RNA interference (RNAi) mediated inhibition of baculoviral infection in lepidoptera

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

by
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TO

My Family and Friends

For constant support and encouragement

CERTIFICATE

This is to certify that Mr. Sriramana Kanginakudru has carried out the research work in the present thesis under my supervision and guidance for a full period prescribed under the PhD ordinance of this university. I recommend his thesis entitled **"RNA interference (RNAi) mediated inhibition of baculoviral infection in lepidoptera"** for submission for the degree of Doctor of Philosophy of this university.

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STATEMENT

I hearby state that the work presented in this thesis entitled **"RNA interference (RNAi) mediated inhibition of baculoviral infection in lepidoptera"** has been carried out by me under the supervision of Dr. J. Nagaraju at Centre for DNA Fingerprinting and Diagnostics, Hyderabad and that this work is original and has not been submitted in part or full for any degree or diploma of any other university earlier.

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RNA interference (RNAi) mediated inhibition of baculoviral infection in lepidoptera

Do not believe in anything because you have heard it. Do not believe in anything because it is spoken and rumored by many. Do not believe in anything because it is found written in your religious books. Do not believe in anything merely on the authority of your teachers and elders. Do not believe in traditions because they have been handed down for many generations. But after observation and analysis, when you find that anything agrees with reason and is conducive to the good and benefit of one and all, then accept it and live up to it – Gautama Buddha (563 –483 BC)

Abbreviations and terminologies used

AcNPV: *Autographa californica* multiple nuclear polyhedrosis virus, also abbreviated as AcMNPV, also some times referred as *Autographa californica* multiple nucleopolyhedrovirus or *Autographa californica* multicapsid nucleopolyhedrovirus

AU: Arbitrary unit(s) used in luminescence, autoradiography and flow cytometric studies.

aa: Amino acid(s).

bp: Base pair(s).

BmNPV: *Bombyx mori* nuclear polyhedrosis virus, also called as *Bombyx mori* nucleopolyhedrovirus.

BV: Budded virus, a form of baculovirus that brings within the host or within the culture infection. Also known as free virus.

°C: degree centigrade(s).

Co-suppression: Silencing of an endogenous gene because of the presence of a homologous transgene or virus. It can occur at transcriptional (TGS) or post-transcriptional level (PTGS).

Da: Dalton and its variant *kDa* - kilo-dalton.

FACS: Fluorescence activated cell sorter (machine) or FACSorting (process).

FBS: Fetal bovine serum.

FF: An unconventional abbreviation used in the present study to indicate the presence of sense and antisense *ie-1* gene fragments (in 5' to 3' direction) under a single promoter, referred as 'flip-flop'.

g: grams. Its variants include *µg* - micrograms and *mg* – milligrams.

GFP (= eGFP): Enhanced green fluorescent protein, a selection marker used in the present study.

hpi: hours post infection- It is the duration in hours, since the initial inoculum of virus has been removed from the culture.

ie-1: Baculoviral *immediate early-1* gene used as a target in the present study.

siRNA: small interfering RNA. These are ~22nt RNA molecules processed either from exogenous dsRNA or directly added as siRNA.

SV 40: Simian virus 40.

TGS: Transcriptional Gene Silencing. Well observed in plants but has also been seen in animals, wherein the gene expression is reduced at the transcriptional level by chromatin modifications like DNA methylation.

UTR: Un-Translated Region.

VIGS: Virus Induced Gene Silencing. A phenomenon most well understood in plants, where in presence of replicating virus (mostly RNA virus) induces gene silencing.

wt: wild type.

Contents

Chapter I

Prologue

Nothing in biology makes sense except in the light of evolution - Theodosius Dobzhansky (1900-1975)

Introduction

The fight for existence and natural selection are the two fundamental components of the Darwinian theory. While non-Darwinism and anti-Darwinism protagonists question many of the assumptions of Darwinism, some of the basic attribute of Darwin's principles still holds good. Fight for existence between the pathogen as a predator and their host as a prey, for example, is an assumption of Darwinian theory. The predator–prey relationship hastens evolution by introducing means of protecting the interests of both the pathogen and its host. As implied in his book "Andromeda Strain" by the physician turned novelist Michael Crichton, 'it is a bad pathogen that destroys its host, and it is a bad host that is totally destroyed by the pathogen'. In the long run of host-pathogen co-existence, hosts have developed a number of pathways that prevent viruses from replicating and eliminating them. They use the strategies like protein shutdown, prevention of viral movements and apoptosis to protect their interest, while viruses have developed mechanisms to counter them including prevention of apoptosis, suppressing the viral antagonist proteins and prevention of spreading of 'anti-viral message'. A better understanding of pathogen-host interactions will not only help in designing new strategies for combating pathogens infecting the economically important organisms but also give an insight into the process of host-pathogen co-evolution. Researchers try to exploit these pathways as a tool to suit the need of either of the pathogen or the host, for improving the output from an economic perspective.

The domesticated silkworm *Bombyx mori* L, is one of a very few organisms that has both economic and scientific importance. The sericulture industry based on this insect provides means of subsistence to millions of farmers in India. The sericulture industry incurs huge losses due to infection of *B. mori* with a number of pathogens and pests like baculovirus (virus), Bacillus (bacteria), Nosema (microsporidia), and uzi fly (insect). Amongst different viral

pathogens of the silkworm, the baculovirus, *Bombyx mori* nucleopolyhedrovirus (BmNPV) causes 40-60% of the total cocoon crop loss. In the absence of effective management practices, development of the BmNPV resistant silkworm strain is the only practical alternative for enhancing the silk output and improving the standard of living of the farmers.

B. mori and baculovirus

B. mori as a lepidopteran molecular model

Over the past few years fast pace of technology improvement in the field of molecular biology has led to tremendous progress in the field of genetics and genomics. The adoptions of simpler molecular "role model" organisms have paved ways to understand the intricate mysteries of life. Because of its historical, cultural and economic perspective and due to availability of a repertoire of mutants/strains, the silkworm *B. mori* is considered as an ideal lepidopteran model system for molecular and genetic studies (Nagaraju et al. 2000, Nagaraju and Goldsmith 2002).

Life cycle of B. mori

The domesticated silkworm strains could be categorized into two varieties, diapause and non-diapause. While diapause strains of the silkworm hibernate in the embryonic stage during winter, the non-diapause embryos continue to grow without any hibernation. The diapause strains of the silkworms have one or two life cycles per year (hence are termed 'uni' or 'bivoltine') while non-diapause strains will have multiple generations in a year, hence are also known as 'poly or multivoltine' strains. Life cycle of the silkworm could be classified into embryonic, larval, pupal and moth stages extending for about 35-45 days (Fig 1). In the embryonic stage a number of major decisions like sex determination, organ development and germ line formation takes place. The freshly hatched larvae are called as 'ants'. The larval stage extends into five instars and this is the only stage in the life cycle when the silkworm feeds on its

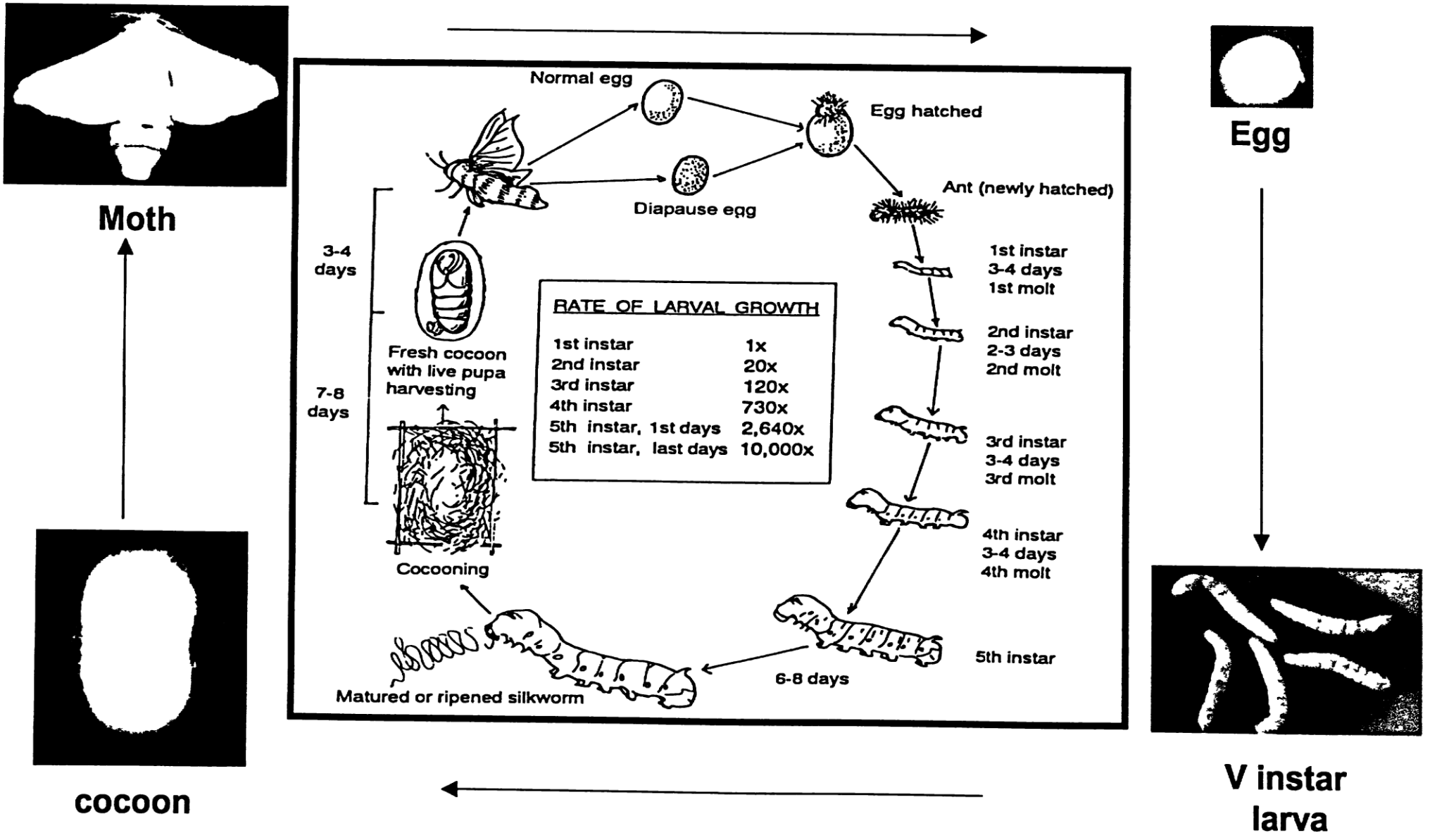


Fig 1 Life cycle of the domesticated silkworm *B. mori*. The photgraphs of the major stages of silkworm are shown while life cycle is shown as a cartoon.

sole food, leaves of the mulberry (*Morus alba).* The process of molting, during which larvae temporarily cease to eat and shed their skin, separates each instar. This periodic shedding of the exoskeleton is called as 'ecdysis' and is regulated by two hormones, one produced by neurosecretory cells in the brain (prothoracicotropic hormone) and another by the prothoracic glands (ecdysone). The larval stage is also the time when silkworms are highly vulnerable to pathogens. On successful completion of the larval growth silkworm starts the process of spinning. During this stage silkworm secretes the silk to ensheath itself in a cocoon. Inside the cocoon, pupation takes place, during which extensive degeneration of mid gut and other tissues takes place. After successful pupation, the moths emerge from the cocoons. Mating, reproduction and egg laying are the tasks of silk moths.

Genome

In the silkworm *B. mori*, there are more than 400 characterized mutations and approximately 3000 strains. The haploid genome size of *B. mori* is 428.7 million base pairs and is distributed over 28 holocentric chromosomes. The whole genome sequencing of *B. mori* has been accomplished by two independent research groups (Mita et al. 2004, Xia et al. 2004). Construction and sequencing of 32 tissue-specific cDNA library for EST database has been completed and is now available in the public domain (Mita et. al., 2002, 2003, see section B of references for the web addresses). The genetic linkage maps of the silkworm have been constructed using RAPD, RFLP and AFLP markers (Nagaraju and Goldsmith 2002). Availability of a wealth of information and a number of molecular markers like RAPD, RFLP, ISSR and SSR makes the silkworm a favourite organism for genetics studies. According to an estimate, there are more than 18,000 genes in the silkworm (Xia et al. 2004).

Transgenesis

Besides its usefulness as an economic insect and a genetic tool, the industrial application of the silkworm has been augmented by the successful germline

transgenesis. piggyBac transposon mediated germline transgenesis has been successfully achieved in the silkworm. Fluorescent transgenic silkworms were successfully obtained by co-injection of *helper* plasmid that expresses piggyBac transposase along with a GFP-fusion construct under constitutively active *B. mori* A3 cytoplasmic actin promoter, (Tamura et al. 2000). This success has opened new ways to introduce exogenous genes for expression of recombinant proteins. Further, it is now envisaged that, use of tissue specific promoters, in the near future will help in developing transgenic silkworm strains with enhanced capacity to express desired traits like silk production with disease resistance capacity.

Pathogens

There are a number of silkworm pathogens that a sericulturist is worried about. From an economic point of view, amongst different pathogens, microsporidian infection and baculoviral infection are of serious consequences. These pathogens not only distress the generation infected, but also affect the next generation as they are transovarially transmitted (Khurad et al. 2004, Rao et al. 2004). *Nosema* and *Vairimorpha* are the two major microsporidian pathogens infecting *B. mori.* The molecular events occurring during microsporidian infection is yet to be systematically analyzed. Amongst viruses, BmNPV is the primary pathogen of academic and economic significance. The other viruses that infect *Bombyx* include Cytoplasmic polyhedrosis virus (BmCPV) that belongs to *Reoviridae*, Densovirus (BmDNV) of *parvoviridae* and *Infectious flacherie virus* (Watanabe 2002). In India, BmNPV infection is the major viral threat that affects the economy of silk cocoon production. As the molecular events associated with BmNPV infection are well understood, it warrants utilization of this knowledge in development of the silkworm strain resistant to BmNPV infection. A few strains of the silkworms show a natural resistance to baculovirus. Non-diapause strains of the silkworm are comparatively more resistant than the diapausing strains. However, the molecular basis for this kind

of natural viral resistance between the strains is poorly understood. Recently a couple of anti-viral proteins have been isolated from the silkworm. These belong to the family of serine protease (*B. mori* serine protease-2 (BmSP-2) isolated from the digestive juice of the silkworm (Nakazawa et al. 2004) and lipase (Ponnuvel et al. 2003). However, the molecular mechanism by which they wield their activity is yet to be investigated.

Life cycle and molecular biology of the baculovirus

Classification of baculovirus

Baculoviridae is one of the fourteen major families of invertebrate virus, which infect arthropods, primarily insects (Murphy et al. 1995, van Regenmortel et al. 2000). The term '*Baculo*' comes from *baculum*, meaning 'stick', which refers to the morphology of the nucleocapsid enveloping the virions. Baculoviridae consists of two genus, Granulovirus (GV) and nucleopolyhedrovirus (NPV). Granulovirus has granular appearance of occlusion bodies and is smaller in size, while Nucleopolyhedrovirus has polyhedral occlusion bodies. There are about twelve species of Granulovirus, with *Cydia pomonella* granulovirus as the type species from this genus. However, there is no report of any granulovirus infection in the silkworm. There are 24 well-characterized species and about 8 tentative species in Nucleopolyhedrosis genus (Table 1). *Autographa californica* multiple nucleopolyhedrovirus (AcNPV) is considered as the type species of this genus (Blissard et al. 2002). Most baculoviruses are host specific, thus they are named according to the host they infect. For example BmNPV is highly infectious to *Bombyx mori* N (BmN) cells, but does not replicate in *Spodoptera frugiperda 21* (Sf21) cells (Kondo and Maeda 1991). However, a few of them (e.g., AcNPV) can infect more than one host. With its influence on economy, BmNPV is also considered as an important species for studies of NPVs, especially in developing countries like India. On the positive note, BmNPV- *B. mori* system is used for large-scale expression of recombinant proteins (Tada et al. 1988, Rahman and Gopinathan 2003).

