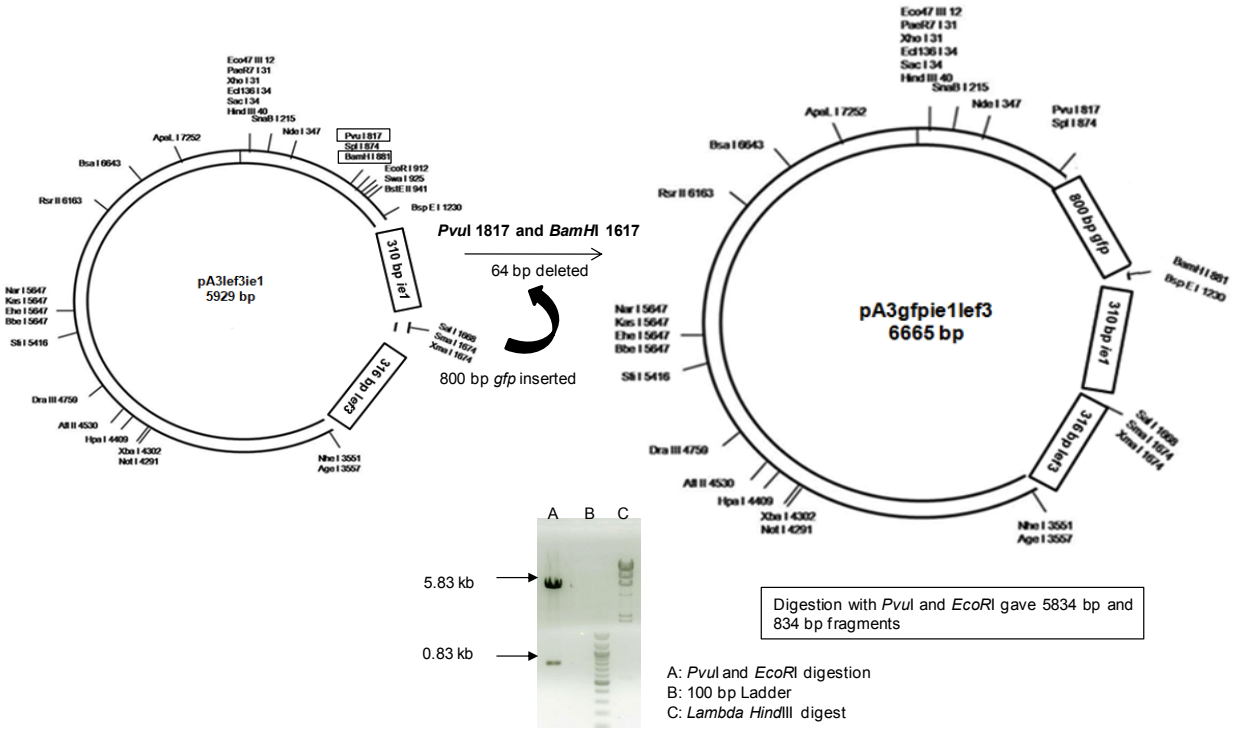
Step 2

A 310 bp fragment of *ie1* was cloned into pA3Δlef3 vector backbone using *Bsp*EI and *Sal*I sites to generate 2-gene construct, pA3Δie1lef3



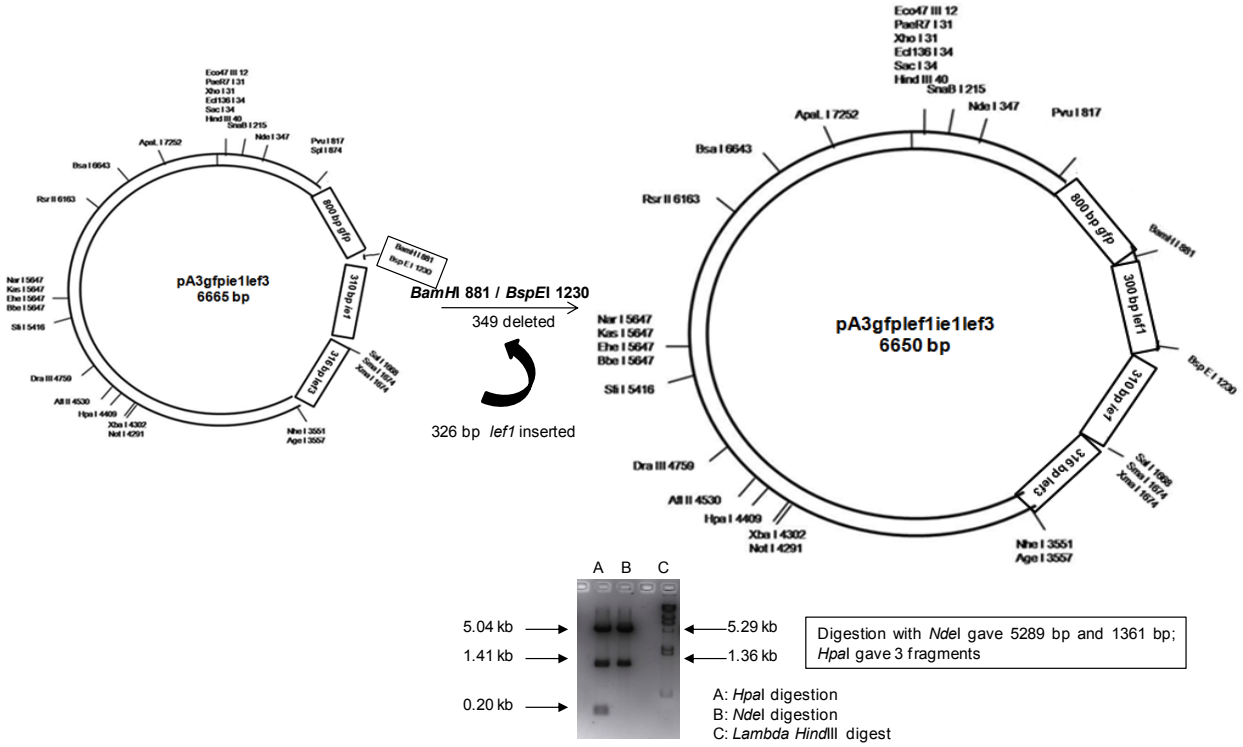
Step 3

A 800 bp fragment of *gfp* was cloned into pA3Δie1lef3 vector backbone using *Pvu*I and *BamH*I sites to generate 3-gene construct, pA3Δgfpie1lef3



