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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 more than 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 combine 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 non-transgenic 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 (BONFIM *et al.* 2007; BUCHER *et al.* 2006; ZHANG 2010a; ZHANG 2010b) 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 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 (HOOPES and ROHRMANN 1991; HUH and WEAVER 1990a; HUH and WEAVER 1990b). Transient plasmid based analyses have led to recognize 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 either 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-long double-stranded 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 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 combine 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 1000rpm 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 hours 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 4 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 the File S2. 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 (ROYER *et al.* 2005; TAMURA *et al.* 2000) 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°C 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(+) x 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 till 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 till 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 x CSR2 and TAFib6 x 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 till 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 5th 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 2X 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°C and 10 min at 95°C followed by 40 cycles at 95°C for 15 sec and 60°C 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 was analyzed by using RT-7500 system (Applied Biosystems®) 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 (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 more than 120 bp that comprises of 120 bp of 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**;** 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 colour were selected as donors of the antiviral trait, for further breeding. BC_1 generation was carried forward to BC_4 based on 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 5^{th} 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 square 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 the day 1 of the 4^{th} instar. A total of 30 larvae were used for each dosage. The larval mortality was recorded from day 1 of post infection till 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 4.9 version. The pairwise comparisons for various parameters among groups was done 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-stranded RNA of the viral gene sequences. Germline transgenesis was carried out as described earlier (ROYER *et al.* 2005; TAMURA *et al.* 2000) 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(+) x MG(-) hybrids, reflecting the effectiveness of the RNAi process in the transgenic silkworm lines.

BmNPV challenges show reduced mortality in transgenics: We analysed 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 x 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 found no difference in the survivability upon viral infection between MG(+/-) and MG(+) x 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; TABLE S5). The viral accumulation in MG(+/-) lines was comparable to MG(+) x MG(-) ($P < 0.6732$) lines

The viral titre 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).

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.

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(+) x 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(+) x 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 backcross 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 >3 fold reduction in viral load in the CSR2 transgenic lines as compared with the nontransgenic lines (FIGURE 6B).

In addition to the transgene marker (DsRed eye color), ten 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 CSR_2 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; 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:** In order 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 involved in multiplication of so-called budded viruses (BV) that establish infection within the host and a second phase where occlusion-derived viruses (ODV) 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*. (JIANG *et al.* 2012) revealed an anti-BmNPV activity in the gut juice of transgenic silkworm that could be enhanced by 33% by over expressing the *Bmlipase-1* which is shown to have antiviral activity. Modifying host gene could help developing control strategies, but, at the moment, the effects are measurable only at sub-lethal doses of virus.

Recent developments suggest that RNA interference-based anti-pathogen strategies are efficient to block the activity of genes crucial to the accomplishment of the viral cycle, when there is a lack of other effective methods (DAVIDSON and MCCRAY 2011; MUELLER *et al.* 2010; TAN and YIN 2004). 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*. (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 either the sense, the antisense strand or in 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 budded virus (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 stranded 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 stranded RNA. The resistance that we measured was mostly the consequence of the coincident inhibition of the IE1, LEF1 and LEF3 accumulation which impacted 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.* (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.* (WANG *et al.* 2009) have shown that P74 of AcMNPV has 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*. (PENG *et al.* 2011) reported 3-fold 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 two fold 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, CSR_2 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% CSR_2 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*. (HUNTER *et al.* 2010) whereby feeding bees (*Apis mellifera*) with dsRNA of Israeli Acute Paralysis Virus (IAPV) protects them from Colony Collapse Disorder (CCD).

In conclusion, we succeeded in getting 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.

FIGURE LEGENDS

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 stranded RNA of the baculovirus partial gene sequences.