Table 1 List of NPV and GV from ICTVdb index of virus, abbreviations and their complete genome accession numbers. The underlined viruses indicate presence of more than one species of virus for the same host.

ICTVdB virus code	Virus name	Abbreviation	*Strain*	Genome accession number
00.006.0.01.043	*Agrotis ipsilon* nucleopolyhedrovirus	(AgipMNPV)		
00.006.0.01.003.00.000.002	*Anagrapha falcifera* NPV	(AnfaNPV)	ACNPV isolate/strain	
00.006.0.01.002	*Anticarsia gemmatalis* MNPV	(AgMNPV)		
00.006.0.02.002	*Artogeia rapae* granulovirus	(ArGV)		
00.006.0.01.003	*Autographa californica* MNPV	(AcMNPV)		NC_001623
00.006.0.01.004	*Bombyx mori* NPV	(BmNPV)		NC_001962
00.006.0.01.105	*Buzura suppressaria* nucleopolyhedrovirus	(BusuNPV)		
00.006.0.01.005	***Choristoneura fumiferana* MNPV**	**(CfMNPV)**		NC_004778
00.006.0.01.006	***Choristoneura fumiferana* DEF NPV**	**(CfDefNPV)**		NC_005137
00.006.0.02.028	***Choristoneura fumiferana* granulavirus**	**(ChfuGV)**		
00.006.0.01.131	*Choristoneura rosaceana* nucleopolyhedrovirus	(ChroNPV)		
00.006.0.02.037	*Cryptophlebia leucotreta* granulavirus	(CrleGV)		NC_005068
00.006.0.01.012	*Culex nigripalpus* nucleopolyhedrovirus	(CuniNPV)		NC_003084
00.006.0.02.003	*Cydia pomonella* granulovirus	(CpGV)		NC_002816
00.006.0.01.209	*Epiphyas postvittana* nucleopolyhedrovirus	(EppoNPV)		NC_003083
00.006.0.01.003.00.000.003	*Galleria mellonella* MNPV	(GmMNPV)	ACNPV-isolate/strain	
00.006.0.02.064	*Harrisina brillians* granulavirus	(HabrGV)		
00.006.0.01.238	***Helicoverpa armigera* nucleopolyhedrovirus**	**(HearNPV)**		NC_003094, NC_002654
00.006.0.02.065	***Helicoverpa armigera* granulavirus**	**(HearGV)**		
00.006.0.01.007	*Helicoverpa zea* single NPV	(HzSNPV)		NC_003349
00.006 0.02.077	*Lathronympha phaseoli* granulavirus	(LaphGV)		
00.006.0.01.008	*Lymantria dispar* MNPV	(LdMNPV)		NC_001973
00.006 0.01.009	Mamestra brassicae MNPV	(MbMNPV)		
00.006.0.01.309	*Mamestra configurata* nucleopolyhedrovirus	(MacoNPV)	A and B	NC_003529, NC_004117
00.006 0.01.323	*Neodiprion leconti* nucleopolyhedrovirus	(NeleNPV)		NC_005906
00.006.0.01.017	*Neodiprion sertifer* NPV	(NeseNPV)		NC_005905
00.006.0.01.010	*Orgyia pseudotsugata* MNPV	(OpMNPV)		NC_001875
00.006 0.02 002 00 000.00[illegible]	*Pieris brassicae* granulovirus	(PbGV)	(ArGV)	
00.006.0 02.004	*Phdia interpunctella* granulovirus	(PiGV)		
00.006.0.02.109	*Plutella xylostella* granulavirus	(PlxyGV)		NC_002593
00.006.0.02.114	*Pseudaletia unipuncta* granulavirus	(PsunGV)		
00.006.0.01.003.00.000.004	*Rachiplusia ou* MNPV	(RoMNPV)	ACNPV-isolate/strain	NC_004323
00.006.0.01.003.00.000.005	*Spodoptera exempta* MNPV	(SpexMNPV)	ACNPV-isolate/strain	
00.006.0.01.013	*Spodoptera exigua* MNPV	(SeMNPV)		NC_002169
00.006.0.01.014	*Spodoptera frugiperda* MNPV	(SfMNPV)		
00.006.0.01.457	*Spodoptera littoralis* nucleopolyhedrovirus	(SpliNPV)		
00.006.0.01.015	*Thysanoplusia orichalcea* nucleopolyhedrovirus	(ThorNPV)		
00.006.0.01.003.00.000.006	***Trichoplusia ni* MNPV**	**(TnMNPV)**	ACNPV-isolate/strain	
00.006.0.01.016	***Trichoplusia ni* single NPV**	**(TnSNPV)**		
00.006.0.02.005	***Trichoplusia ni* granulovirus**	**(TnGV)**		
00.006.0.01.491	*Wiseana signata* nucleopolyhedrovirus	(WisiNPV)		
00.006.0.02.006	*Xestia c-nigrum* granulovirus	(XecnGV)		NC_002331

Genome

Average size of the double stranded DNA genome of baculoviruses is 130 kb. On an average baculoviruses contain 150 Open Reading Frames (ORFs) (Ayres et al. 1994, Gomi et al. 1999, Cheng et al. 2002). Many of the baculoviral genes are well conserved across the species (> 55%) suggesting possibility of evolution of these viruses from a common ancestral species. The BmNPV genome is over 90% identical to about three-quarters of the genome of AcNPV (Gomi et al. 1999), while the average amino acid sequence identities between BmNPV and *Orgyia pseudotsugata* MNPV (OpMNPV), another well studied baculovirus, is about 55%, suggesting the divergence of these two viruses within NPV genera. The major difference of BmNPV from AcNPV is the presence of BmNPV specific genes, named as baculovirus repeated ORFs (*bro* genes). However the role of these genes is not known (Gomi et al. 1999). Similarly, there are 16 ORFs present in AcNPV that are missing in BmNPV. Some of the important genes of BmNPV are listed in Table 2.

Life cycle and molecular events during viral infection

NPVs are unique in that they produce two types of virions that have differences in structure and mode of transmission. The first one is occlusion-derived virus (ODV- also known as polyhedral inclusion body - PIB) that transmits between the hosts. Structurally it is covered by a proteinaceous sheath called 'polyhedra', which renders stability to virions. ODVs are assumed to enter the midgut columnar epithelial cells through membrane fusion (Kawanishi et al. 1972, Adams and McClintock1991), The other one is known as budded virus (BV) that transmits within the host. These are unstable outside the host or cell culture media and enter the target tissues by fusion of BV envelop with endocytic membranes. Most of our knowledge about molecular events inside the host cells comes from the studies of AcNPV, which is probably similar in other closely related baculoviruses like BmNPV.

Table 2 A few major BmNPV proteins and their properties. (See Gomi et al. 1999)

NAME	aa Length	Mol. Wt. (Da)	Function*	Note
POLYHEDRIN	245	28.8	Structural protein	
LEF-1	270	31.1	DNA primase	
IAP-1	292	34.0	Inhibitor of apoptosis	
LEF-6	173	20.3	Late gene expression	M
LEF-11	112	13.1	Late gene activation	M
P47	399	47.3	Viral RNA polymerase component	
LEF-12	183	21.1	Late gene expression	
LEF-8	877	101.8	Viral RNA polymerase component	M
LEF-10	78	8.6	Late gene expression	
LEF-9	490	56.4	Viral RNA polymerase component	M
DNAPOL	986	114.4	DNA polymerase	M
LEF-3	385	44.9	Single strand DNA binding	M
IAP-2	249	28.7	Inhibitor of apoptosis	M
LEF-4	465	54.0	Viral RNA polymerase component	M
DNAHEL	1222	143.6	DNA helicase	M
LEF-5	265	31.1	Initiation factor	
LEF-7	227	26.6	Involved in DNA replication	
GP64/67	530	60.6	Major protein of budded virus	
P35	299	34.9	Inhibitor of apoptosis	
HR5	-	-	Replication origin	
P74	645	74.0	Virion envelop protein	
IE-0	261	30.1	Transcriptional activator	M
IE-1	584	66.9	Trans-activator of early genes	
IE-2	422	48.8	"	
HR1	-	-	Replication origin	
LEF-2	210	23.8	Late gene expression	

Note: M = multiple forms of the protein, * Function based on biochemical assays, or by homology to other baculoviral proteins.

The life cycle of baculovirus starts when the caterpillars eat environmentally resistant polyhedra that are deposited on the mulberry leaves. Voracious feeding nature of the *B. mori* ensures consumption of virus in sufficient dose to start the life cycle. Once inside the gut, the polyhedral cover surrounding the virions are dissolved by the extreme alkaline pH (appx. 10) of the midgut digestive juice (Fig 2). The virions thus released enter the midgut by membrane fusion. Once inside the midgut cell, the viral genes start transcribing in a temporal fashion (Huh and Weaver 1990a). The early genes like *ie-1*, *ie-2* are transcribed from the early promoters by the Amanitin sensitive host RNA polymerase II (Huh and Weaver 1990b, Glocker et al. 1993, Hoopes and Rohrmann 1991). This is followed by the expression of late effector factors (*lefs*) that are produced in the 'late stage' of viral replication. The transcription of very late genes are initiated by α–Amanitin resistant viral RNA polymerase that starts transcribing approximately six hours post infection (hpi) (Huh and Weaver 1990b). Most of the very late gene products are involved in viral packaging and cell lysis. Ultimately, the virions encapsulated within the polyhedral envelope are released to environment by cell-lysis causing complete dissolution of the infected larvae. These polyhedra can cause infection in fresh larvae by entering the digestive tract of the new larvae. The liberated free virions multiply in the epidermis, tracheal matrix, fat bodies, and hemocytes of the larvae. In case of AcNPV, it is known that the larval tracheal system provides the major channel for the virus to pass through basal laminae thus spreading the infection (Engelhard et al. 1994).

By using plasmid DNA replication it was shown that six of the *lefs* (*ie-1*, *lef-1*, *lef-2*, *lef-3*, *p143* and *p35*) were essential for replication, while three of them (*ie-2*, *lef-7* and *dnapol)* stimulated replication (Lu and Miller 1995). By contrast, Kool et al.(1994) observed that *p35* was stimulatory but not essential, while *dnapol* was essential for DNA replication. These genes may thus be essential for viral replication *in vivo*. The viral DNA replication is believed to originate from the

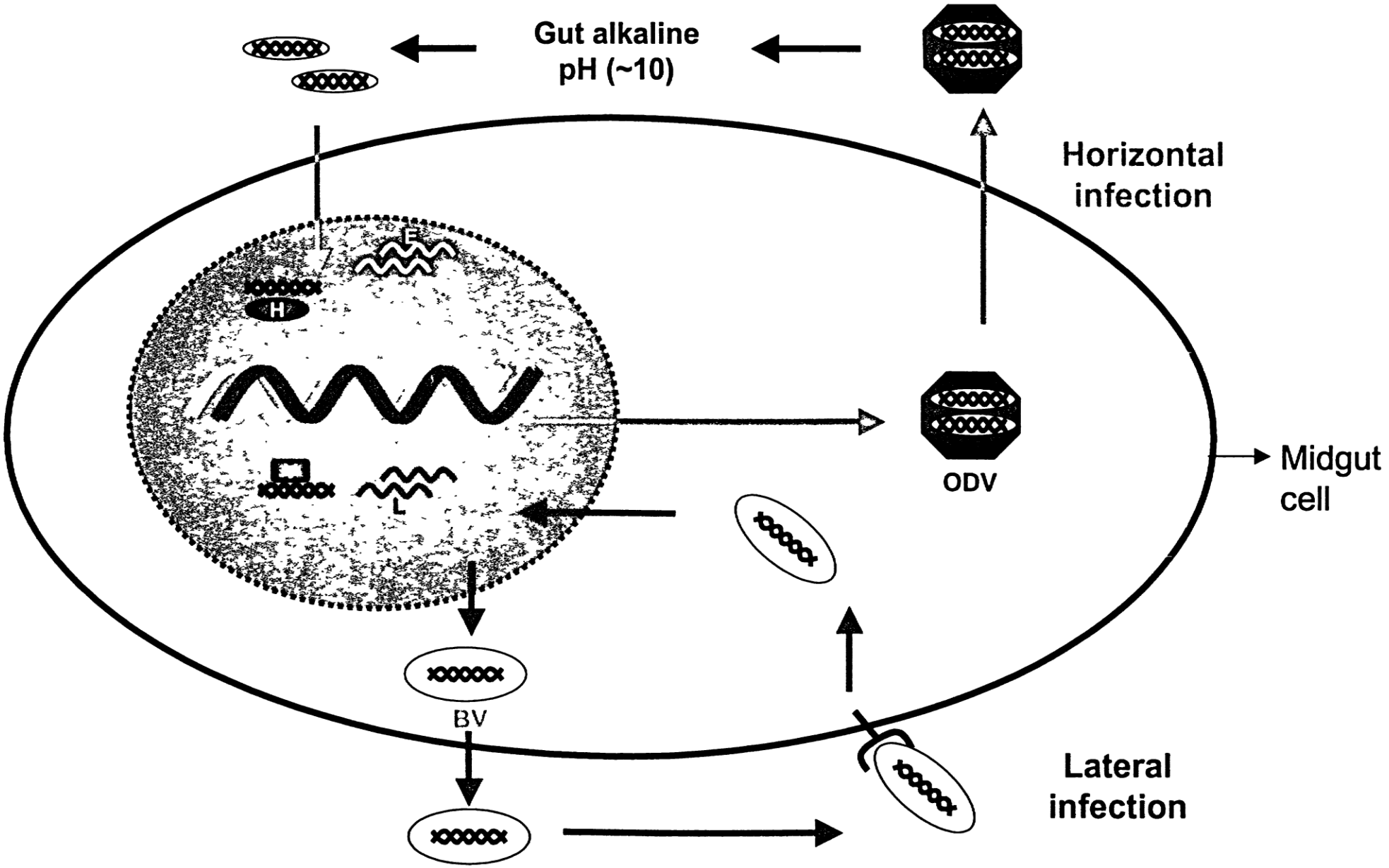


Fig 2 Baculoviral life cycle inside the cell. There are two forms of virus, Occlusion Derived Virus (ODV) brings host-to-host infection, while Budded Virus (BV) causes with-in the host infection. Virions are released from ODV by alkaline pH of the gut, enter the cell. Early (E) class of genes are transgcribed by the host (H) RNA polymerase and later virus produces its own RNA Polymerase (V) that transcribes late (L) class of genes.

homologous regions (*hrs*) (Kool et al. 1993a, Kool et al. 1993b, Ahrens et al. 1995). These hrs are the imperfect palindrome repeats with *Eco*RI at the core and are interspersed throughout the baculoviral genomes (Leisy and Rohrmann 1993, Pearson et al. 1993). They also act as strong enhancers of certain early genes. In BmNPV there are seven hrs (hr1, hr2L, 2R, hr3, hr4L, 4R and hr5).