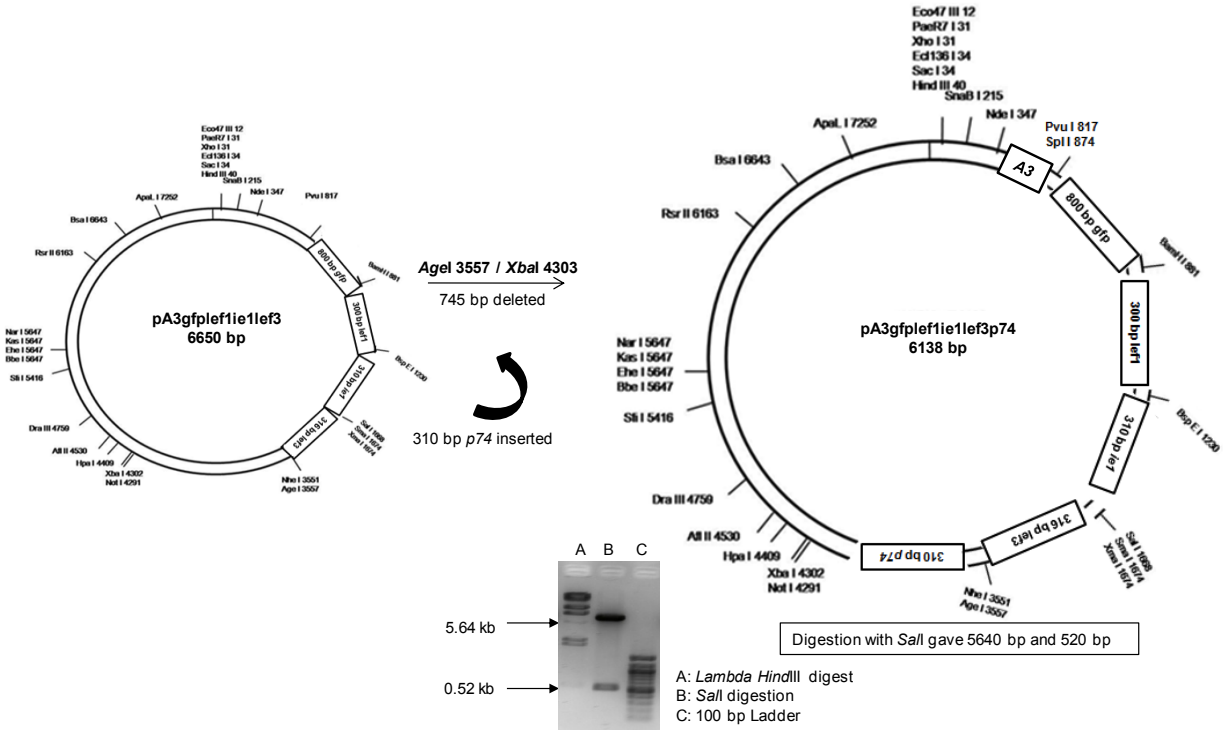
A 326 bp fragment of *lef1* was cloned into pA3Δgfpie1lef3 vector backbone using *Bam*HI and *Bsp*EI sites to generate 4-gene construct, pA3Δgfplef1ie1lef3

Step 4



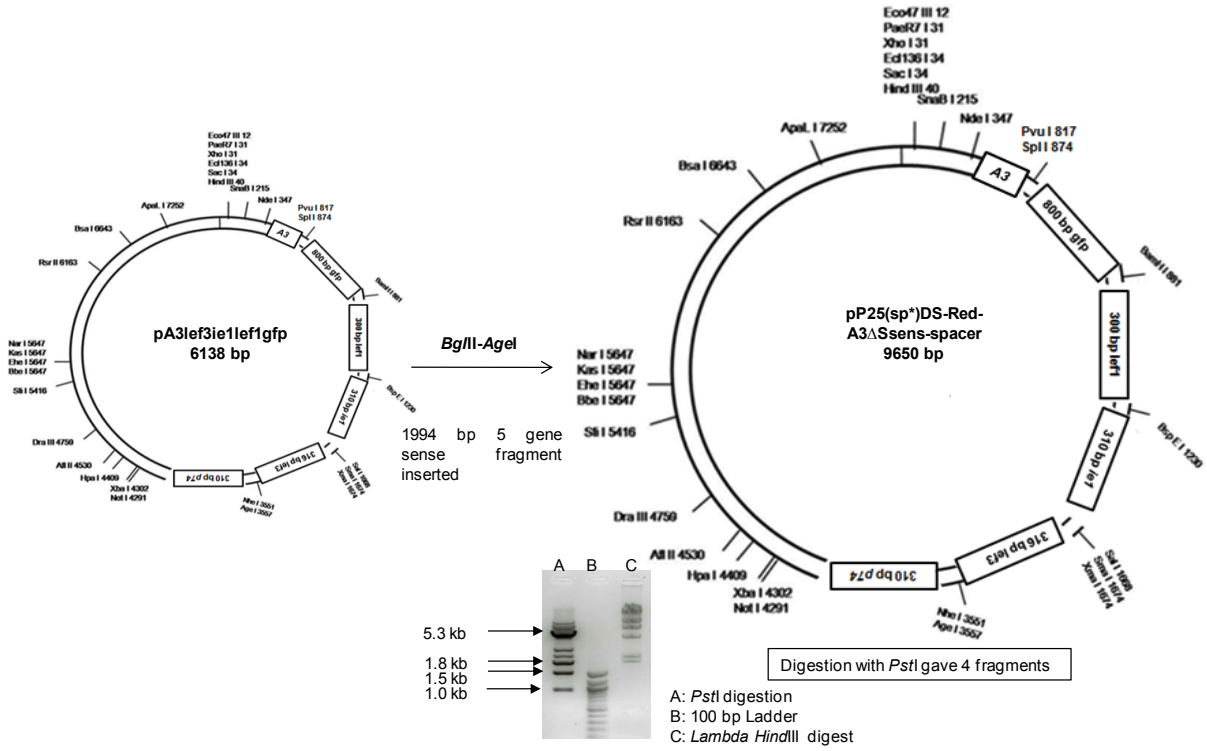
Step 5

A 310 bp fragment of *p74* was cloned into pA3Δgfplef1ie1lef3 vector backbone using *Age*I and *Xba*I sites to generate 5-gene construct, pA3Δgfplef1ie1lef3p74