Figure 2. Transgenics show reduced mortality upon BmNPV infection. Histogram showing percentage mortality in the transgenic Nistari, nontransgenic and control lines. TAFib6,

transgenic line that expresses non-target 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(+) x 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 standard deviation, N = 48. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Figure 3. Viral accumulation as measured by occlusion bodies (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 standard deviation, N = 36. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (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 5 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 done using anti-GP64 antibody. Lane 1, control CSR2 line; Lane 2**,** TAFib, a transgenic line that expresses non-target 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 x 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 non-target dsRNA; IE, transgenic line with single viral gene target; MG(+), transgenic lines with segments of four viral target genes transcribed in sense orientation; AS, 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(+) x 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 standard deviation, N = 48. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Figure 5. Schematic diagram 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 standard deviation, 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 5 larvae each, and the results were normalized against endogenous 18S rRNA gene. The bars denoted by different letters differ significantly ($P < 0.001$).

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TABLES

TABLE 1

A two-way analysis of variance for the percentage mortality of the transgenic and control lines under normal and BmNPV infected conditions

Source of Variation	Sum of Squares	df	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 x 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(+) x MG(-) and control lines under normal and BmNPV infected conditions. Degrees of freedom (df) 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. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE 2

A one-way analysis of variance for the rate of viral proliferation of the transgenic and control lines under BmNPV infected conditions

Source of Variation	Sum of Squares	df	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(+) x MG(-) and control lines under BmNPV infected conditions. Degrees of freedom (df) and sum of squares for each source of variation is shown. N = 48. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE 3

Cocoon and silk characteristics of CSR2 transgenic lines and CSR nontransgenic (Control) line

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 ± standard deviation.

TABLE 4

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

Type of OBs used	Test strain	LD_{50}	95% Fiducial limit		1/Slope	Regression equation	Standard error
			Upper	Lower			
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 post infection till 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 ($P < 0.05$).