Baculoviral immediate early-1 (ie-1) gene and IE-1protein

The baculoviral *immediate early-1* gene is one of the five essential genes needed for viral replication. The 1.75 kb *ie-1* gene of BmNPV shows > 90% homology to AcNPV *ie-1* gene. The size of IE-1 is 66.9 kDa. Like many other baculoviral early transcript, *ie-1* gene has a CAGT in the promoter region from where transcription is initiated (Guarino and Summers 1986, Chisholm and Henner 1988, Kovacs et al. 1991a, 1991b). The transcription of *ie-1* continues into the late phases of infection. In case of AcNPV *ie-1* gene, removal of sequences from –13 to +24 eliminates late transcription from *ie-1* promoter. Further, the sequences between +11 to +24 are also required for maximum levels of late *ie-1* expression (Pullen and Friesen 1995).

The protein product of *ie-1* gene, IE-1 plays a major role in control of expression from early promoters. AcNPV IE-1 has been extensively studied for its function and has been suggested to activate promoters of early genes including *ie-2*, *39K* and *p35* (Guarino and Summers 1986, Nissen and Friesen 1989, Carson et al. 1991) as well as its own promoter. IE-1 is directly or indirectly involved in expression of late genes (Passarelli and Miller 1993). The N-terminal acidic domain of this highly conserved protein is required for the *trans*-activation functions, while a 70 aa C-terminus is required for DNA binding and *trans*-inhibition from *ie-0* and *ie-2* promoters (Kovacs et al. 1991a, Yoo and Guarino 1994). Two copies of the Nuclear Localization Element (NLE) at the C-terminus of IE-1, located in a separate monomer of an IE-1 dimer, are essential for nuclear import of IE-1. It has been hypothesized that the IE-1 oligomerizes in the cytosol (Olson et al. 2002) and subsequently

enters the nucleus where it gets involved with the viral DNA replication factories (Okano et al. 1999). Transactivation by IE-1 is dramatically stimulated by *cis* linkage of the promoter to AcNPV *hr* elements. This *hr* dependent trans-activation requires the conserved basic domain of IE-1 (Olson et al. 2003). Like many early genes of viruses, baculoviral IE-1 induces apoptosis (Prikhod'ko and Miller 1996), which is augmented by PE38 (Prikhod'ko and Miller 1999), and suppressed by p35, an anti-apoptotic protein (Clem and Miller 1994, Miller 1997). It is essential for the virus to suppress the apoptosis as otherwise it reduces *in vivo* infectivity (Clem and Miller 1993).

Presence of a temperature sensitive mutant of *ie-1* (Ribeiro et al. 1994) suggests the survival of the mutant, probably because of the leaky function of IE-1. This may advocate that very low level of functional IE-1 is sufficient for survival and successful replication of the baculovirus. However, in this mutant the DNA replication and viral proliferation were delayed.

Though we have a good understanding of some of the molecular events that take place during baculoviral infection *in culturo*, the exact sequence of events is far from complete *in vivo* conditions. Most of our knowledge on molecular biology of virus comes from the study of AcNPV in *S. frugiperda* cell lines. However, further investigations of host-pathogen interaction needs to be carried out for a clear understanding of BmNPV infection in *B. mori*. Suitable strains of *B. mori* with BmNPV resistance not only necessary for improved economy of silk cocoon production, but also for having more scientific knowledge about molecular biology of the virus *in vivo*.

Gene regulation by RNA interference

MicroRNA (miRNA), double strand RNA (dsRNA) and RNA interference (RNAi)

The presence of endogenous antisense (asRNA) and dsRNA has long been noted in eukaryotes (Reviewed in Kumar and Carmichael 1998). While exogenously added asRNA were known to down-regulate the gene expression,

the role of microRNA (miRNA), a duplex small molecular RNA of ~22 nucleotide has come into picture recently (Reinhart , et al. 2002). After the initial report of regulation of gene-expression by miRNA became known, the rate at which new miRNAs are being discovered is astonishing. Bio-computational analysis has greatly helped to identify the potential miRNA coding genes. In *Caenorhabditis elegans* alone, 88 validated miRNA genes have been detected representing 48 gene families. Of these, 46 families (comprising 86 of the 88 genes) are conserved in *C. briggsae*, and 22 families are conserved in humans (Lim et al. 2003b). Further, by using computational approach Lim et al.(2003a) estimated the presence of ~255 miRNA genes in humans representing nearly 1% of the predicted genes. The abundance of miRNA genes strongly supports their importance in normal cellular functioning. In *Arabidopsis* 16 miRNAs were reported by Reinhart et al. (2002). Now it is speculated that miRNA may be present in most of the organisms and potentially in viroids also. It is also likely that most of these miRNA have developmental function, as a prime regulator of gene expression and probably viroid induced disease symptoms. The rapid progress in computational biology and fast accumulation of genome sequence information has augmented the discovery of miRNA to be ubiquitous. However, biochemical, genetical and functional importance of many of these miRNAs and their metabolism need to be investigated.

RNA interference (RNAi) is central to a number of natural RNA-based silencing processes and is becoming a common tool used in a wide range of studies in eukaryotes. The first hint of co-suppression by dsRNA was serendipity than a programmed experimentation. The unexpected observation of suppression of *par-1* gene by both sense and anti-sense strands injected separately into *C. elegans* (Guo and Kemphues 1995) was initially regarded as a technical aberration. Similarly, a few years later the observation of Angell and Baulcombe (1997) suggested the probability of RNA induced viral suppression

by over expression of sense strand RNA. Apparent influence of dsRNA in gene-knockdown was noticed by the report of Waterhouse et al.(1998). In this study it was shown that when a viral protease gene was expressed either in sense-strand or anti-sense strand, it repressed the viral growth, but the viral inhibition was more profound in the hybrid plants that produced dsRNA. However, it was Fire et al. (1998) who showed for the first time that the dsRNA was more potent in inducing gene knockdown than the sense or antisense RNA alone. They suppressed the *unc* gene of the *C. elegans* by injecting *dsunc* RNA and coined the word 'RNA interference' (RNAi) for this phenomenon. Later they showed that by soaking in dsRNA or by feeding *C. elegans* with bacteria harbouring dsRNA-producing construct, RNAi could be induced. Subsequently, there has been an explosion of information regarding the RNAi mechanism and the genes knocked down by this technique. RNAi is now believed to be an ancient anti-viral strategy (Lindenbach and Rice 2002, Downward 2004), probably a part of which the cellular machine also utilizes for gene regulation mechanism (Denli and Hannon 2003, Agrawal et al. 2003) in the form of miRNA pathway. The complexity and interactive nature of these two pathways is far from clear. For example, Dicer enzyme that is necessary for the initiation of RNAi is also involved in miRNA processing from the stem-loop precursors (Tijsterman and Plasterk 2004). Mutation of *Arabidopsis* homologue of Dicer prevents accumulation of miRNAs (Reinhart et al. 2002). The precursor miRNA transcripts (pre-miRNA) are processed by a RNaseIII nuclease (e.g. Drosha), and are exported from the nucleus (by Exportin-5). Dicer cleaves miRNAs into their mature forms to initiate gene silencing. The 3' two-nucleotide overhang structure has been identified as the specificity determinant in targeting and maintaining small RNAs in the RNAi pathway. Thus the miRNA analyses by genetic and biochemical studies are providing a glimpse at the range of biological processes and phenomena regulated by RNAi (Murchison and Hannon 2004). RNAi has been

demonstrated in many arthropod species including the silkworm *B. mori* (Riu et al. 2004). Further, the mechanism of RNAi and its therapeutic use has been reviewed (Bosher and Labouesse 2000, Brantl 2002, Caplen 2003, Kittler and Buchholz 2003, Berkhout 2004, Duxbury and Whang 2004, Lehner et al. 2004).

To briefly summarize the mechanism of RNAi, most of the eukaryotic cells recognize the presence of dsRNA and treat them as a potential hazard to the cell. RNAi pathway eliminates this dsRNA systematically, especially in lower animals. The RNAi can be categorized into three steps - initiator, effector and maintenance. In the initiator step, the Dicer enzyme or its homologue cleaves any dsRNA into smaller ~22 nucleotide fragments. These 'small interfering RNA' (siRNA) or 'microRNA' (miRNA) then become the co-substrate for an enzyme complex called 'RISC' (RNA Induced Silencing Complex). In the effector step, RISC, by complementary base pairing between si/miRNA and homologues mRNA destroys them totally, resulting in gene-knockdown. Technically, the RISC complex requires at least helicase, ribonuclease, single stranded and dsRNA binding activities. In the maintenance step, RNAi is maintained by a poorly understood mechanism. This step may involve spreading of RNAi throughout the organism (Fig 3). The enzymes or proteins involved in three steps of RNAi or related PTGS (Post Transcriptional Gene Silencing) are named differently in various organisms. The major genes and proteins involved in RNAi are given in the Table 3. Further, RNAi of RNAi pathway in *C. elegans* showed the need of nearly 90 components with lethal and viable phenotypes (Kim et al. 2005). However, exact roles of many of these proteins are yet to be clearly demonstrated. Only Argonaute family of proteins, one of the components of RISC complex, is the well-studied enzyme of RNAi pathway. These proteins contain PAZ (*Piwi/Argonaute/Zwille*) and PIWI domains (Cerutti et al. 2000) and directly interact with siRNA in RISC (Lingel et al. 2003, Yan et al. 2003, Ma et al. 2004 and Lingel et al. 2004). The crystal

Table 3 A few of the proteins/Genes Involved in RNAi or PTGS

Protein/Gene (or their homologue)	Organism(s)	Function/ property
MUT-7	*C. elegans*	*RnaseD* homologue
MUT-14	*C. elegans*	DEAD box RNA helicase
RDE-1	*C. elegans*	Initiation of RNAi (dsRNA binding protein)
EGO-1, RRF-1	*C. elegans*	*RNA dependent RNA polymerase* (RdRP).
RDE-4	*C. elegans*	Initiator of RNAi (dsRNA binding protein)
SID-1	*C. elegans*	Transmembrane protein, needed for spreading of RNAi
SMG-2	*C. elegans*	RNA *helicase*, needed for persistence RNAi
DICER (or DCR-1)	*Drosophila melanogaster, Homo sapiens, C. elegans*	Processing of long dsRNA into small RNA molecules.
Aubergine	*Drosophila*	Needed for RNAi like silencing of *stellate* locus
AGO-4	*Arabidopsis*	Needed for maintenance of siRNA and DNA methylation
CAF	*Arabidopsis*	Dicer homologue
SDE-1/SGS-2	*A. thaliana*	RdRP. (EGO-1 homologue)
eIF2C	*H. sapiens*	Component of RISC (RDE-1 homologue)
MUT-6	Plants	RNA helicase
AGO-1	Plants	eIF2C homologue
QDE-2	Fungi	eIF2C homologue

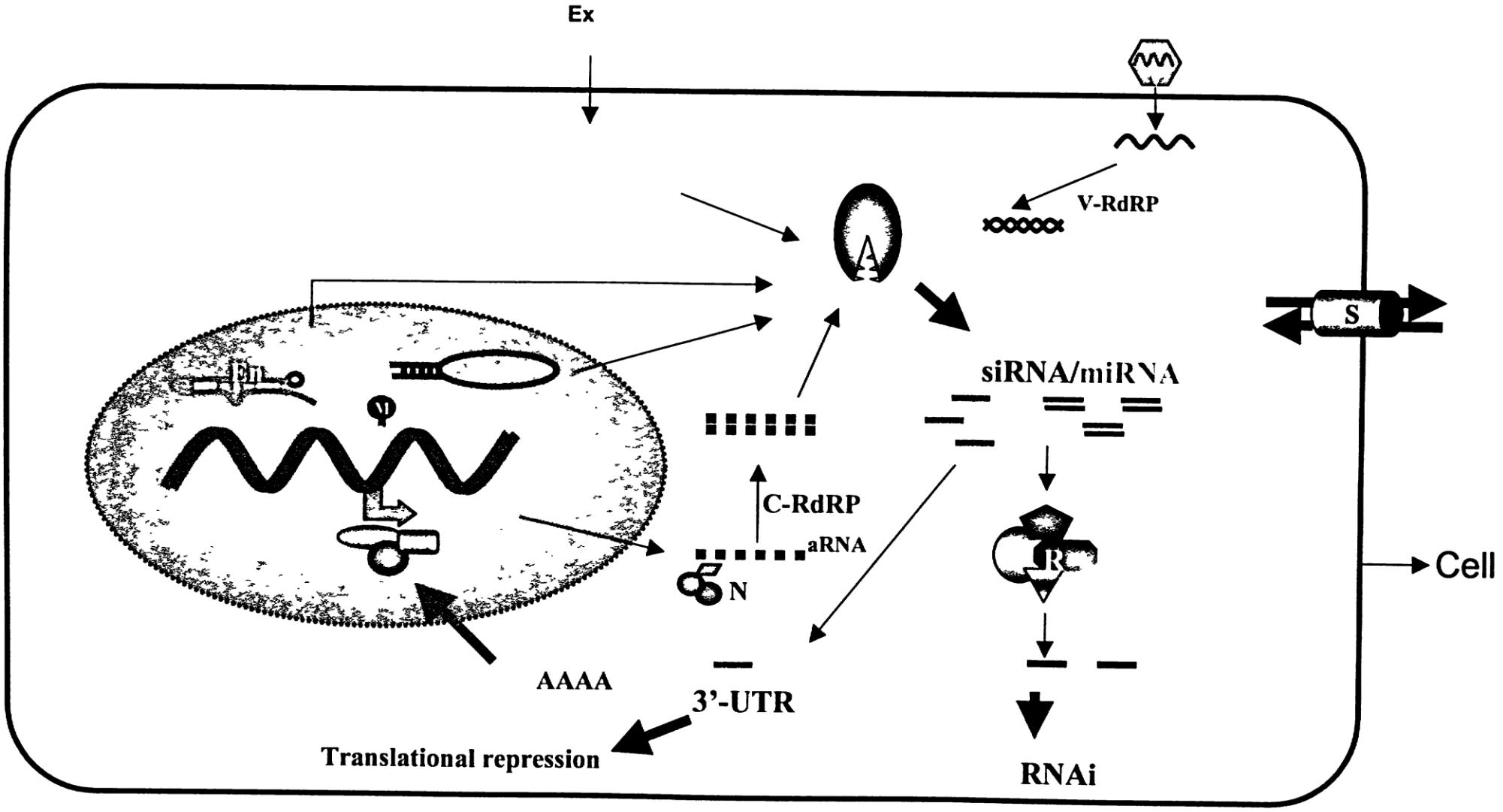


Fig 3 RNAi and related phenomena at a glance. Exogenous RNA (Ex), Virus (V) or si/mi RNA and other double stranded (ds) RNA trigger RNAi mediated gene silencing. These triggers and other endogenous (En) pre-pro miRNA, Transposon (T) are the substrate for Dicer (D), which cleaves them into small interfering (si) or micro (mi) RNA of ~22 nt length. These small RNA are then incorporated into RISC (R) complex and silence the homologous mRNA. miRNA causes translational repression by targeting 3' Untranslated region (UTR). Further, the endogenous genes that produce such dsRNA are silenced by Chromatin modification (C) that may also include Methylation (M). Viral and Cytoplasmic RNA dependent RNA polymerase (RdRP) also gets involved to produce dsRNA. Suppressor of Gene Silencing, SGS (S) proteins are probably involved in spreading of the RNAi signal.

may wedge the use of RNAi mechanism as a tool against virus/viroids that trigger RNA silencing to establish infection.