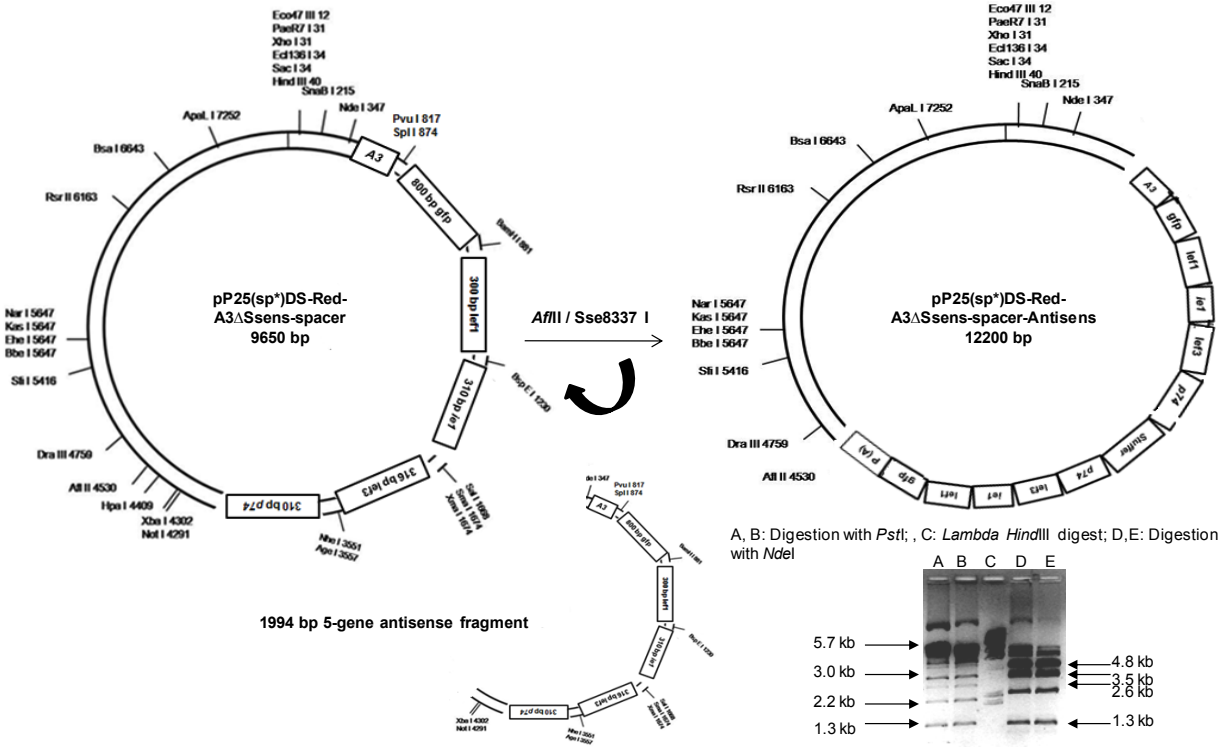
Step 6

1994 bp 5-gene sense fragment cloned to pP25(sp*)DsRed vector backbone using *Bgl*II and *Age*I sites to generate pP25(sp*)DsRed-A3ΔSsens-Spacer



Step 7

Antisense fragment of five genes was cloned into intermediary form of sense-spacer vector backbone using *Afl*II – *Sse8337*I sites to generate pP25(sp*)DsRed-A3ΔS Sense.Spacer.Antisense-Poly A region



A, B: Digestion with *Pst*I; , C: *Lambda Hind*III digest; D,E: Digestion with *Nde*I

Step 8

pP25(sp*)DsRed-A3ΔSSense.Spacer.Antisense-Poly A construct and PiggyP25IL2-(3xP3 DsRed2) TQ backbone vector were digested using *Bgl*II – *Sfi*I sites to generate PiggyA3ΔSSense-Spacer-Antisense(3xP3.DsRed2) multiple flip-flop piggyBac vector