FIGURE 1

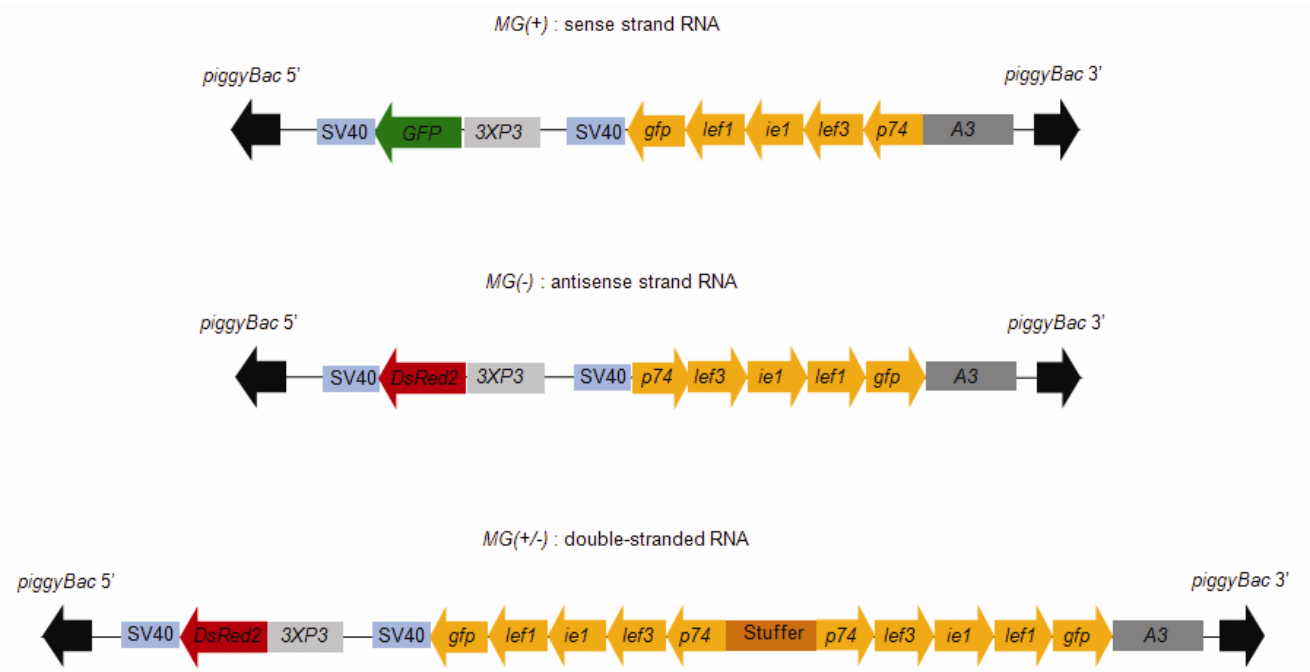

MG(+) : sense strand RNA
piggyBac 5'
piggyBac 3'
SV40
GFP
3XP3
SV40
gfp
lef1
ie1
lef3
p74
A3
MG(-) : antisense strand RNA
piggyBac 5'
piggyBac 3'
SV40
DsRed2
3XP3
SV40
p74
lef3
ie1
lef1
gfp
A3
MG(+/-) : double-stranded RNA
piggyBac 5'
piggyBac 3'
SV40
DsRed2
3XP3
SV40
gfp
lef1
ie1
lef3
p74
Stuffer
p74
lef3
ie1
lef1
gfp
A3