Relationship between RNAi, PTGS, TGS, VIGS and mRNA surveillance

During the evolutionary pathway, the RNAi has been introduced as a mean to counter the proliferation of 'foreign' genetic materials such as transposable elements and viruses. The role of RNAi in controlling transposable element was revealed immediately after the discovery of RNAi. Knocking down of *rde-1* gene resulted in the perturbation of RNAi and increased transposon mobilization (Tabara et al. 1999). This indicated that natural role of RNAi is to repress transposon mobility, particularly in germ cells.

In plants and fungi, PTGS (known in different forms as antisense suppression, co-suppression in plants, quelling in *Neurospora*, VIGS if induced by virus) could be induced by transgenes or viruses. Characteristically, 22-25 nucleotide (nt) small RNAs specific to transgenes were noticed in transgene induced PTGS (Hamilton and Baulcombe 1999). Further, the virus induced transgene silencing (VIGS), also produces ~25 nt products. VIGS in plants takes place if there is a sequence similarity between the virus and either a transgene or an endogenous nuclear gene (Dougherty et al. 1994, Kumagai et al. 1995). VIGS, like RNAi, could be used as a technique to selectively knockdown a gene for high-throughput genomics (Lu et al. 2003).

The dsRNA can also induce transcriptional gene silencing (TGS). This phenomenon involves the methylation of the DNA homologous to the siRNA/miRNA or other chromatin modification (Matzke and Matzke. 2004). The RNAi machinery is also known to be essential for silencing and heterochromatin assembly at centromeric location in fission yeast (Hall et al. 2002). Now it is established beyond doubt that the chromatin could be modified by RNAi (Allshire 2002).

The eukaryotes have evolved nonsense-mediated RNA decay (NMD) as a surveillance mechanism that is linked to translation, for eliminating errors in

mRNA biogenesis. The NMD pathway results in decay of transcripts containing premature termination codons to prevent the expression of truncated proteins. The role of NMD in RNAi was signified with the discovery that a subset of *smg* genes that are essential for NMD, are also necessary for persistent RNAi. Out of seven *smg* genes involved in NMD, 3 were also needed for persistent RNAi (Domeier et al. 2000). However, the function of individual genes in RNAi is yet to be established (Fig 3).

Viral suppression of RNAi or PTGS

The RNAi/PTGS mechanism itself is not full proof. Because of ancient nature of the mechanism, in the course of evolution, the viruses, particularly plant viruses, have developed strategies to suppress the RNAi directly or indirectly. For example, the p19 protein from the tomato bush virus (tombus virus) is such a viral suppressor of RNA silencing and has been shown to bind specifically to siRNA (Ye et al. 2003). Plants over expressing Helper component-protease (HC-Pro), RNA silencing suppressor from a virus (Anandalakshmi et al. 1998), show increased susceptibility to pathogens (Pruss et al. 2004). PTGS is suppressed by a number of plant viral suppressors like, 2b protein encoded by cucumber mosaic virus (cucumovirus) (Li et al. 1999), rgs-CaM, a calmodulin protein (Anandalakshmi et al. 2000), coat protein of turnip crinkle virus etc. Most of these proteins interfere in any step of PTGS like preventing dsRNA formation, inhibiting Dicer or preventing spreading of siRNA. Two invertebrate suppressor of RNAi have also been identified. These are FHV B2 and nodaviral B2 (reviewed in Riu 2004). However, exact mechanism of their role in preventing RNAi is yet to be ascertained.

The development of RNAi to counter viral replication and its neutralization by virus complements the idea put forward by Darwin in a broader sense. The fight for existence in the long run between predator (=viruses) and the prey (=hosts) incorporates newer strategies in different pathways to protect the interest of each one. Humans with their superior intellectual capacity are

exploiting the host-pathogen relationship to suit their need. While RNAi/PTGS strategy is utilized to increase the productivity from economically important 'hosts', the recombinant or wild type viruses are used as 'predators' to control the insect pest population or to study functional genomics.

Objectives of the present study

In view of the existing information, we hypothesized that by RNAi mediated knockdown of an essential baculoviral gene *ie-1*, it should be possible to inhibit the growth of BmNPV either in cell culture system or in the silkworms. Further, if we develop a transgenic silkworm that constitutively expresses *dsie-1*, it is likely to show baculoviral resistance. Thus the objective of the present study was to analyze the feasibility of RNAi induced baculoviral resistance *in culturo* and *in vivo* in the silkworm, *B. mori*. These objectives were studied independently here in two ways - transient study with transfection or injection of *dsie-1* and viral infection (Chapter II) and heritable study with transgenesis and viral infection (Fig 4)(Chapter III). Finally an over all discussion of RNAi mediated inhibition of baculoviral infection is discussed in the chapter IV.

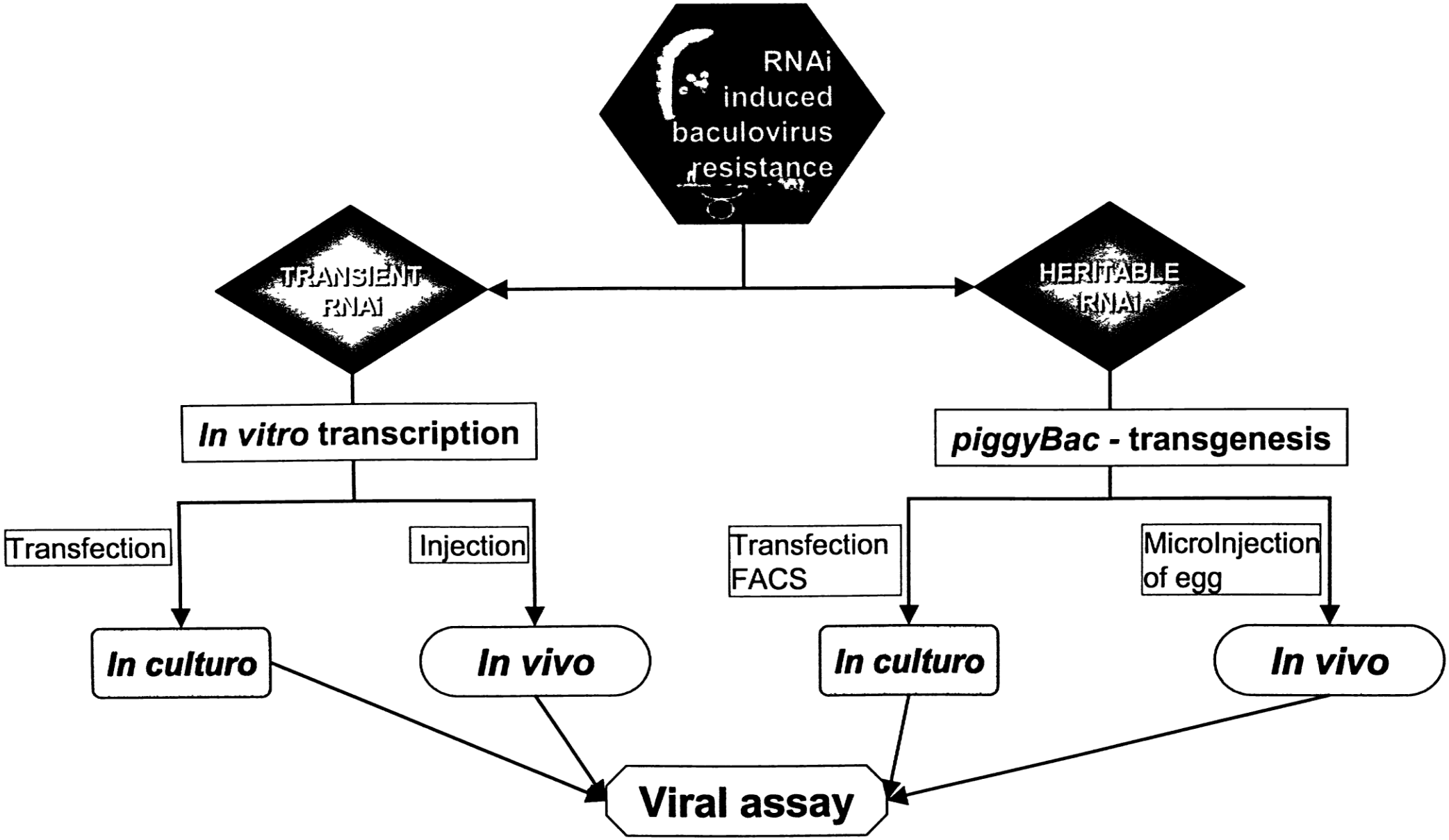


Fig 4 Flow chart of the work carried out to achieve the objective of development of silkworm strains with RNAi mediated inhibition of baculoviral infection. Transient assays are described in chapter II, while transgenic approach is described in chapter III. Pitfalls and future course is described in chapter IV.

Chapter II

Transient *in culturo* and *in vivo RNA*i mediated inhibition of baculoviral infection

Without speculation there is no good and original observation – Charles Darwin (1809-1882)

Introduction

The transfection of cell culture with dsRNA for viral target gene(s) and subsequent assay for viral infection is an easy and rapid approach to test the efficacy of RNAi in inhibiting the baculoviral proliferation. Hence, in transient assays we targeted baculoviral immediate early-1 (*ie-1*) gene for suppression in two different lepidopteran cell lines as well as in the domesticated silkworm, *B. mori*. The vector for *in vitro* RNA synthesis, *pSKGene* was constructed from the plasmid having the complete gene sequence of BmNPV *ie-1* gene, *pBmNPVIEG* (a gift from Dr. Huybrechts – see ref. Huybrechts et al. 1992). For transient RNAi, the double strand *ie-1* was synthesized using *pSKGene* by *in vitro* transcription. In cell culture studies, *dsie-1* RNA was transfected in two lepidopteran cell-lines (BmN - from *B. mori* and Sf9 - from *S. frugiperda*), followed by viral infection with BmNPV or AcNPV. We followed the viral proliferation with western blot, FACS analysis and other conventional methods. We also tested the optimum conditions for initiating the RNAi mediated baculoviral suppression *in culturo*. To test the effectiveness of RNAi *in vivo*, *dsie-1* was also injected into a strain of silkworm (CSR2) susceptible to BmNPV infection and tested for BmNPV resistance. This chapter summarizes the effectiveness and pitfalls of the transient assays.

Materials and Methods

Cell lines, Virus stock, Silkworm strain

BmN cell-line: These cells are derivatives of *B. mori* and are permissive to BmNPV infection. The cells were cultured in TC-100 media (GIBCO-BRL/Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS-Invitrogen) and 40µg/ml of Kanamycin Sulphate (Kan).

Sf9 cell-line: These cells are derivatives of *Spodoptera frugiperda* and are permissive to AcNPV infection. The cells were maintained in Grace Media (Invitrogen) supplemented with 3.33g/Ltr. Yeast extract, 3.33g/Ltr. Lactalbumin

Hydrolysate, 10% FBS and 1X Antimycotic- Antibiotic mix (Invitrogen). The cells were maintained at 26 ± 1°C in a BOD incubator.

Wild type AcNPV (wtAcNPV) was obtained from Saint-Christol-les-Ales, France. Two recombinant BmNPV viruses (BmNPV-polhGFP and BmNPV-P10GFP) were a generous gift from Prof. K.P. Gopinathan (Indian Institute of Science, Bangalore, India). While BmNPV-polhGFP does not produce any PIBs, BmNPV-P10GFP produces PIBs. The baculovirus were amplified in BmN cells and the free virus in cell-culture media was used for experimental purpose. For obtaining BmNPV-P10GFP PIBs, the free virus in cell culture media were injected to the larval hemocoel of the CSR2 strain and five to six days post-infection, the hemolymph was collected from the infected larvae. The fluorescent PIBs were purified by repeated washing and counted using a hemocytometer. Wild type BmNPV were harvested from the hemolymph of the infected silkworms collected from the sericulture farmers. The hemolymph was used for obtaining the free virus. The titre of the free virus was calculated by plaque assay and diluted to get a final concentration of 1X 10^7 BV/ml. The PIBs were used in the experiments involving *per os* inoculation of the silkworms.

The CSR2 silkworm strain used in the present study is a diapausing strain and was obtained from the Andhra Pradesh State Sericulture Research and Development Institute (APSSRDI), Hindupur, India. Larvae were reared on mulberry leaves under standard conditions of silkworm rearing. Healthy fifth instar larvae of were used for experimental purpose after due examination for presence of any infection or injury.

pSKGene Vector

The 470 bp *ie-1* gene fragment was PCR amplified using the following primers and cloned between *Xba*I and *Hind*III sites of *pBluescriptSK*$^+$.