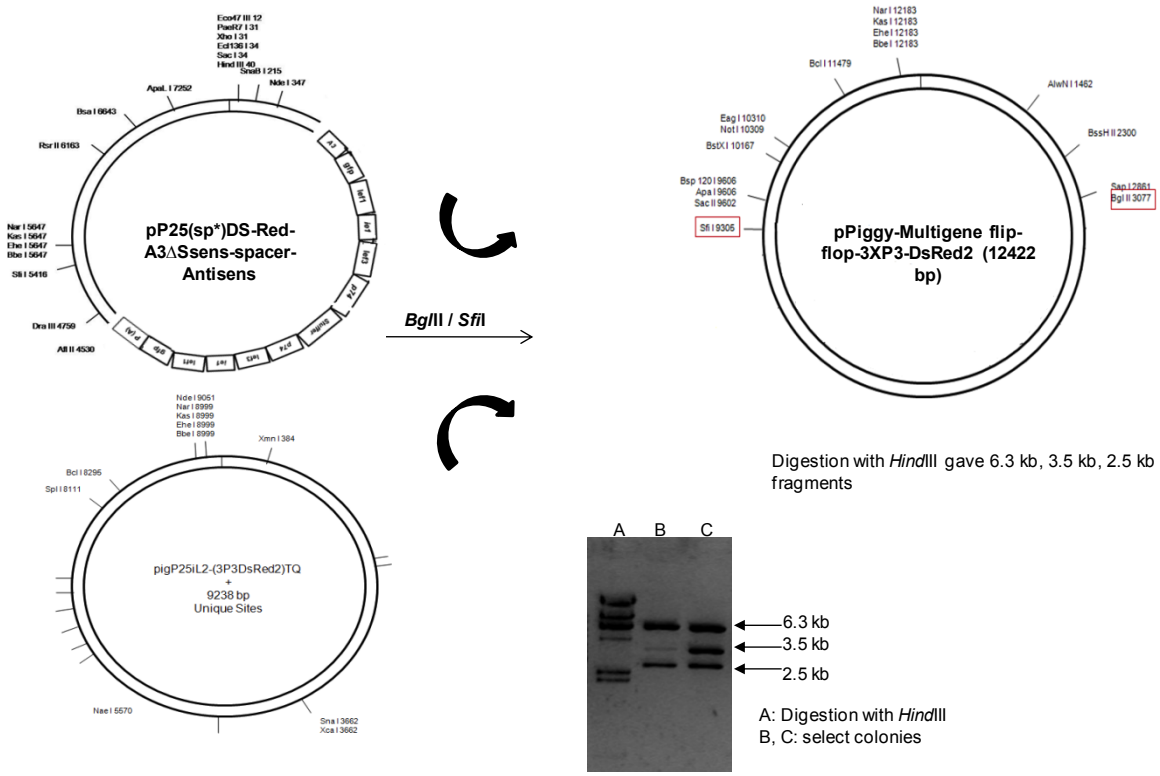


Figure S1 The four baculoviral genes *ie1*, *lef1*, *lef3* and *p74* were initially cloned into pCRII TOPO vector (Invitrogen). The resultant plasmids were labeled as Topo-*ie1*, Topo-*lef1*, Topo-*lef3*, and Topo-*p74*.These vectors having right inserts were confirmed by restriction digestions and DNA sequencing for later use in cloning steps. The various steps involved in the construction of *pPiggyMG(+)3XP3-GFP*, *pPiggyMG(-)3XP3-DsRed2* and *pPiggyMG(+/-)3XP3-DsRed2* were schematically represented. The stepwise description is provided in the text.

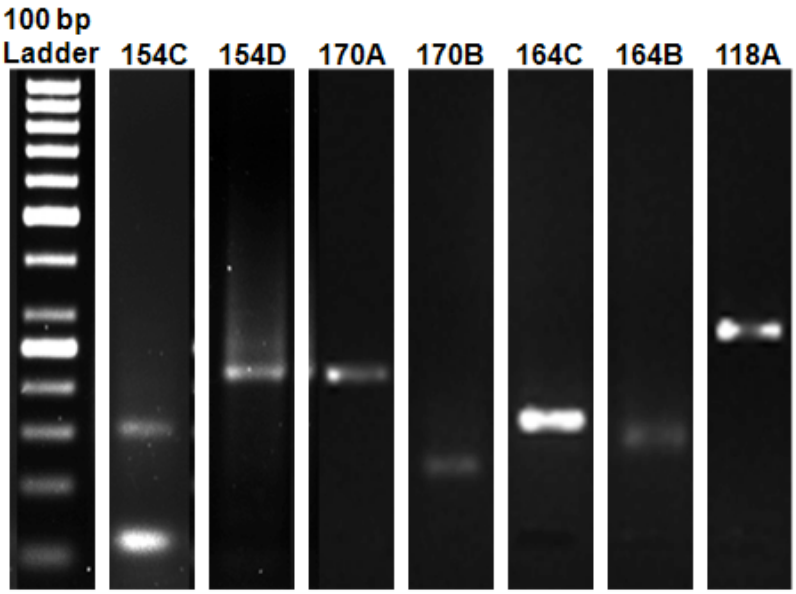


Figure S2 Copy number and site of insertion of transgenes in transgenic lines were characterized using TED assay. Seven Nistari transgenic lines (MG(+/-) 170A, 170B, 164C, 164B, 154C, 154D and 118A) showed single copy insertions.

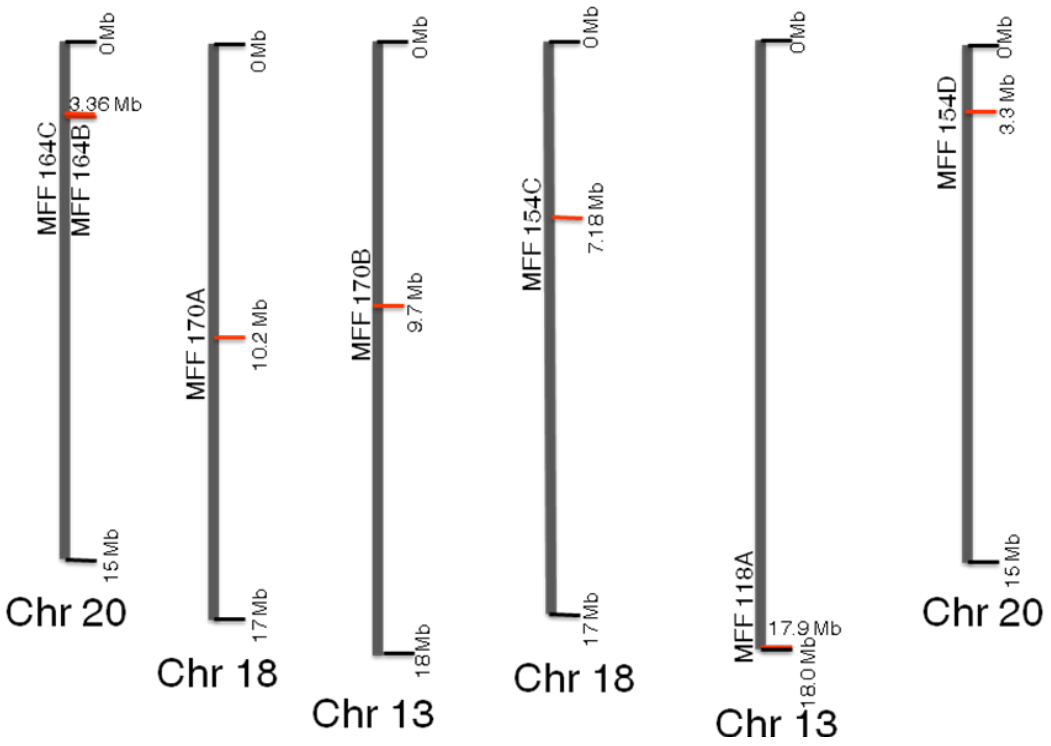


Figure S3 Mapping of transgenes to chromosome locations in different transgenic lines expressing dsRNA for essential baculoviral genes.