FIGURE 2

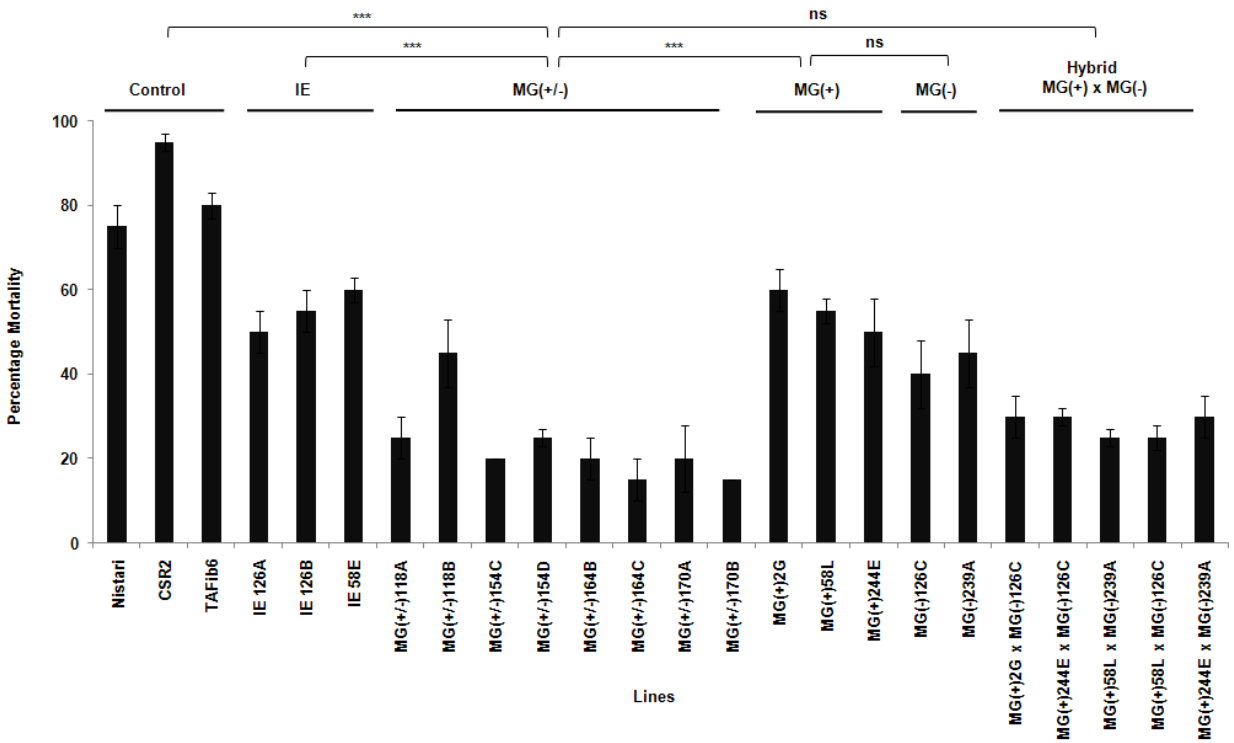

***
ns
***
***
ns
Control
IE
MG(+/-)
MG(+)
MG(-)
Hybrid
MG(+) x MG(-)
Percentage Mortality
100
80
60
40
20
0
Nistari
CSR2
TAFIb6
IE 126A
IE 126B
IE 58E
MG(+/-)118A
MG(+/-)118B
MG(+/-)154C
MG(+/-)154D
MG(+/-)164B
MG(+/-)164C
MG(+/-)170A
MG(+/-)170B
MG(+)2G
MG(+)58L
MG(+)244E
MG(-)126C
MG(-)239A
MG(+)2G x MG(-)126C
MG(+)244E x MG(-)126C
MG(+)58L x MG(-)239A
MG(+)58L x MG(-)126C
MG(+)244E x MG(-)239A
Lines