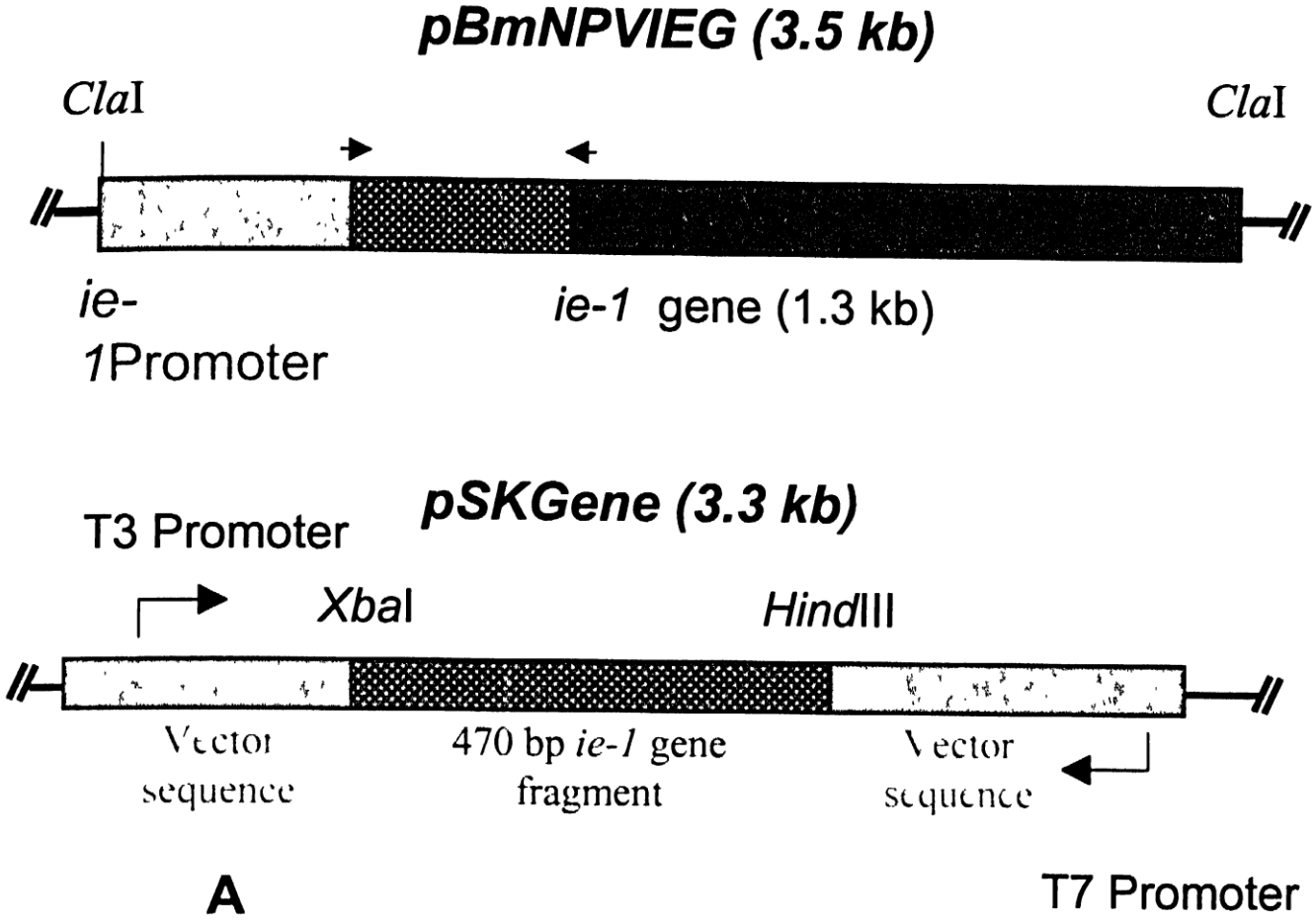
ie-1 Gene F: CCAAACGACTATGACGCAAATT

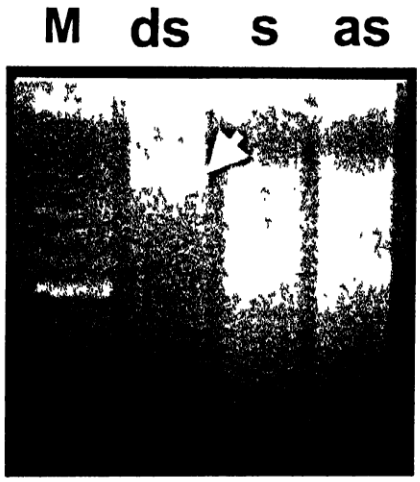
ie-1 Gene R: TTGTTAAATTGGCCCACCAC

The PCR reaction was carried out in 20µl of final volume containing 10 picomoles each of forward and reverse primers, 100 ng of *pBmNPVIEG* as the template in 10 mM Tris-HCl, (pH 8.3 containing 50 mM KCl/1.5 mM $MgCl_2$/0.01% gelatin/0.01% Triton X-100), 1 mM dNTPs, and 0.5 U of IMMOLASE™ DNA Polymerase (Bioline, Germany) per reaction. Thermal cycling was carried out in a thermal cycler (PE9700, Applied Biosystems) using the following conditions: initial denaturation of 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 52°C (48°C for poly A signal), and 2 min at 72°C, and final extension of 10 min at 72°C. The PCR products were separated on 1.0% Agarose gel, purified using Qiagen PCR purification kit according to the manufacturer's protocol. PCR product was confirmed by restriction digestion and DNA sequencing. For DNA sequencing, 250 ng of plasmid was used in a sequencing reaction that contained 8 µl of Ready reaction mix (BDT v 3.0, Applied Biosystems, Foster City, CA) and 5 picomoles of M13 primer. The cycling conditions used were as follows: 25 cycles of 96°C for 10 s, 50°C 5 s, 60°C 4 min. Samples were ethanol precipitated, washed with 70% ethanol and resuspended in Hi-Di™ formamide (Applied Biosystems). The sequencing was carried out in ABI Prism 3100 Genetic Analyzer (Applied Biosystems) to obtain *pSKGene*. This vector was used for *in vitro* RNA synthesis. The graphical representation of the vectors used is shown in Fig 5A.

In vitro transcription and dsRNA synthesis

For in vitro transcription, the *pSKGene* was linearized with either *Xba*I or *Hind*III enzymes. Three µg of the linearized plasmid was used as a template in a reaction volume of 50 µl that contained 400 µM each of NTPs, 1 mM of $MgCl_2$, 1 mM DTT and 10 U of T7 (for *Xba*I template) or T3 (for *Hind*III) RNA polymerase. RNAsin, a commercial RNAse inhibitor (Promega) was used at a final concentration of 0.5U/ml. The *in vitro* reaction was carried out at 37°C for 6-8 hrs and was stopped by extracting the RNA using TRIZOL® reagent. RNA was ethanol precipitated, washed with 80% ethanol, dissolved in





B

Fig 5 *In vitro* synthesis of *dsie-1* for transient RNAi mediated inhibition of baculoviral infection.

A Vectors used for transient RNAi assays. *pBmNPVIEG* has a complete ORF of BmNPV *immediate early –1* gene cloned between *Cla*I sites. Gene segment is depicted without vector back-bone. *pSKGene* was constructed for *in vitro* transcription, has 470 bp of N-terminal *ie-1* gene fragment cloned between *Xba*I and *Hind*III sites of *pBluescript SK*$^{+}$ vector.

B. *In vitro* transcribed s - sense, as - anti-sense and ds - double stranded *ie-1* RNA. M – 50 bp marker. Yellow arrow indicates the expected *dsie-1* band.

DEPC-treated water and stored at -70°C. RNA was quantified by spectrophotometric method and quality was confirmed by electrophoresis. For double strand RNA synthesis, equi-molar concentration of the sense and anti-sense strand RNAs were mixed together in an Eppendorf tube in 1X Insect Buffered Saline (160mM NaCl, 10mM KCl, 4 mM $CaCl_2$, pH 6.2) and denatured in a boiling water bath for 2 min and cooled slowly to room temperature. The dsRNA was verified by running the sample on agarose gel and *RNAse A* resistance (Fig 5B). The *dsie-1* was stored at -20°C until used.

In culturo RNAi mediated viral resistance in Sf9 and BmN cell lines

Transfection of Sf9 or BmN cells

The exponentially growing Sf9 or BmN cells were transfected with the desired amount of *dsie-1* or mock transfected using the transfection reagent DAC-30 according to the manufacturer's protocol. Briefly, the *dsie-1* and DAC-30 mixture was prepared in the Grace-media or TC-100 media without serum and antibiotics and layered onto the 50% confluent cells. After four hours of incubation, the transfection media was replaced by fresh media containing 10% serum and antibiotics. Other transfection reagents like Escort (Sigma-Aldrich) or Lipofectamine (MBI Fermentas) were also tried in place of DAC-30 reagent.

In vitro RNAi of anti-sense ie-1 (asie-1) RNA

For demonstration of specific effect of RNAi, 10 X 10^6 Sf9 cells were transfected with ten µg of *dsie-1* or mock transfected using the transfection reagent lipofectamine. Twenty-four hours post transfection, the cells were washed and lysed in hypotonic solution (10 mM HEPES pH 7.3, 6 mM β-mercaptoethanol with Protease inhibitor cocktail (Sigma) and 0.5 U/ml RNAsin) according to Hammond et al. (2000). The lysate was incubated with radiolabelled *asie-1* RNA. For synthesizing the radiolabelled *asie-1*, *in vitro* transcription reaction was carried out using *pSKGene* digested with *Xba*I as a template and T7 RNA polymerase with P35- α-ATP (3500 Ci/mmol) as the sole source of ATP. The radiolabelled *asie-1* was gel purified and used for

incubation with the lysate at 37°C for 30 min. Each reaction consisted of 5 µl of the cell lysate in reaction buffer (20 mM HEPES pH 7.3, 110 mM KOAc, 1 mM $Mg(OAc)_2$, 3 mM EGTA, 2 mM $CaCl_2$, 1 mM DTT and 0.5 U/ml RNAsin) and 10,000 c.p.m. *asie-1*. The reaction was terminated by extracting the RNA by Trizol® reagent and precipitating small molecular RNA in ethanol and 1.0% Glycogen. The RNA was then run in 12% denaturing (8M urea) PAGE in 1X Tris-Borate-EDTA buffer (89mM Tris base, 89mM Boric acid, 2 mM EDTA pH 8.0) according to Sambrook and Russel (2001). The gel was vacuum dried and exposed to X-ray film.

Viral infection protocol

Viral infection was carried out according to Summers and Smith (1987). Briefly, exponentially growing cells were detached by gentle shaking and the cell count was carried out using a hemocytometer. The cells were diluted to desired number (5 X 10^4 to 1 X 10^6) in serum free media, seeded into six well plate or 35-mm dish and were allowed to attach to the culture dish for an hour. The virus of known titre was then layered onto the cells, allowed for attachment to cells for one hour at room temperature. After this period of incubation, the virus containing media was removed and replaced with fresh media containing 10% FBS. The time point at which the fresh media was added was considered as 0 hpi, and the cells were maintained without replacing the media in an incubator at 26 $\pm$ 1°C for known duration of time expressed as hpi.

Western blot by using antibodies against GP64 or LEF-3

A commercial anti-body against GP64 coat protein of baculovirus was obtained from Novagen. This monoclonal antibody can cross react with the GP64 of BmNPV. LEF-3 monoclonal antibody was a kind gift of Linda Guarino. These antibodies were used for measuring the GP64 and LEF-3 proteins, respectively, in the cells infected with the virus as a measure of viral proliferation.

Cell lysate was prepared by suspending the cells in lysis buffer (50 mM Tris-Cl, pH 8.0, 120 mM NaCl, 0.5% (v/v) NP-40, 0.2 mM sodium orthovanadate, 100 mM NaF) containing 1 X protease inhibitor cocktail (Sigma). The cells were repeatedly freeze-thawed by dipping in liquid nitrogen and warming to 37°C. The protein concentration in the lysate was measured by using BioRad protein estimation reagent in a BioRad ELISA plate reader. Briefly, the commercial reagent was diluted 1:5 with water. The lysate was diluted 50 times in water. 50 µl of diluted protein sample was mixed with 200 µl of diluted BioRad reagent. The absorbance was measured at 595 nm in an ELISA reader along with Bovine Serum Albumin (BSA) as a standard. Equal amount of protein was denatured in SDS-Loading dye, separated on 10% SDS-PAGE in Tris-Glycine buffer (25 mM Tris, 250 mM Glycine, pH 8.3, 0.1% SDS) and the proteins were electro-blotted to Hybond P+ membrane (Sambrook and Russel, 2001). Briefly, the membrane was cut into the size of the gel, and then placed on the gel avoiding air bubbles. The gel-membrane was sandwiched between wet Whatmann papers, and placed inside the blotting cassette (BioRad). The cassette was placed inside the blotting apparatus such that the membrane was facing the positive electrode. The electro-blotting was carried out in immersion buffer (48 mM Tris base, 39 mM Glycine, 20% Ethanol, 0.0375% SDS) for four hours at 75 V in a cold chamber. The protein transfer was confirmed by staining the membrane with Ponseu S stain (0.1% in 5% Acetic acid) and the gel with Coomassie Brilliant Blue R-250 stain (0.25 g/100 ml).

The blot was then probed with the respective antibody. The stained membrane was repeatedly washed with the 1XTBS (140mM NaCl, 10mM Tris/HCl, 0.02% (w/v) NaN_3 (pH 7.4), then blocked with 5% skimmed milk in 1X TBST (TBS with 0.05% (v/v) Tween-20 (polyoxyethylene sorbitan monolaurate) for one hour, washed once with 1X TBST, then incubated in primary antibody at a dilution of 1:5000 in 1X TBST overnight. At the end of the incubation period, the membrane was washed four times in 1X TBST and was incubated for an

hour in appropriate secondary antibody (anti-mouse for GP64 and LEF-3) conjugated to horseradish peroxidase suspended in 5% skimmed milk in 1X TBST at a dilution of 1:1000. The membrane was then washed six times in 1X TBST, incubated in chemiluminiscent detection reagent (ECL detection kit, Amersham) according to manufacturer's protocol. It was then exposed to an autoradiogram film and the autoradiogram was developed using commercially available developer and fixer. The band was quantified densitometrically by using QuantityOne software of BioRad gel documentation system and a graph was plotted.

RT-PCR of ie-1 and β-Actin transcripts

For RT-PCR the RNA was extracted from the cells infected with the respective virus by using Trizol® reagent (Invitrogen) and suspended in water. For cDNA synthesis, about 200 ng of DNA free total RNA was incubated with MMLV reverse transcriptase (MBI fermentas) and the reaction was carried out according to the manufacturer's instructions. β-Actin was used as an internal control for RT-PCR. The PCR reaction was carried out at 52°C (*ie-1*) or 59°C (β-Actin) of annealing temperature, as described above for 25 cycles using equal amounts of cDNA. The sequence of the primers used are 5'-CACTGAGGCTCCCCTGAAC – 3' (forward) and 5' – GGAGTGCGTATC CCTCGTA – 3' (reverse). The intensity of the PCR products was estimated densitometrically using QuantityOne software (BioRad). The ratio between the band intensity was calculated by the following formula.

Ratio = band intensity of *ie-1* (AU) / band intensity of β-Actin (AU)

Interval between transfection and infection

Sf9 cells were seeded at a density of 1 X 10^6 cells per well of 6 well plates and transfected with the 2 µg *dsie-1*per well. The wtAcNPV was added during the transfection of *dsie-1* (simultaneously - SI) or 24 hours post-transfection (Prior transfection - PI). At 48 hpi, the experiment was terminated and western blot analysis was carried out using GP64 antibody.

Amount of dsie-1

The confluent Sf9 cells were seeded in 12 well plates at a density of 5 X 10^5 cells per well. The cells were transfected with 0.5 to 5 μg of *dsie-1* RNA using Escort ™ transfection reagent (Sigma, USA) according to the manufacturer's protocol. Briefly, the *dsie-1* RNA was mixed with the appropriate volume of transfection reagent (2 volumes of reagent to 1 volume of *dsie-1* RNA of concentration 1μg/μl) and incomplete medium. The mix was incubated at room temperature for 10-15 min and layered on to the cells and allowed for RNA uptake overnight. The transfection reagent was removed and replaced with fresh complete media. Twenty-four hours post transfection, the cells were infected with 1 multiplicity of infection (MOI) of wt AcNPV. Cell lysate was prepared at 48 hpi and western blot experiments were carried out.

Progression of infection

Confluent BmN cells were seeded at a density of 1 X 10^6 in each well of six well plates, transfected with *dsie-1* at a concentration of 2μg/well. Cells were then infected with 1 MOI of BmNPV. The infection was allowed to progress for different time point from 24-96 hpi. At the termination of each time-point, the cells were lysed and western blot analysis was carried out as mentioned earlier. Microscopic observation was also carried out for the signs of infection..

FACS analysis of BmN cells infected with recombinant BmNPV (BmNPV-polhGFP)

The BmN cells were seeded at a density of half a million cells per well. The cells were transfected with one μg dsie-1per well or mock transfected. After 24 hr incubation, the cells were infected with a recombinant BmNPV having GFP under polyhedrin promoter (BmNPV-polhGFP) at a titre of one MOI. The cells were analyzed for the presence of BmNPV at 2 to 4 days pi by FACS analyses as follows:

The cells were removed gently, washed with 1 X PBS (100 mM NaCl, 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH 7.4) and suspended in the sheath fluid (1x PBS with 0.02% Sodium Azide). For identifying the FACS event occurring in

the 'dead cell zone' that reflects dead components (like membrane, nuclei or other debris), transformed cells were lysed in water by endo-osmosis, vortexed and their FSC and SSC values were recorded. Based on this observation, the FACS machine was calibrated such that the viable cells were defined by a FSC value between 200 AU and 1000 AU. The number of viable events was counted and partitioned into transformed (high fluorescence) and non-transformed (auto-fluorescing) regions.

BmNPV resistance in dsie-1 injected silkworms

Healthy silkworm larvae of CSR2 strain that were molting from fourth to fifth instar were used for *in vivo* viral inhibition assay. After the fourth molt, each larva was injected with either water (control) or 10 or 25 μg of *dsie-1* RNA near the thoracic segment. Thirty larvae were injected in control and 25 μg groups, while 9 larvae were used for 10 μg group. A day after the *dsie-1* injection, the larvae were fed with 10^4 polyhedral inclusion bodies (PIBs)/ml (sub-lethal dose) or 10^7 PIBs/ml smeared on to a square piece of mulberry leaf. Only those larvae that completely ate the PIBs smeared leaves were maintained. Each larva ate ~ 400 PIBs (sub-lethal) or 4 x 10^5 PIBs (lethal). The larvae were maintained on mulberry leaves until five days post-infection. The dead larvae were removed immediately to prevent secondary infection. The percentage mortality was calculated until the fifth day of pi.