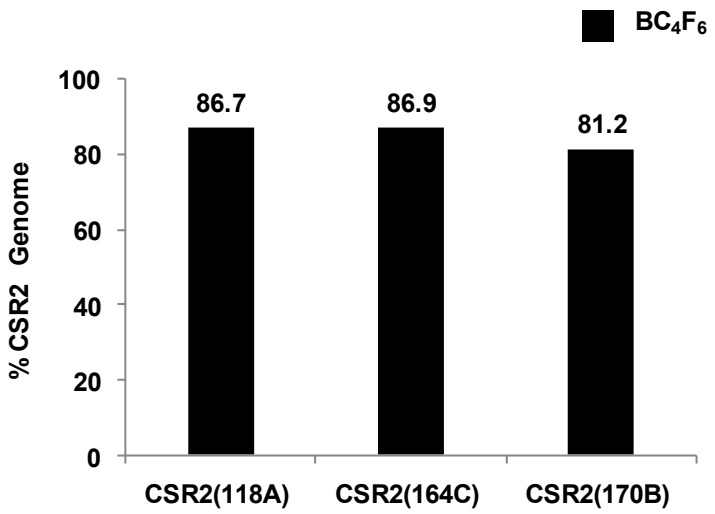


Figure S4 Histogram representing percentage incorporation of the CSR2 genome in the BC_4F_6 population of transgenic lines, CSR2(118A), CSR2(164C) and CSR2(170B) obtained through recurrent backcross strategy, as measured by CSR2-specific SSR alleles from 10 loci.

File S1

ADDITIONAL METHODS

Construction of the vectors: The vectors were constructed by amplifying the portions of *ie1*, *lef1*, *lef3* and *p74* genes encoding the *immediate early 1* gene, late expression factors 1, 3 and envelope protein of BmNPV, respectively, using primers carrying specific restriction enzyme sites. Similarly, *gfp* gene of pPIGA3GFP (Kanginakudru *et al.* 2007) was amplified. The primers used for the amplification of the gens are listed in the TABLE S1**.** The amplicons were cloned into the T-overhangs of pCRII TOPO vector (Invitrogen) (FIGURE S1). The resultant plasmids were labeled as Topo-*ie1*, Topo-*lef1*, Topo-*lef3*, Topo-*p74* and Topo-*gfp.* The targeted regions of *ie1*, *lef1*, *lef3*, and *p74* are as mentioned in File S2. These vectors having right inserts were confirmed by restriction digestions and DNA sequencing. These baculoviral genes produce virus specific proteins and do not share any homology to known insect or human genes.

The pTopo clones were transformed into one shot INV110 chemically competent *E. coli* cells (Invitrogen). Blue/White screening was performed on LB agar plates containing 40 µl of X-Gal (40 mg/ml) and 40 µl of isopropyl β-D-thiogalactoside (IPTG, 100 mM). White colonies were picked up for insert analysis through digestion. Colonies that gave right inserts of expected size were picked up. For DNA sequencing, 250 ng of plasmid was used in a sequencing reaction that contained 8 µl of Ready reaction mix (BigDye terminator, BDTv 3.0, Applied Biosystems, Foster City, CA) and 5 picomoles of M13 sequencing primers. The cycling conditions used were as follows: 25 cycles of 96°C for 10 sec, 50°C 5 sec, 60°C 4 min. Samples were ethanol precipitated, washed with 70% ethanol and resuspended in Hi-Di^{TM}formamide (Applied Biosystems). The sequencing was carried out in ABI Prism 3100 Genetic Analyzer (Applied Biosystems) and the intactness of each gene fragment was confirmed. The constructs were obtained in a series of cloning steps as described below:

1. A 316 bp fragment of *lef-3* was cloned into pA3DS-FSG vector backbone (7624 bp) using *Sal*I-*Nhe*I sites to generate single gene construct, pA3ΔS-lef3 (6057 bp). The transformants were verified by *Xmn*I (an internal site of *lef3*) restriction digestion and *Xmn*I/*Sca*I double digestion. *Xmn*I digestion linearizes to 6.05 kb fragment of 1-gene construct and double digestion with *Sca*I (a site in backbone) released 323 bp product or insert size and 5.73 kb fragment as per DNA strider map pattern.
2. A 310 bp fragment of *ie-1* was cloned in to pA3ΔS-lef3 vector using *Bsp*EI-*Sal*I sites to generate pA3ΔS-ie1lef3SG, a two gene construct (5929 bp). The transformants were checked upon sequential digestion using *Xmn*I*/Hpa*I restriction system. *Xmn*I linearization of 2-gene construct gave rise to 5929 bp fragment and *Xmn*I/*Hpa*I sequential digestion gave rise to expected three bands of 339 bp, 1071 bp and 4519 bp fragments.

3. An 800 bp fragment of *gfp* was cloned into pA3ΔS-ie1lef3 vector backbone using *Pvu*I-*Bam*HI sites to generate pA3ΔS-gfpie1lef3, a three gene construct (6665 bp). The transformants were checked upon *Pvu*I/*Eco*RI restriction analysis which gave rise to 834 bp insert and 5834 bp backbone.

4. A 326 bp *lef-1* clone was added to pA3ΔS-gfpie1lef3 vector backbone using *Bsp*EI/*Bam*HI to generate pA3ΔS-gfplef1ie1lef3 (6650 bp) a four gene product. The transformants were confirmed through *Hpa*I and *Nde*I restriction digestions which gave rise to 3 fragments of expected lengths 188 bp, 1410 bp, 5040 bp and 2 fragments of expected length 1361 bp and 5289 bp, respectively.

5. A 310 bp fragment of *p74* was cloned in to pA3ΔS-gfplef1ie1lef3 vector backbone using *Age*I-*Xba*I sites to generate a five gene construct, pA3ΔS-gfple1ie1lef3p74 (6160 bp). The transformants were confirmed through *Sal*I restriction digestion. It gave rise to 520 bp insert and 5640 bp backbone.