FIGURE 3

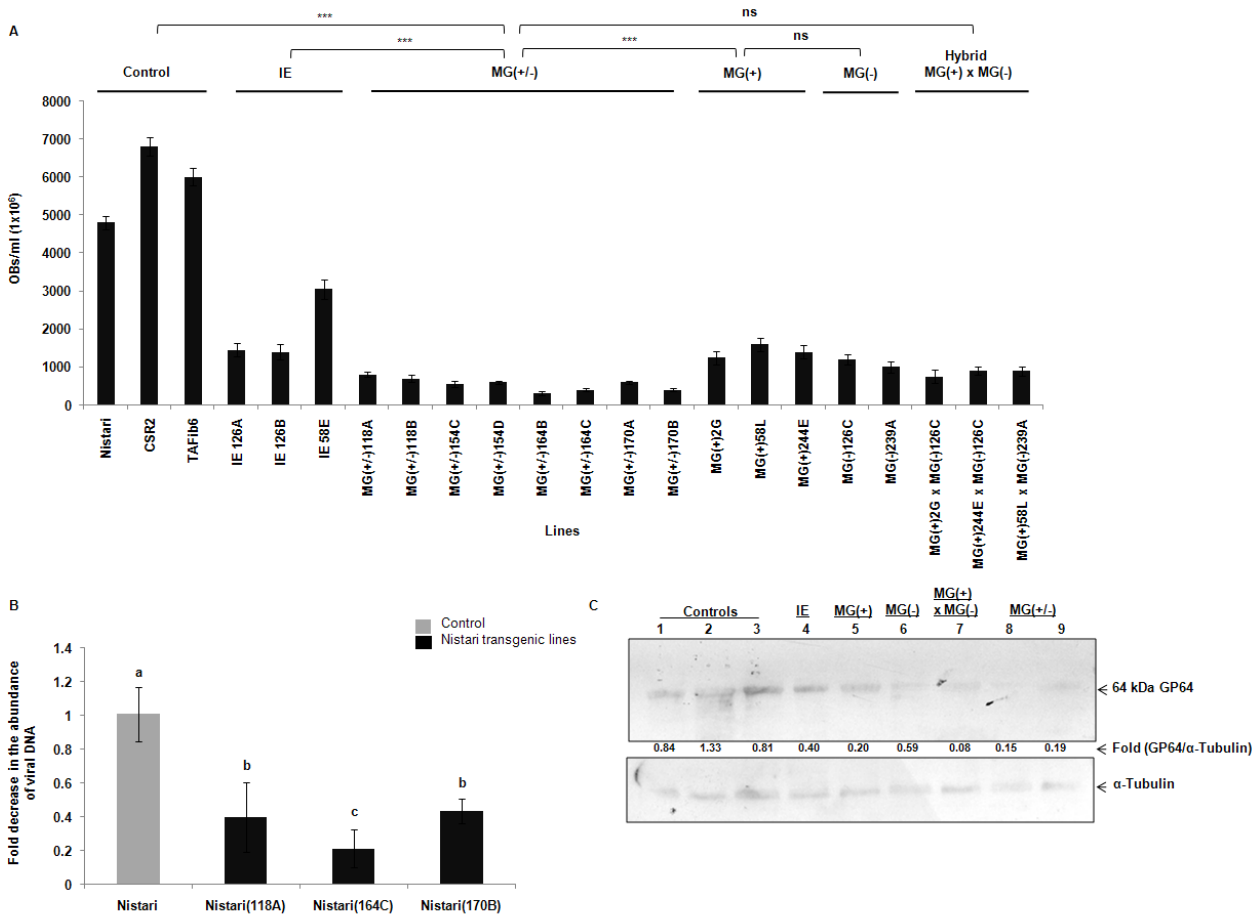

A
***
***
ns
***
ns
Control
IE
MG(+/-)
MG(+)
MG(-)
Hybrid MG(+) x MG(-)
OBs/ml (1x10⁶)
8000
7000
6000
5000
4000
3000
2000
1000
0
Nistari
CSR2
TAFib6
IE 126A
IE 126B
IE 58E
MG(+/-)118A
MG(+/-)118B
MG(+/-)154C
MG(+/-)154D
MG(+/-)164B
MG(+/-)164C
MG(+/-)170A
MG(+/-)170B
MG(+)2G
MG(+)58L
MG(+)244E
MG(-)126C
MG(-)239A
MG(+)2G x MG(-)126C
MG(+)244E x MG(-)126C
MG(+)58L x MG(-)239A
Lines
B
Control
Nistari transgenic lines
Fold decrease in the abundance of viral DNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
c
b
Nistari
Nistari(118A)
Nistari(164C)
Nistari(170B)
C
Controls
IE
MG(+)
MG(-)
MG(+) x MG(-)
MG(+/-)
1
2
3
4
5
6
7
8
9
64 kDa GP64
0.84
1.33
0.81
0.40
0.20
0.59
0.08
0.15
0.19
Fold (GP64/α-Tubulin)
α-Tubulin