Results

In vitro lysis of anti-sense ie-1 to small molecular RNA was observed only in dsie-1 transfected cells

To verify whether the *dsie-1* could initiate *in vitro* RNAi, radiolabelled *asie-1* was incubated with the lysates from *dsie-1* treated cells or mock-transfected cells. On incubation of *asie-1*for half an hour at 37°C, we observed the characteristic 21~ 22 nt small molecular RNA products only in the reaction that had lysate from *dsie-1* treated cells, but not in mock transfected cells. Since the *asie-1* was

fragmented only in *dsie-1* treated cells and not in mock transfected cells, it suggests the specific need for prior activation of RNAi machinery (Fig 6) by *dsie-1*. This also suggests the induction/activation of RNAi machinery by the transfected *dsie-1*.

Prior activation of RNAi machinery is necessary to initiate viral suppression in Sf9 cells

In order to confirm the necessity of prior activation of RNAi machinery in suppressing the viral proliferation, the *dsie-1* was transfected either 24 hours prior to the viral challenge or along with the virus. The western blot results indicate that, simultaneous transfection with infection (SI) did not trigger viral suppression while there was 3 fold decrease in the GP64 protein level when the cells were pre-incubated with *dsie-1* (Fig 7) suggesting the necessity for prior activation of RNAi machinery.

Higher amount of dsie-1 does not increase efficiency of viral inhibition

For determining the minimum amount of *dsie-1* that could inhibit viral growth, half a million Sf9 cells were infected with 1 MOI of AcNPV. The western blot analysis carried out 24 hpi using the anti-GP64 antibody showed that under a given experimental condition, the minimum amount of dsRNA that could elicit the maximum viral inhibition was about 1 μg for half a million cells. Increasing the dsRNA did not increase the inhibition (Fig 8). However, the amount of *dsie-1* that could elicit the RNAi mediated viral suppression may depend on the transfection efficiency and the cell number apart from other experimental conditions.

Transient transfection with dsie-1 protects cells only at early stages of infection in BmN cells

BmN cells were incubated with *dsie-1* for 24 hours following which, they were infected with wtBmNPV and maintained for a duration of 24 –96 hours. The western blots carried out at the end of known hpi using anti-GP64 antibody

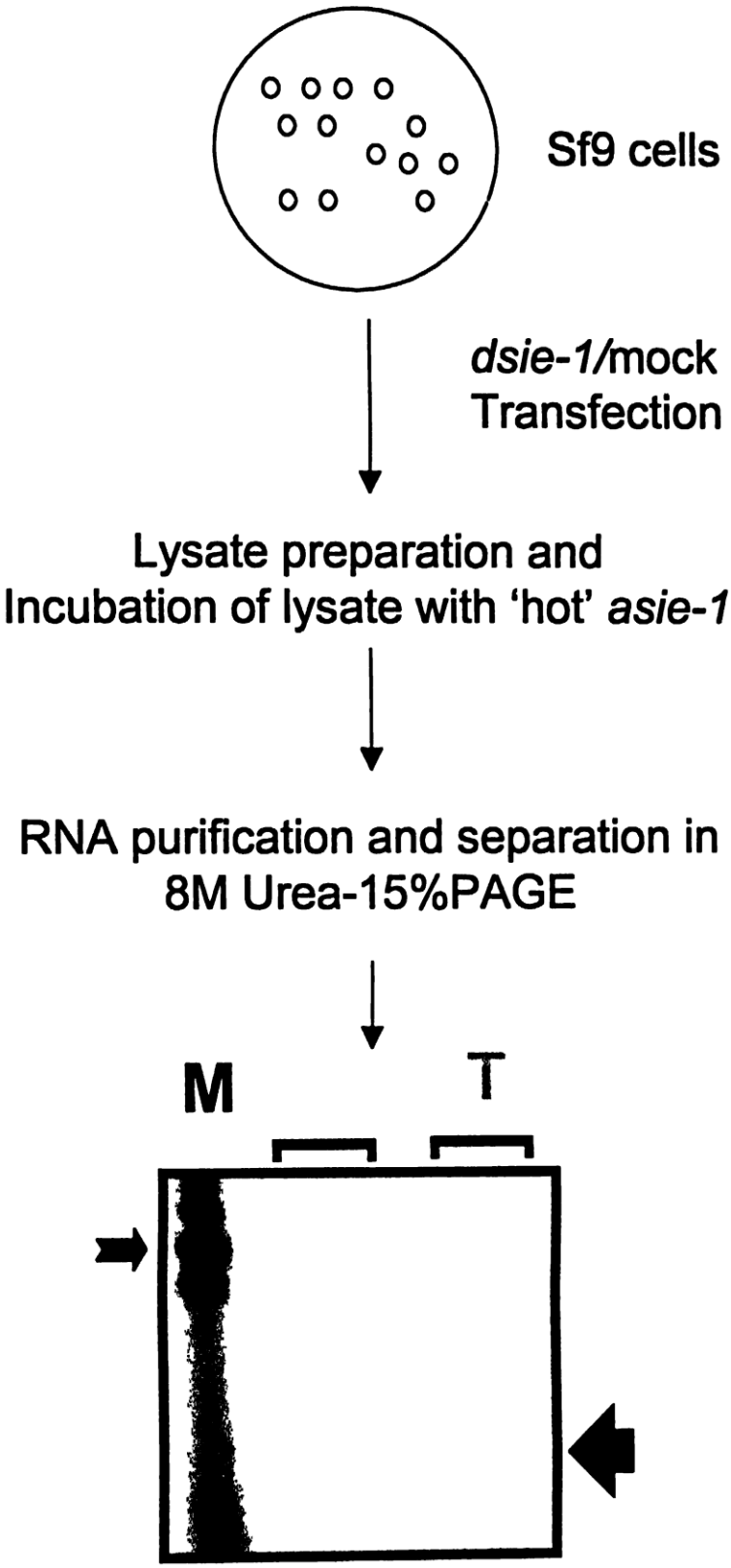


Fig 6 *In vitro* RNAi of radiolabelled *ie-1* RNA. Sf9 cells were incubated in cold *dsie-1*, the cell lysate was prepared and incubated with radiolabelled *asie-1*. After the incubation, RNA was extracted, separated on a 8 M Urea- 15% PAGE and autoradiogram was developed. C and T indicate lysate from mock and *dsie-1* transfected cells respectively. The small arrow indicates 50 bp in marker lane (M) and the large arrow indicates the small molecular RNA of ~21-22 nt in length in T alone.

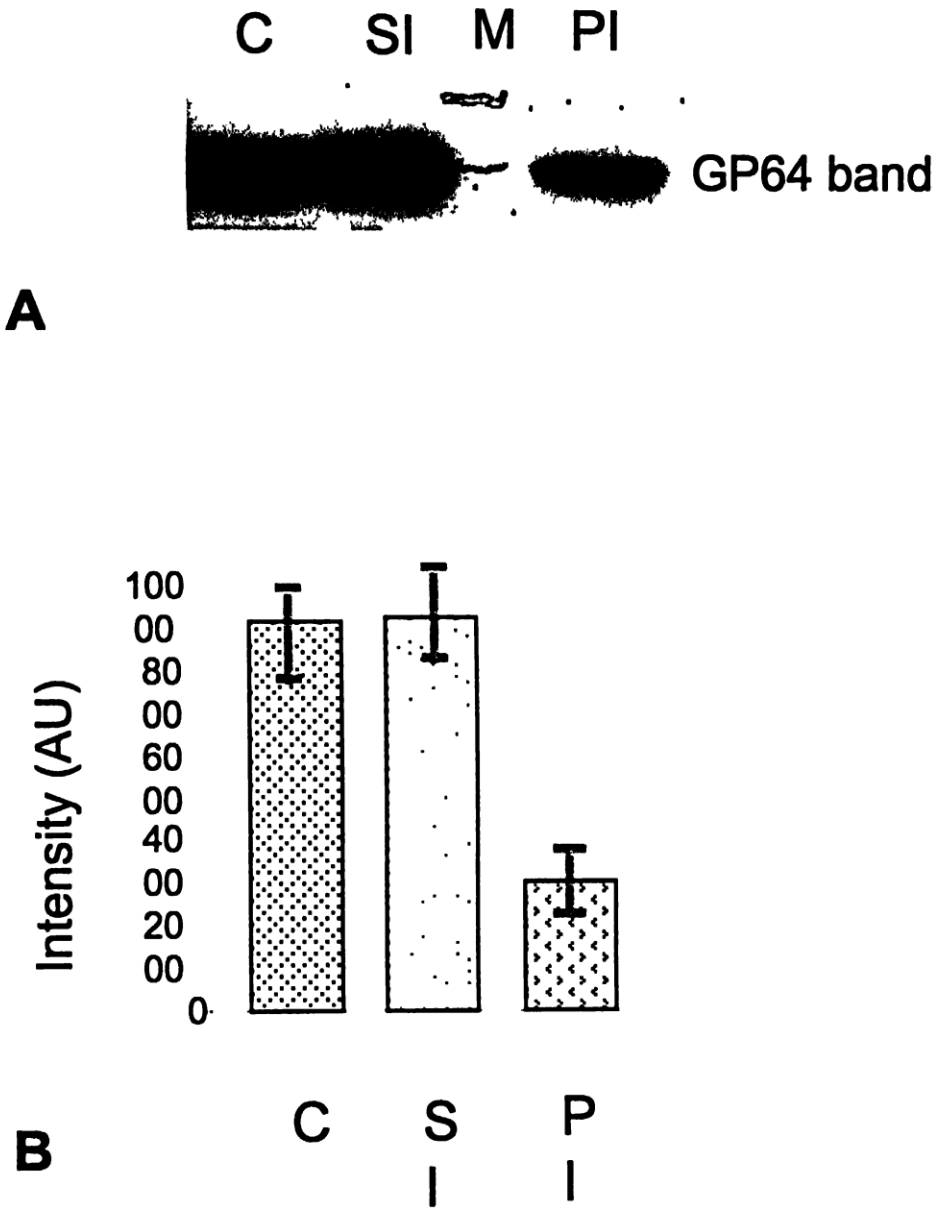


Fig 7 Prior activation of RNAi pathway is necessary to induce *dsie-1* mediated AcNPV resistance in Sf9 cells as indicated by GP64 based western blot. Viral inhibition was noticed only when cells were prior transfected with *dsie-1* (PI) and not when virus infection and transfection were carried out simultaneously (SI). C indicates mock transfection. M is protein marker.

A. Western blot of lysates from virus infected cells.

B. Densitometric estimate of the GP64 band.

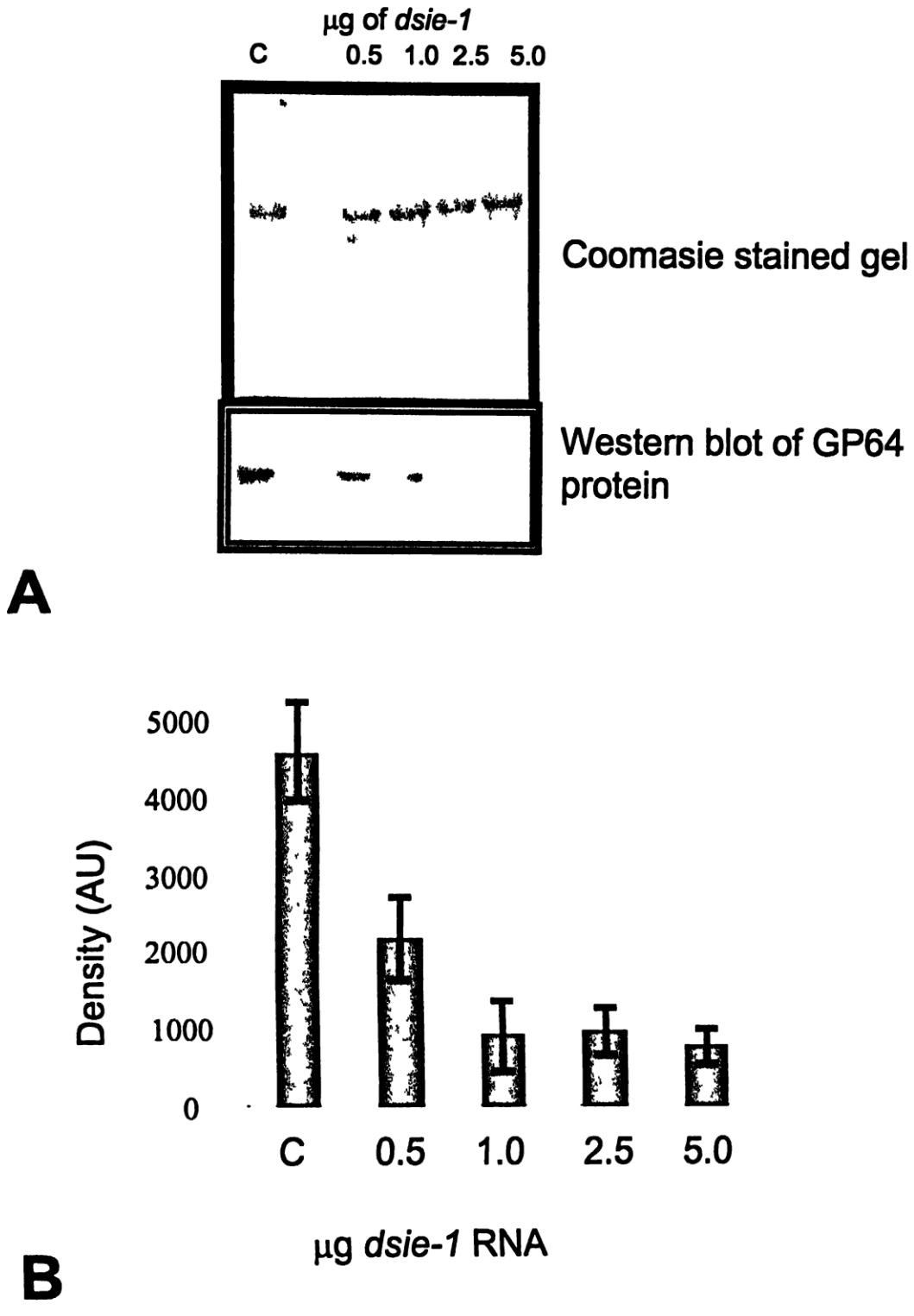


Fig 8 Higher amount of *dsie-1* does not increase viral inhibition. Sf9 cells were incubated with increasing amounts of *dsie-1* and 48 hpi, cells were lysed and equal amount of protein was loaded and western blot was carried out. The results show that at a given condition, 1 µg of *dsie-1* induced as much effect as 5 µg treated group suggesting increasing the *dsie-1* above a threshold level does not increase the viral inhibition.

A. Coomasie-blue stained protein gel and corresponding western blot

B. Densitometric estimate of GP64 band.

indicated that there was a 50% reduction in the GP64 protein level in *dsie*-1 treated cells as compared to the control mock transfected cells at 24 hpi. However, the level of viral protein started to increase and reached that of control level by 96 hpi. At this stage there was no difference between the control and *dsie-1* transfected cells (Fig 9).