The five gene sense oriented construct, pA3ΔS-gfplef1ie1lef3p74 was digested with internal *Nhe*I-*Xba*I sites to generate sense and antisense oriented pool of fragment of five genes. Transformants were screened for antisense oriented plasmids. This was confirmed by *HindIII/XmnI* digestion, wherein the antisense colony gave rise to 1.3 kb fragment whereas sense oriented colony gave rise to 2.2 kb fragment.

Sense construct - The *A3:gfp* fused promoter along with four genes and poly A tail were cloned into *pPiggyP25il2-(3XP3-GFP)TQ* using *BglII-NheI* and *BglII-XbaI* sites to generate *pPiggyMG(+)3XP3-GFP* vector (FIGURE 1).

Antisense construct - The antisense fragment obtained as mentioned above was cloned into *pPiggyP25il2-(3XP3-DsRed2)TQ* to generate *pPiggyMG(-)3XP3-DsRed2* vector (FIGURE 1).

6. Inverted Repeat Construct - The above *A3:gfp* fused promoter along with four genes was cloned into pP25(sp*)DsRed vector backbone using *Bgl*II-*Age*I sites to generate sense fragment with spacer region, an intermediary vector form pP25(sp*)DsRed-A3ΔS Sense.Spacer.

7. The antisense fragment of five genes was cloned into intermediary form of sense-spacer vector backbone using *Sse*8337I-*Afl*II sites to generate flip-flop form of multigene construct, pP25(sp*)DsRed-A3ΔS Sense.Spacer.Antisense.Poly A region.

8. The intermediary shuttle vector, pP25(sp*)Sense-Spacer-Antisense vector was digested with *BglII* and *SfiI* sites to generate inverted repeat. This insert was cloned into *PiggyP25IL2-(3XP3-DsRed2)TQ* using *BglII/SfiI* sites to generate *pPiggyA3gfplef1ie1lef3p74.spacer.p74lef3ie1lef1gfp.3XP3-DsRed2 (pPiggyMG(+/-)3XP3-DsRed2)* vector with *DsRed2* as the selection marker gene (FIGURE 1).

Polymerase Chain Reaction: A typical PCR reaction consisted of 20 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 *Taq* DNA Polymerase (NEB, UK) per reaction. Thermal cycling was carried out in a thermal cycler (PE9700, Applied Biosystems) using the following conditions: initial denaturation of 3 min at 95°C; 35 cycles of 30 sec at 94°C; 30 s at 52°C (as per the *Tm* of the respective gene) and 2 min at 72°C, and final extension of 10 min at 72°C. The PCR products were quantified on agarose gel, purified using Qiagen PCR purification columns, according to the manufacturer's protocol.

File S2

Sequence of the selected viral genes

1. The entire *ie1* of BmNPV is 1755 bp, the targeted region is 141 – 450 bp (310 bp)

ie1 **(Accession Number L33180)**

TGGAGCCGACACGGTAGTATCTGACAGCGAGACTGCAGCAGCTTCAAACTTTTTGGCAAGCGTCAATTCGTTAACTGATGATAACGAT

ATAATGGAATGTTTGCTCAAGACCACTGATAATCTCGGAGAAGCAGTTAGTTCTGCTTATTATTCGGAATCCCTTGAGCTGCCTGTTGC

GGAGCAACCATCGCCCAGTTCTGCTTATAATGCGGAATCTTTTGAGCAGTCTGTTGGTGTGAACCAACCATCGGCAGCTGGAACTAAAC

GGAAGCTGGACGAATACTTGGACGATTCACAAAGTGTGGTGGGC

2. The entire *lef1* of BmNPV is 813 bp, the targeted region is 80 – 405 bp (326 bp)

lef1 **(Accession Number L33180):**

GTCACCGGACCACATCCACCAATTCCATAACCAGGATAGCATTGCTTTAATTTGTCTAGCAATTCCTTTGTTATACAACGAGAAAATTTAGTTC

CCTTATAATTATAGCTGTACGGTGCGCGTATTTGTTTGTTAACGTTACAAAAAATATCCCTGTCCACGTCCGGCCAATACTGCAACGTGAGCGC

GTCCAAGTTTGAATCTTGCATATGCGGAATGTACAAACGTACGGCCTCTCTCACACAATGCGCAAAACTGCCCGGCTGAATGCAATCACTATC

CAACTTTGCAGGTTTTTCGAAGGCCTTGTACCGATGCACGCGAAC

3. The entire *lef3* of BmNPV is 1158 bp, the targeted region is 391 – 706 bp (316 bp)

lef3 **(Accession Number L33180):**

TCTTGCGTTTGTGCAATTTTGCAAATTTGAATAGGCTTCGTTTTCTTTAGCCTCGCACAATTCGATGCGCGTAGAGTTGACCACGTTCCAATTCA

TGTACACGTTTGCTCCATTGAAAATTTGTTGACATTTTATATTGTAAATGGTAAAGATTTGGTTTTCATTGTCTTTTAAATATTTAAACACCTCAT

TGATGTCGTCAGACCCCTTTATATTGTTTTTGAATAGATTCATCAGTGTTTTCGCATTGACAAAACATTCCACTTGAACCACGTCGGAATCGTC

GTTAAAATTTTTGTACGCGACTTCAAAAACA

4. The entire *p74* of BmNPV is 1938 bp, the targeted region is 1243 – 1552 bp (310 bp)

p74 **(Accession Number L33180):**

GTTGGCCAGCATTTTGAAAGTGACAAGAATCGTGTCGCCCAGCACGAATCCGACGAGCGATTCCCACCATCTAAACGAACAGCCGCCGTTGA

ATAGATTTCTGCCGAATCGTCGACAGTAGGCTTCGTTGAATTCGCCTTTAAAGCGTTCGGGAAACAAGGGATCGGGATCGGGCCGAACGTTA

AAAGCCGGCACATCGTCCACGCCCATGATCGTGTGTTCTTCGGTGCGCAAGTATGGGCTGTTAAAGTACATTTTGGACAGCGAGTCCACCAA

GATGCATCTGTTGTCGGGCGTGTATCTAAACTCG

File S3

Genomic DNA sequences of different MG(+/-) transgenic lines surrounding *piggyBac* insertions, with the TTAA duplicated sequence appearing at all 5' and 3' insert boundaries. TTAA sequences which are characteristic of *piggyBac* mediated transposition are shown in red colour.