FIGURE 4

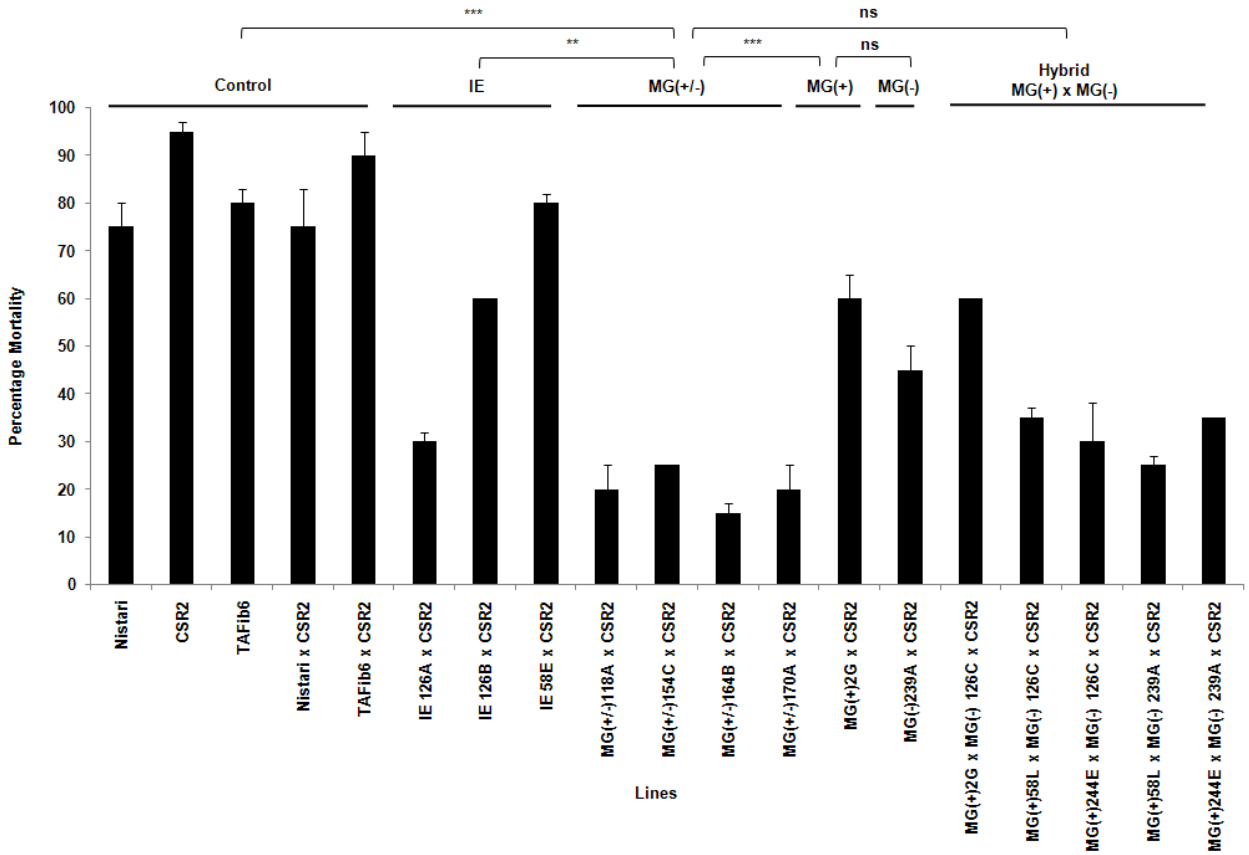

***
ns
**
***
ns
Control
IE
MG(+/-)
MG(+)
MG(-)
Hybrid
MG(+) x MG(-)
Percentage Mortality
100
90
80
70
60
50
40
30
20
10
0
Nistari
CSR2
TAFib6
Nistari x CSR2
TAFib6 x CSR2
IE 126A x CSR2
IE 126B x CSR2
IE 58E x CSR2
MG(+/-)118A x CSR2
MG(+/-)154C x CSR2
MG(+/-)164B x CSR2
MG(+/-)170A x CSR2
MG(+)2G x CSR2
MG(-)239A x CSR2
MG(+)2G x MG(-) 126C x CSR2
MG(+)58L x MG(-) 126C x CSR2
MG(+)244E x MG(-) 126C x CSR2
MG(+)58L x MG(-) 239A x CSR2
MG(+)244E x MG(-) 239A x CSR2
Lines