To confirm these results, RT-PCR was carried out using *ie-1* specific primers with β-Actin as an internal control. The resulting PCR bands were densitometrically quantified and represented as the ratio between the band intensity of *ie-1* and band intensity of β-Actin. The results of RT-PCR show that, the ratio was much lower in *dsie-1* treated cells than in mock transfected controls at 24 hpi and the ratio steadily increased and reached to that of mock transfected cells by 72 hpi. At 72 hpi there was no difference in the ratio between the *dsie-1* treated and untreated cells. Thus RT-PCR results also confirm the initial suppression of BmNPV proliferation at 24 hpi, with a subsequent recovery at 72 hpi in *dsie-1* treated cells (Fig 10).

Microscopic and FACS analysis also confirm initial suppression of BmNPV proliferation in dsie-1 treated cells

The fluorescent microscopic observation of BmN cells that were infected with recombinant BmNPV-polhGFP shows that the *dsie-1* transfected cells had lesser number of fluorescent cells on second day of infection, but showed same degree of virus proliferation by 72 hpi as compared to the mock transfected cells (Fig 11A). The FACS assay of the cells infected with this recombinant BmNPV reflected the similar trend. Only 20% of the *dsie-1* transfected cells were having GFP fluorescence as compared to 30% in the mock-infected cells at 48 hpi. However, such difference in fluorescent cells was not observed by 72 hpi, suggesting the recovery of the viral proliferation to a near normal level (Fig 11B).

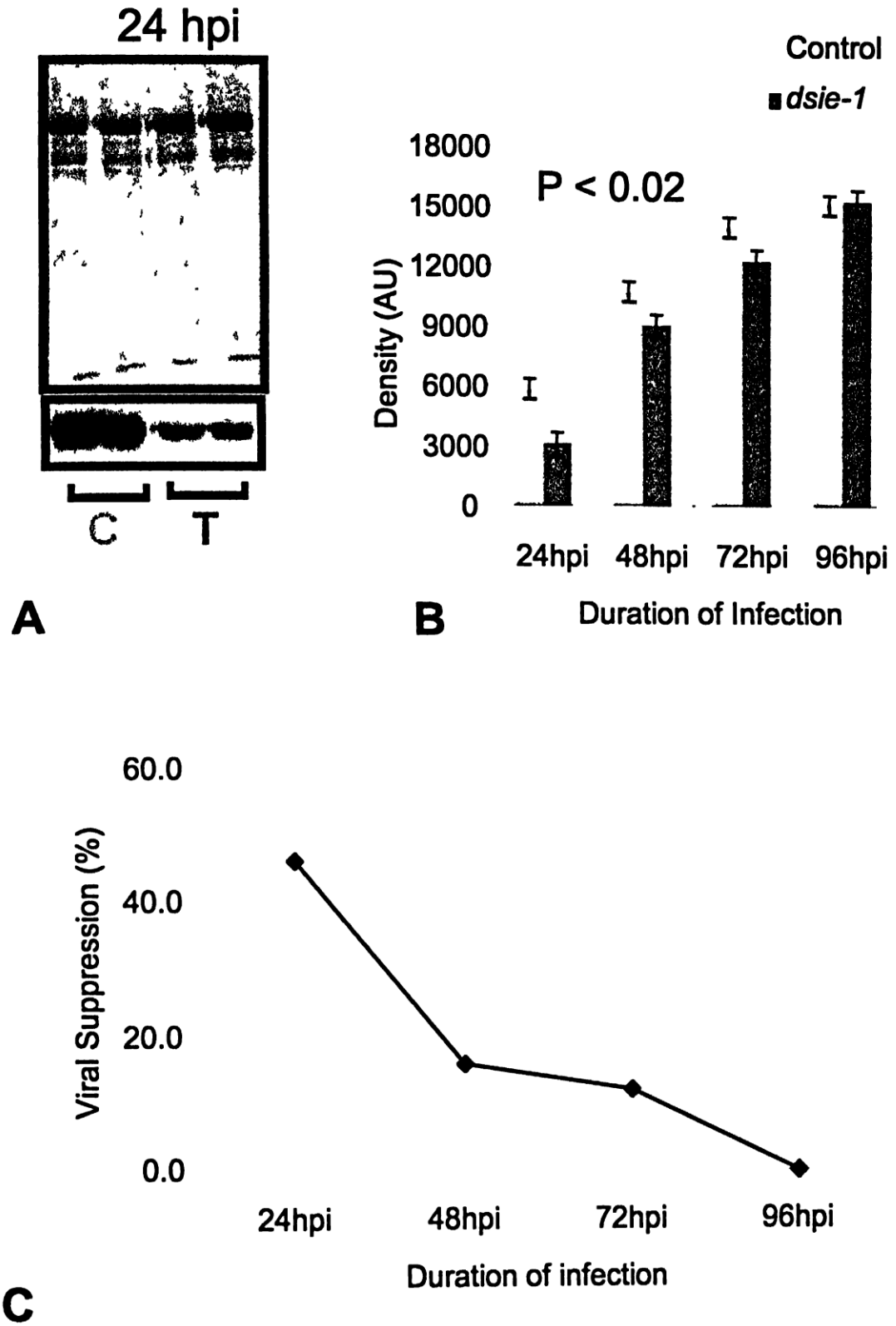


Fig 9 Western blot analysis of *dsie-1* treated cells show reduced GP64 expression at early stages of infection but subsequent recovery to that of control transfection.

A. Coomasie stained protein gel of lysate prepared from infected cells and the corresponding chemiluminiscent band of western blot from mock transfectd (C) or *dsie-1* treated cells (T). Only 24 hpi figure is representated.

B. Densitometric analysis of the western blot. Y-axis represents arbitrary units (AU)

C. The percentage viral suppression for 24 – 96 hpi.

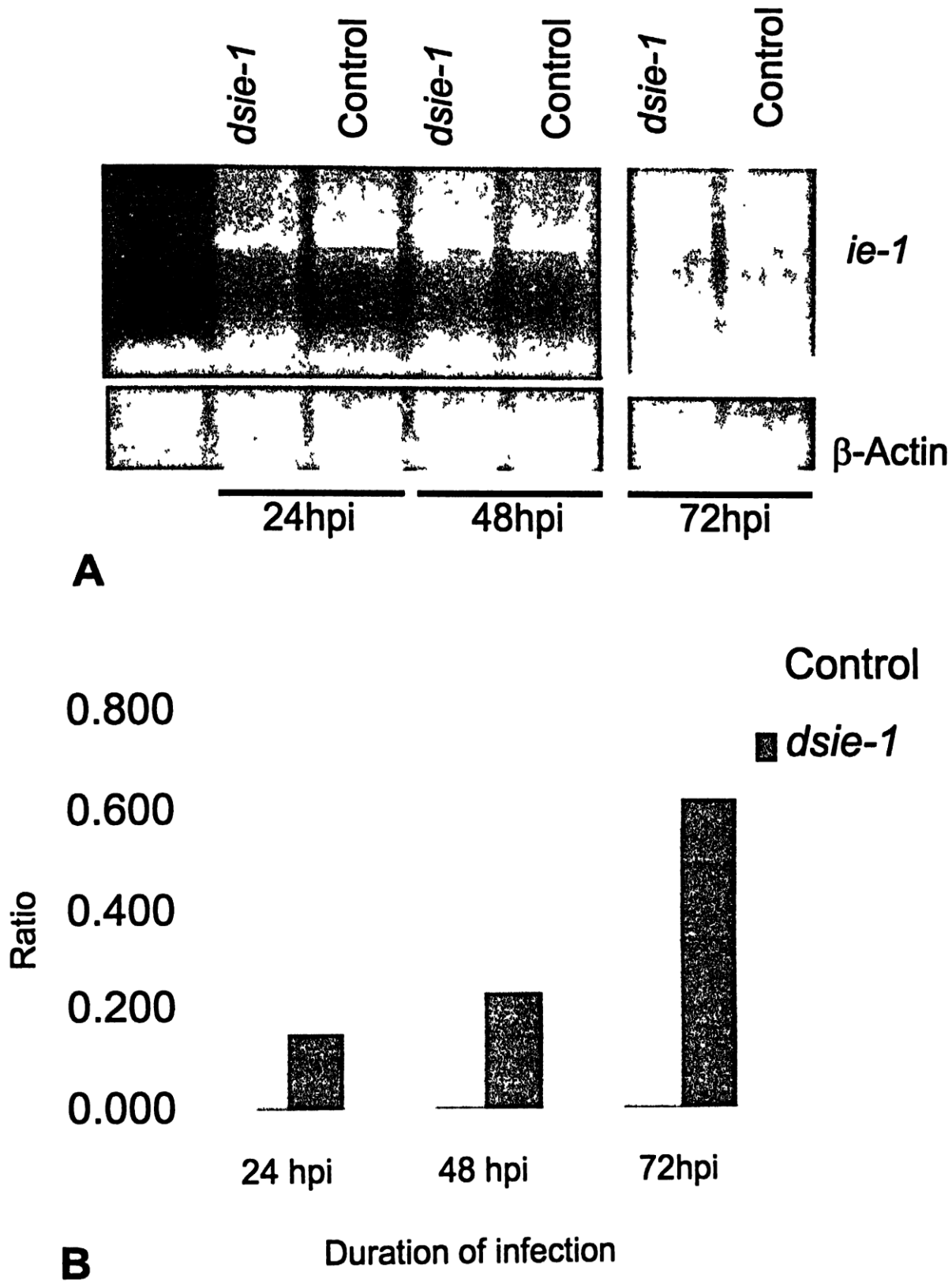
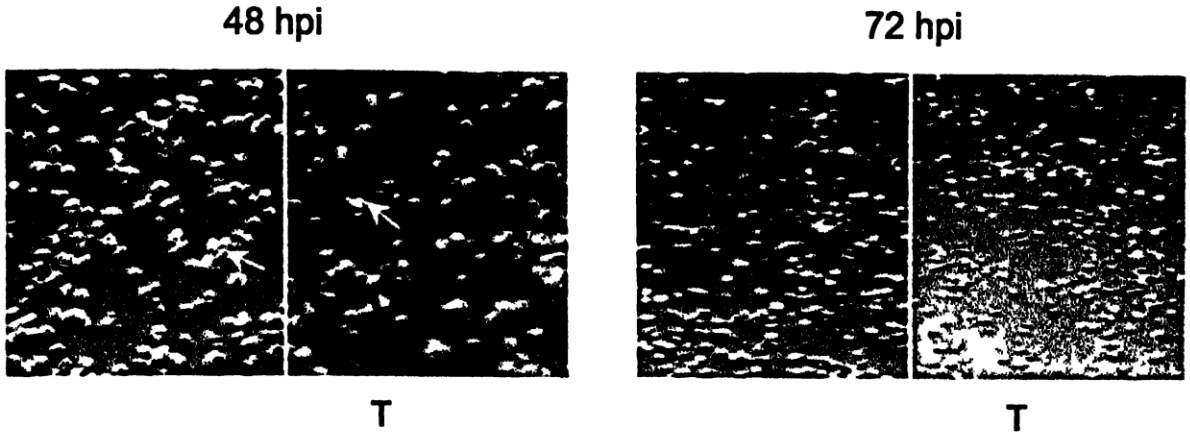


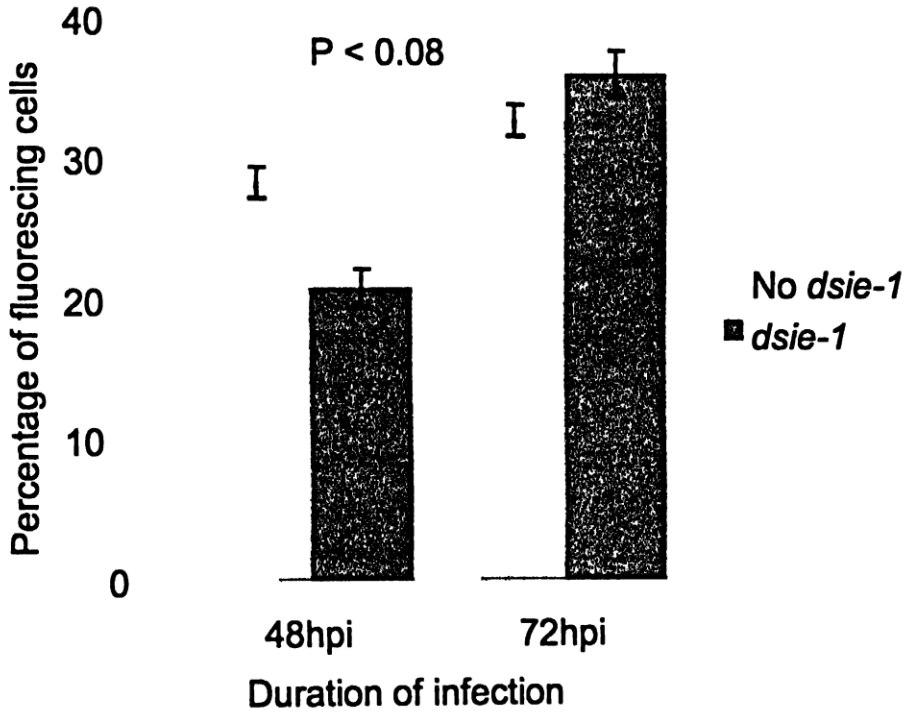
Fig 10 RT-PCR of *ie-1* and β-Actin mRNA in mock transfected or *dsie-1* treated cells. BmN Cells were infected with BmNPV and RNA was isolated at 24-, 48- and 72- hpi.

A. Amplified products of 470 bp *ie-1* and 350 bp β-Actin.

B. Densitometric analyses of RT-PCR products. The band intensities were represented as a ratio of *ie-1* band (numerator) to that of β-actin band (denominator).



A



B

Fig 11 BmNPV-polhGFP resistance in *dsie-1* transfected (T) and mock transfected (C) BmN cells. Results indicate reduced virus at 48 hpi, but recovery by 72 hpi

A. Stereomicroscopic observation of cells under dual light (white and blue) after 48 and 72 hpi. Cells with recombinant virus show green fluorescence (arrow) while uninfected cells do not show such fluorescence.

B. Graphical representation of the FACS analysis of the cells 48 and 72 hpi.

Mortality was reduced in dsie-1 administered and BmNPV infected silkworms

To test the efficiency of the injection of *dsie-1* in silkworm larvae, two different doses of dsRNA were injected into the hemocoel of the CSR2 larvae. These larvae are sensitive to viral infection (LD_{50} ~ 400 PIBs/larva). Twenty-four hours post *dsie-1* injection, the fifth instar larvae of day one were fed with either lethal dose of wtBmNPV, or sub-lethal dose or no virus at all. The dead larvae were scored and the number of surviving and dead larvae in each treatment group was tabulated (Table 4). The result indicates that at a lethal dose of 10^7 PIBs/ml, the *dsie-1* could not protect the silkworms efficiently. However, no lethality was observed in the larvae injected with *dsie-1* and fed with sub-lethal dose of BmNPV. In the same treatment group, there was 20% mortality in the control larvae, indicating that *dsie-1* could protect the larvae at low levels of infection.

Table 4 Percentage survival in *dsie-1* injected and virally challenged silkworm larvae of fifth instar first day.

Amount of *dsie-1*	Viral dose (PIBs/ml)	Viral dose (PIBs/larva)	Total treated (Dead larvae)	% Mortality	% Survival
Water control	10^4	4 X 10^2	30 (6)	20	80
10 µg	10^4	4 X 10^2	9 (0)	0	100
25 µg	10^4	4 X 10^2	30 (0)	0	100
Water control	10^7	4 X 10^5	25 (15)	60	40
10 µg	10^7	4 X 10^5	7 (5)	72	28
25 µg	10^7	4 X 10^5	30 (12)	60	40

Discussion

With the objective of exploring the feasibility of RNAi in suppressing the baculoviral proliferation, we carried out transient transfection/injection assays.