MG(+/-)118A

ACTATCTTTCTAGGGTTAATGATTGAATGCAAACATCAGCGATATGAGAGTAACAAATCAGCTTTAGCGATAAACCTCTACAAGAACAAACGA
ACTACAATAGGTACTTATTATGTTTTTAATAAACTTTTATTATCTTCACACGTTTATTACTATGTATGTATGTNACGGAAACTTTGAACATGATTT
TCACCCCCTTCAAAACGTCGGACTAACTCAAAATTTGGCATACGTATTTATGATTGATGACAATTCAATATTTTAATCAGTTAGATCCAAGGAG
AGGACAGCGATTCTCGTACANACGGTCGCG

MG(+/-)154C

ATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAAGCAAAAGCGGACGTGCGGGCCACAATAGTCGTGCTCATACAAATTTGTGGG
TAGATAGTATATGGCACGATCCAAGGCGAGGACGGCGATTC

MG(+/-)154D

AGCGATATTTCAAGCATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAAGCAGAAGCGGACGTGCGGGCCACAATAGTCGTGCTC
ATACAAATTTGTGGGTAGATAGTATATGGCACGATCCAAGGCGAGGACCGCGATTCTCGTACAAGGGGGGGG

MG(+/-)164B

GGGCGTTTCGCGCTATTTGGAAAGAGAGAGCAATATTTCAAGAATGCATGCGTCAATTTTACGCAGGACTATCTTTCTAGGGTTAAGCAAAA
GCGGACGTGCGGGCCACAATAGTCGTGCTCATACAAATTTGTGGGTAGATAGTTATATGTCACGATCCAAGGAGAGGACAGCGATTCTCGTA
CACGAGGACAGCGATTCTCGTACAC

MG(+/-)164C

TCGCGCTATTTGGAAAGAGAGAGCAATTATTTCAAGAATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAAGCAAAAGCGGACGT
GCGGGCCACAATAGTCGTGCTCATACAAATTTGTGGGTAGATAGTTATATGTCACGATCCAAGGAGAGGACAGCGATTCTCGTACAAACGGT
TACGATTCGAGAAGGG

MG(+/-)170A

ATTTCAGAATGCATGCGTCATTTTACGCAGACTATCTTTCTAGGGTTAAATAAATATGTTAAATGAACAAAAAACCGAATTCCGAGTTTTCAGT

ACAAATCGGCAATTTCATACTTTAACCCCTTATATTATCGTTACGATAATACATTTATATATAGCAATTATTATAAAATACTAAAATATCTTCAAC

AAAATTTACTTACAGAAATTCAATCGAAAACATATTTCTTTAGATCCAAGGAGAGGACAGCGGATTCTCGTACAGCCGGCGGCGATTCGTGAA

GGG

MG(+/-)170B

CGGGCGGATTCGCGCTATTTAGAAAGAGAGAGCAATATTTCAAGNAATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAATAAGC

GTGTTCACGTTTTGGACATGGTTTGCACTGAAACGAGATCCAAGGAGAGGACAGCGATTCTCGTACAAACGGTTACGATTCGAGAAGGGAG

AG

Table S1 PCR primers used for amplification of the different indicated genes/constructs

Gene/construct	Sequence 5’ to 3’
gfp F	ATCGATCGCTAGCCCCGGGATGGTGAGCAAGGGCGAG
gfp R	CGCGGATCCTTACTTGTACAGCTCGTCCA
3XP3GFP F	CCGAATTCAGATCTTCCCACAATGGTTAATTCGAGCTC
3XP3GFP R	CCGGATCCGCCGGCGTACGCGTATCGATAAGCTTTA
DsRed F	CAAGATCTAATTCGAGCTCGCCCGGGGATCTAATTC
DsRed R	TAGCAGATCTGTACGCGTATCGATAAGCTTTAAG
ie1 gene F	CGCTCCGGAGCCGACACGGTAGTATC
ie1 gene R	ACGCGTCGACGGCCCACCACACTTTGTGAAT
lef1 gene F	CGCGGATCCGGTACAAGGCCTTCGAAA
lef1 gene R	CCGTCCGGACCGGACCACATCCACCAA TTCCAT
lef3 gene F	ACGCGTCGACTTGTTTTTGAAGTCGCGTACA
lef3 gene R	TGCTCTAGACTTGCGTTTGTGCAATTTTGCA
p74 gene F	TCCCCCCGGGTAGATACACGCCCGACAACA
p74 gene R	TGCTCTAGACCTGCAGGACCGGTTGGCCAGCATTTTGAA
Viral *lef3* F	GGAGGAAGGCAAGTGCTATG
Viral *lef3* R	CTCAGGGGGATTGCAGTTT
18S rRNA F	CGATCCGCCGACGTTACTACA
18S rRNA R	GTCCGGGCCTGGTGAGATTT

F – Forward; R - Reverse

Table S2 List of transgenic lines obtained

Vector	Injected embryos[a]	Hatched embryos	Fertile moths[b]	G_1 broods with GFP/DsRed positive larvae[c]	Percent G_0 transformed moths	Name of the transgenic lines
pPiggyMG(+) 3XP3-GFP	800	618	550	6	1.1	2G, 58L, 244E
pPiggyMG(-) 3XP3-DsRed2	1380	806	508	6	1.2	126C, 239A
pPiggyMG(+/-) 3XP3-DsRed2	1500	821	600	8	1.3	118A, 118B, 154C, 154D, 164B, 164C, 170A, 170B

[a] *B. mori* eggs were injected with a mixture of *pHA3PIG* and sense, antisense or MG(+/-) constructs
[b] Hatched larvae (G_0) were allowed to develop to moths
[c] The moths were intercrossed and the resulting G_1 broods were screened for GFP/DsRed expression

Table S3 Location of insertion of transgenes

Transgenic Line	Accession Number	Description
MG(+/-)118A	BAAB01060705.1	*Bombyx mori* DNA, contig26425, whole genome shotgun sequence
MG(+/-)164C	AADK01013361.1	*Bombyx mori* strain Dazao Ctg013361, whole genome shotgun sequence
MG(+/-)164B	AADK01013361.1	*Bombyx mori* strain Dazao Ctg013361, whole genome shotgun sequence
MG(+/-)170A	X58446.1	*Bombyx mori* ErA.3 gene for chorion protein
MG(+/-)170B	AADK01004442.1	*Bombyx mori* strain Dazao Ctg004442, whole genome shotgun sequence

Table S4 Pairwise comparisons using Tukey HSD among groups on percentage mortality (values arcsin transformed) in transgenic lines, and their hybrids with CSR2 upon BmNPV treatment done after post-hoc analysis of the corresponding treatment factor using main effects in a one-way ANOVA.