FIGURE 5

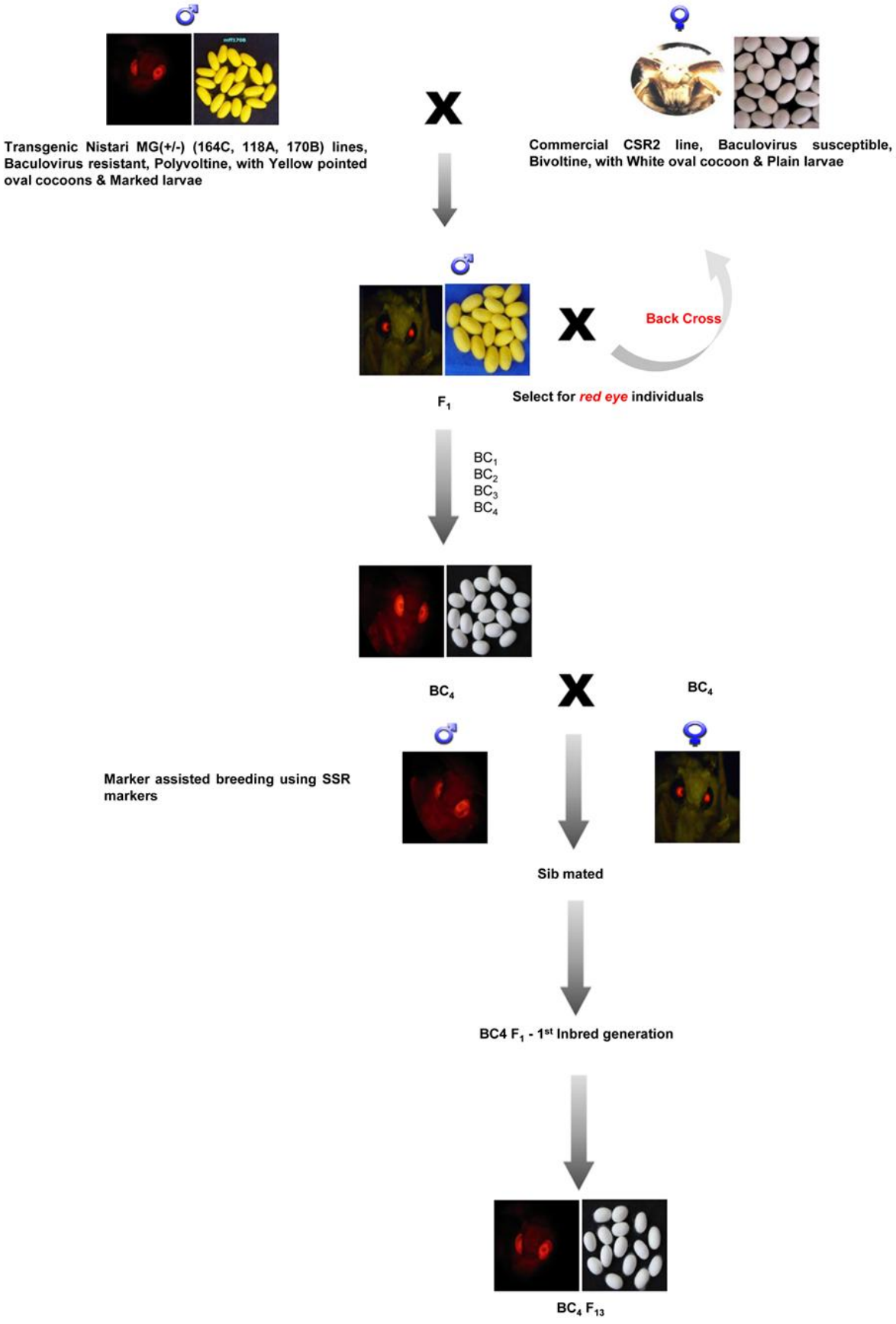
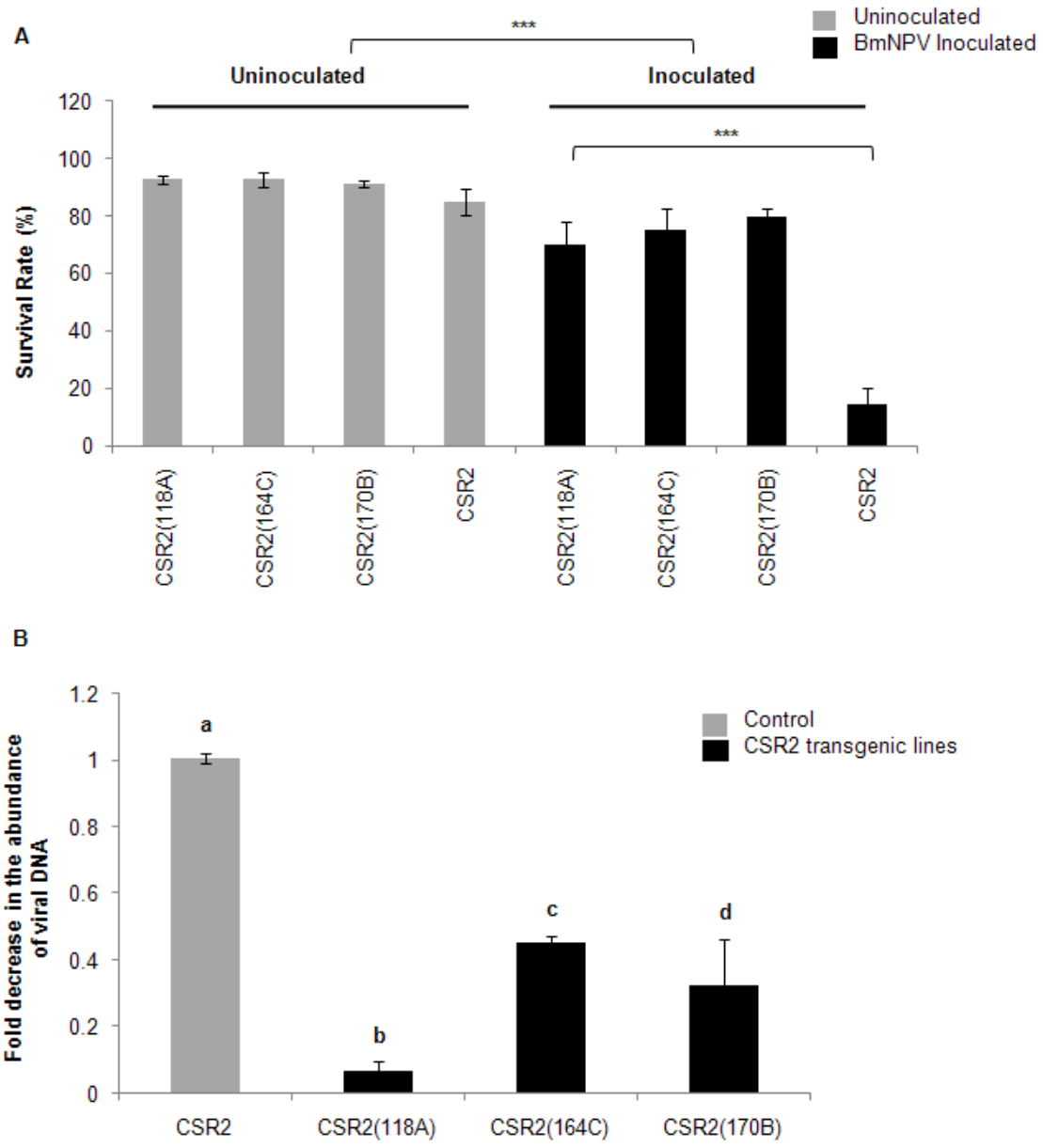

♂
Transgenic Nistari MG(+/-) (164C, 118A, 170B) lines, Baculovirus resistant, Polyvoltine, with Yellow pointed oval cocoons & Marked larvae
X
♀
Commercial CSR2 line, Baculovirus susceptible, Bivoltine, with White oval cocoon & Plain larvae
♂
X
Back Cross
F1
Select for red eye individuals
BC1
BC2
BC3
BC4
BC4
X
BC4
Marker assisted breeding using SSR markers
♂
♀
Sib mated
BC4 F1 - 1st Inbred generation
BC4 F13

FIGURE 6


A
Uninoculated
BmNPV Inoculated
***
Uninoculated
Inoculated
***
Survival Rate (%)
120
100
80
60
40
20
0
CSR2(118A)
CSR2(164C)
CSR2(170B)
CSR2
CSR2(118A)
CSR2(164C)
CSR2(170B)
CSR2
B
Fold decrease in the abundance of viral DNA
1.2
1
0.8
0.6
0.4
0.2
0
a
b
c
d
Control
CSR2 transgenic lines
CSR2
CSR2(118A)
CSR2(164C)
CSR2(170B)