Lines		Mean Difference	Test Statistics	P level	Significance
Transgenic lines					
Control	IE	17.6925	7.8087	0.0001	***
	MG(+/-)	41.0488	18.1171	0.0001	***
	MG(+)	19.7788	8.7295	0.0001	***
	MG(-)	26.6300	11.7533	0.0001	***
	MG(+) x MG(-)	32.2100	14.2160	0.0001	***
IE	MG(+/-)	23.3563	10.3084	0.0001	***
	MG(+)	2.0863	0.9208	0.9863	
	MG(-)	8.9375	3.9446	0.0791	
	MG(+) x MG(-)	14.5175	6.4074	0.0007	***
MG(+/-)	MG(+)	-21.2700	9.3876	0.0001	***
	MG(-)	-14.4188	6.3638	0.0008	***
	MG(+) x MG(-)	-8.8388	3.9010	0.0848	
MG(+)	MG(-)	6.8512	3.0238	0.2882	
	MG(+) x MG(-)	12.4313	5.4866	0.0047	**
MG(-)	MG(+) x MG(-)	5.5800	2.4628	0.5132	
Hybrids of transgenic lines x CSR2					
Control x CSR2	IE x CSR2	21.5750	7.7516	0.0002	***
	MG(+/-) x CSR2	38.8650	13.9637	0.0001	***

	MG(+) x CSR2	14.9100	5.3570	0.0082	**
	MG(-) x CSR2	21.6433	7.7762	0.0002	***
	MG(+) x MG(-) x CSR2	27.6317	9.9277	0.0001	***
IE x CSR2	MG(+/-) x CSR2	17.2900	6.2121	0.0017	**
	MG(+) x CSR2	-6.6650	2.3946	0.5465	
	MG(-) x CSR2	0.0683	0.0246	1.0000	
	MG(+) x MG(-) x CSR2	6.0567	2.1761	0.6431	
MG(+/-) x CSR2	MG(+) x CSR2	-23.9550	8.6067	0.0001	***
	MG(-) x CSR2	-17.2217	6.1875	0.0018	**
	MG(+) x Mg(-) x CSR2	-11.2333	4.0360	0.0758	
MG(+) x CSR2	MG(-) x CSR2	6.7333	2.4192	0.5357	
	MG(+) x MG(-) x CSR2	12.7217	4.5707	0.0322	*
MG(-) x CSR2	MG(+) x MG(-) x CSR2	5.9883	2.1515	0.6538	

The significance between each of the groups was calculated as a *P* value. *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

Table S5 Pairwise comparisons using Tukey HSD among groups on viral accumulation rate (values logarithm transformed) in transgenic and control lines upon BmNPV treatment was done after post-hoc analysis of the corresponding treatment factor using main effects in a one-way ANOVA.

Lines		Mean Difference	Test Statistics	P level	Significance
Control	IE	0.5094	9.0023	0.0001	***
	MG(+/-)	1.0496	18.5470	0.0001	***
	MG(+)	0.5066	8.9521	0.0001	***
	MG(-)	0.7257	12.8241	0.0001	***
	MG(+) x MG(-)	0.9304	16.4417	0.0001	***
IE	MG(+/-)	0.5401	9.5448	0.0001	***
	MG(+)	-0.0028	0.0501	1.0000	
	MG(-)	0.2163	3.8219	0.0961	
	MG(+) x MG(-)	0.4210	7.4394	0.0002	***
MG(+/-)	MG(+)	-0.5430	9.5949	0.0001	***
	MG(-)	-0.3239	5.7229	0.0029	**
	MG(+) x MG(-)	-0.1191	2.1053	0.6732	
MG(+)	MG(-)	0.2191	3.8720	0.0888	
	MG(+) x MG(-)	0.4238	7.4896	0.0002	***
MG(-)	MG(+) x MG(-)	0.2047	3.6176	0.1308	

The significance between each of the groups was calculated as a *P* value. ***P* < 0.01; ****P* < 0.001.

Table S6 List of 10 SSR loci with primer sequences, annealing temperature, $MgCl_2$ concentration and corresponding amplicon size used to genotype the BC_4F_6 population.

Marker	Forward Primer 5' to 3'	Reverse Primer 5' to 3'	Tm (°C)	$MgCl_2$ (mM)	Amplicon size (bp)
Bmg sat001	TTAACCGCGATGTGACTGAA	TGACCGGCACTAAATATGGA	55	1.8	1200 - 1304
Bmg sat015	CATAGACGCCGGGGAATC	CGCGTAATTCGTTTCCAAAT	55	1.8	500 - 540
Bmg sat031	AGACACAGATGGCGATGATG	CTCCAAGGACCTTCGCTTC	55	1.8	700 - 730
Bmg sat084	TCGTGGTAGACCAGCGATAG	GATTTGCCCTCGAAGTTGAC	55	1.8	1145 - 1200
Bmg sat095	GGTCTTCTTCACGTGCTGGT	TGCCGGATCCAAGTTCTTTA	55	1.8	780 - 1035
Bmg sat098	CATGCATTTACCCTCTCCAC	GTGAATTCCGTACTGGCAAA	55	1.8	850 - 1000
Bmg sat108	CCAGGTCATCATGAAGAAGG	ACTGGCTCTGGCAGGTGTT	55	1.8	1005 - 1050
Bmg sat114	CGTCTCTATCTGCGGACGTT	TCAGCTTAGGCAATCCAAGG	55	1.8	627 - 700
Bm sat070	ACCCTCTACGTAGTATATG	CAGCCTTTTTCAGGGCTAA	55	1.8	107 - 148
Bm sat102	GCTCGCCATATGCAATCCTC	CGTCATTGCCTTCATTTCAGTTC	55	1.8	143 - 186