Our result indicated the efficacy of such rapid assay to evaluate the candidate viral genes to target by RNAi. We exposed cells to diverse amount of *dsie-1* and tested them for imparting viral resistance. The experiments involved two different lepidopteran cell-lines and viral inhibition with emphasis to duration of transfection, amount of *dsie-1* and extent of infection. RNAi mediated suppression of viral proliferation was confirmed by three strategies viz. immunoblot assay of GP64 protein, RT-PCR analysis of *ie-1* transcript and FACS analysis of a fluorescent virus. Anti-GP64 antibody is the only commercial antibody available, and it is being used for rapid detection of viral plaques. Since we noticed a direct correlation between western blot using anti-GP64 antibody with other analyses, we used western blot as a rapid method for assessing the viral proliferation. Baculoviral infection is known to cause down regulation of major cellular transcripts like histone, actin and heat shock protein70 (hsp70). (Ooi and Miller 1988) as most of the cellular transcription/translation machinery is shunted to facilitate the viral proliferation. Many housekeeping genes like heat shock proteins, β-Actin are reduced after viral infection. Hence, in the present study we used total protein as a control to observe the equal loading and compared the baculoviral proteins in virus infected or mock infected cells for verifying the viral proliferation. In transient transfection studies, we used *in vitro* synthesized *dsie-1* and carried out the viral proliferation in transfected and mock-transfected cells. By transient transfection of *dsie-1* and measuring the viral load by quantifying the GP64 protein load, we observed that there was an initial suppression of viral proliferation up to 48 hpi, but subsequent recovery of virus to control levels beyond this time point. The results were consistent in BmN and Sf9 cell lines and were independent of higher amount of *dsie-1*. In Sf9 lines, prior activation by *dsie-1* was necessary for inducing the viral resistance. The microscopic and FACS analysis of the transfected BmN cell lines confirm the initial protection against virus by *dsie-1*.

When we challenged the silkworm larvae injected with *dsie-1*, we observed that the larvae were slow to show the symptoms of BmNPV infection, but the lethality was not significantly altered in *dsie-1* injected worms when the viral load was high. No mortality was observed when *dsie-1* injected larvae were fed with 400 PIBs/larva. However, we observed almost 50% lethality in *dsie-1* injected larvae when larvae were fed w 4×10^5 PIBs/larva confirming that protection induced by *dsie-1* is of temporary nature and virus could overcome the RNAi induced suppression of IE-1.

The observed partial inhibition of viral protein expressions during early phase of infection suggests the initial suppression of viral proliferation by targeting essential genes like *ie-1*. However transient studies indicate that the leaky expression of IE-1 was sufficient to establish the subsequent viral proliferation suggesting the inadequacy of *dsie-1* in complete removal of IE-1. We can also assume that during cell cycle, the newly formed daughter cells may not receive the signals of RNAi, hence the initial input of *dsie-1* gets diluted during cell division. Hence the new cells generated are unprotected against baculovirus. Ideal condition for transfection under given experimental condition included the transfection of BmN or Sf9 cells by Escort™ transfection reagent at a *dsie-1* level of 2 μg per million cells, which by any standard could be considered as very high amount. The reason for such a high amount of *dsie-1* could be because of poor transfection efficiency. An initial report suggested the successful suppression of AcNPV proliferation in Sf9 cell lines and *S. frugiperda* (Valdes et al. 2003) by RNAi mediated targeting of IE-1. However, we observed a moderate suppression by baculoviral proliferation only at early stages of infection, but not at later stages of infection. Similarly, the *dsie*-1 could not protect the silkworms in lethal dose of virus. While dilution effect could account for the absence of viral suppression at later stages of infection in both cell lines and silkworms, it also prompted us to believe that the 'amplification' of siRNA signal may not be occurring in the silkworm/lepidoptera. Evidences

indicate that, while such amplification signal exists in *C. elegans* and in plants, amplification is not reported in *Drosophila* and in higher mammals. So it is likely that in lepidoptera such amplification does not exist.

In the course of our study, we also observed that BmN cells are not easily amenable and less efficient in transfection studies than SF9 cells. Hence, even though our objective centers on *B. mori*, the study was continued using Sf9 cells. BmN cells get stressed faster than the Sf9 cells and difficult to carry out the plaque assay with them. Further, the cells could not recover the rigorous treatment during FACS analyses.

In conclusion, the RNAi mediated viral repression was achieved in cell cultures and also we could demonstrate the protective effect of *dsie-1* against BmNPV infection in the silkworm, *B. mori*. Transient assays could be useful in rapid assessment of the viral target genes. However, the inability of the RNAi signals to pass on to next generation makes the transient assay ineffective in obtaining baculoviral resistant strain of the silkworm. Thus it becomes necessary to constitutively and continuously express the *dsie-1* for obtaining RNAi mediated inhibition of baculoviral infection.

Chapter III

Heritable RNAi mediated inhibition of baculoviral infection in Sf9 cells and *B. mori*

He that would have the fruit must climb the tree – M. K. Gandhi (1869-1948)

Introduction

Success of transient assays using both *in culturo* and *in vivo* studies, though partial, has thrown the way for testing the sustained RNAi mediated baculoviral resistance in silkworms. Since transient assay of RNAi mediated baculoviral inhibition is not transmitted to daughter generations, for persistent *dsie-1* production, it becomes necessary to obtain stable transformed cells and silkworms. The success of the Franco-Japanese groups in germline transgenesis of silkworm using piggyBac transposon based vectors (Tamura et al. 2000) forms a basis for obtaining sustained and heritable *dsie-1* production in lepidoptera. The co-injection of the genes to be integrated and helper transposase at early stages of silkworm development has resulted in obtaining a number of transgenic lines of silkworm. This chapter summarizes the successful attempt of generating transformed lines of Sf9 cells and the silkworms using the transposon based vector and the of transformants for RNAi mediated inhibition of baculoviral infection in transgenic cell-lines and silkworm.

Materials and Methods

Cell line, virus stock

Sf9 cells were grown and maintained as mentioned in chapter II.

WtAcNPV and recombinant luciferase expressing AcNPV were used in the current study. Firefly luciferase is under the polyhedrin promoter in the recombinant AcNPV-luc virus. It is a kind gift of Dr. Seyed E. Hasnain of Laboratory of Molecular and Cellular Biology, CDFD.

Construction, confirmation and purification of pPIGA3GFP-FF plasmid

PCR amplification of ie-1

For generation of *pPIGA3GFP-FF* plasmid, sequential cloning strategy was adopted by using a number of shuttle or intermediary plasmids like TA vector

and SK+ vectors. PCR amplification of the *ie-1* promoter, 473 bp *ie-1* gene fragment and SV40 poly A signal was carried out using following primers-

ie-1 promoter-

ie-1 Prom F: GCCGGCCGATTTGCAGTTCGGGA

ie-1 Prom R: TTGTTCACGATCGTGTCCCGCC

ie-1 gene-

ie-1 Gene F: CCAAACGACTATGACGCAAATT

ie-1 Gene R: TTGTTAAATTGGCCCACCAC

SV40 poly A signal sequence

Poly A F: ATCGATAAACGAACGGAATGTT

Poly A R: GGCCGAGGCGGCCACAC

The typical PCR reaction consisted of 10µl of final volume with 5 picomoles 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/0.01% Triton X-100), 1 mM dNTPs, and 0.5 U of IMMOLASE™ DNA Polymerase (Bioline, Germany) per reaction. Thermal cycling was carried out in a thermal cycler (PE9700, Applied Biosystems) using the following conditions: initial denaturation of 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 52°C (48°C for poly A signal), and 2 min at 72°C, and final extension of 10 min at 72°C. The PCR products were quantified on 1.0% Agarose gel, purified using Qiagen PCR purification columns, according to the manufacturer's protocol. For *ie-1* promoter and gene fragment, *pBmNPVIEG* was used as a template, where as for SV40 poly A signal, *pPIGA3-GFP* was used as a template.

For DNA sequencing, 200 ng of plasmid was used in a sequencing reaction that contained 8 µl of Ready reaction mix (BDT v 3.0, Applied Biosystems, Foster City, CA) and 5 picomoles of M13 primers. The cycling conditions used were as follows: 25 cycles of 96°C for 10 s, 50°C 5 s, 60°C 4 min. Samples were ethanol precipitated after the sequencing PCR reaction, washed with 70%

ethanol and resuspended in Hi-Di™ formamide (Applied Biosystems). The sequencing was carried out in ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Cloning and generation of pPIGA3GFP-FF

The purified PCR products were cloned separately into TA vector, transformed in DH5α cells, plated in LB-agar plate according to Sambrook et al. 2001). The positive clones were selected after confirmation of the desired inserts, by PCR, restriction digestion and sequencing. *ie-1* gene product was then moved into SK+ vector by using the appropriate restriction enzymes, to generate the plasmid *pSK-gene* that stands for *ie-1* gene product (of length 473 bp). Subsequently, *ie-1* promoter and SV40 poly A signal were mobilized into this plasmid to obtain *pSKPGA*. Subsequently, the 'PGA' construct was mobilized to *pPIGA3-GFP* plasmid between *NgoM*IV and *Sfi*I sites. This *pPIGA3GFP-PGA* clone has A3- GFP cassette followed by the *ie-1* promoter-*ie-1* gene fragment (in reverse orientation) and SV-40 poly A signal, with a *Bam*HI site in-between. The cloned product was again confirmed by restriction analysis. The final step in cloning the *ie-1* 'Flip-Flop' proved to be the most cumbersome job in the entire cloning process. Many attempts to clone the 'reverse' *ie-1* fragment failed. However, by inserting a fragment of 'stuffer' in between the *ie-1* gene and poly A signal, the cloning of reverse strand of *ie-1* gene fragment was achieved and *pPIGA3GFP-FF* was generated. For the last cloning step, *an E. coli SURE* cell was used. This bacterial cell has very low or no recombination and hence it is a preferred choice for cloning DNA with long stretches of complementary DNA. Here FF stands for 'flip-flop' DNA, which is nothing but the presence of *ie-1* DNA in forward and reverse orientation in 5' to 3' direction with a stuffer fragment in-between.

Confirmation of the clone and purification of pPIGA3GFP-FF

Once pPIGA3GFP-FF was generated, confirming the construct was technically challenging. A number of attempts to sequence the construct

proved futile, and restriction digestion product of the 'flip-flop' often showed erroneous movement in gel. This itself was an indirect evidence for the presence of 'flip-flop' insert in the recombinant plasmid.

The SURE cell colonies were slow growing, if the construct was in 'flip-flop' orientation and were fast growing if the construct was in the same orientation. During final cloning step, only those colonies that were growing slowly were picked up and analyzed for the presence of 'flip-flop'. Once plasmid was isolated from different colonies (Sambrook and Russel 2001), the confirmation of the construct for the flip-flop insert was done both by restriction digestion and sequencing. Sequencing was carried out using the following primer

Sequencing primer.

Upper primer: TATTATTATCGTGTTCGCCATTA

Lower primer: GGTCTTTAGGTTCACGCTTTAAT

Once confirmed, the plasmid was purified by passing it through the Elutip-D columns.

Construction of pPIG3XP3GFP-FF

The construct *pPIGA3GFP-FF* was replaced with *pPIG3XP3GFP-FF* by removing the A3-GFP cassette with 3XP3-GFP cassette. For this, the 3XP3-GFP fragment was PCR amplified using the following primers.

Forward: CCGAATTCAGATCTTCCCACAATGGTTAATTCGAGCTC

Reverse: CCGGATCCGCCGGCGTACGCGTATCGATAAGCTTTA

The template used for this construct was *pBac[3xP3-EGFPaf]*. The PCR reaction was similar as mentioned above, except that, the annealing temperature was 52°C. The PCR product and *pPIGA3GFP-FF* were restriction digested with *NgoM*IV and *Bgl*I enzymes. The PCR products were gel-purified, ligated and transformed into 'SURE' competent cells. The colonies were screened for the presence of the 3XP3-GFP by PCR and confirmed for the successful ligation through restriction digestion. The presence of 3XP3-GFP in *pPIGA3GFP-FF* was also confirmed by sequencing.

Computational prediction of dsie-1 secondary structure

The secondary structure of *dsie-1* was predicted using the online free software Mfold (Zuker 2003). For computational analysis, the sequence immediately downstream of *ie-1* promoter in *pPIGA3GFP-FF* was *in silico* transcribed till the poly A signal and used as the *ie-1* flip-flop transcript. This transcript was submitted to the online RNA folding prediction software "Mfold". Default parameters were used to predict the secondary structure of the transcript.

Transformation of Sf9 cell line with pPIGA3GFP-FF

Transfection of Sf9 cells

The exponentially growing Sf9 and BmN cells were transfected with the *pPIGA3GFP-FF* and *helper* plasmids (1:1 molar ratio) using the transfection reagent DAC-30. Briefly, the 50% confluent cells were rinsed with the media, the two plasmids+DAC-30 mixture was prepared in the Grace-Media or TC-100 media devoid of serum and antibiotics. The mixture was layered on the cells, and the DNA was allowed to enter the cells for four hours. After the incubation period, the transfection media was replaced by fresh media containing 10% serum and antibiotics. The cells were monitored for expression of GFP from the second day onwards. The fluorescent cells were scored and represented graphically as percentage of fluorescing cells versus the days post transfection. As a negative control for transgenesis, only the *pPIGA3GFP* plasmid was added to the cell culture without *helper* plasmid and the fluorescence decay was also verified.

Selection of transformed cells by Fluorescence Activated Cell Sorter (FACS)

After the level of fluorescent cell population was stabilized, which took a month, the cells were sorted aseptically using FACSVantage (Becton and Dickinson). Briefly, the FACS machine was sterilized by passing the alcohol in the FACS machine, rinsed thoroughly with sterile distilled water and used for selecting and sorting the GFP+ve cells. First, a set of control, non-fluorescent cells were run to identify the auto-fluorescence event. Next, by using GFP

specific FL1H filters, transformed cells were sorted at a level outside the auto-fluorescence event. Only those cells with FSC and SSC values > 200 and < 1000 arbitrary units were selected. Approximately 10,000 events (corresponding to cells) in high fluorescent region were sorted. The sorted cells were harvested by centrifuging at 900Xg, then cultured in media containing 15% serum and antibiotics in a conditioned medium. As a 'transformed negative control' cell-line, a transformed Sf9 cells having only *pPIGA3-GFP* were also obtained similarly.

Obtaining the monoclonal population

To obtain the monoclonal populations, the cells were diluted to a final concentration of about 10 cells per ml. 100 µl each of this cell suspension was placed in each well of a 96 well plate. The cells were grown until the well became confluent, and the cells were reseeded in a 6-well plate at a dilution of 3-4 cells per well, and grown in a pre-filtered conditioned medium. The cells that were growing clonally could be seen as a patch, with each patch representing a monoclonal population. This clonal population of cells was picked up and reseeded and confirmed for purity with fluorescent microscope. The monoclonal populations were later used for anti-viral assays.

AcNPV resistance in transformed Sf9 cell line

Viral infection and Plaque assay

One million exponentially growing Sf9 cells were seeded in each well of to six well plates. As a control, trasngenic Sf9 with *pPIGA3-GFP* was used. 5 MOI ($5X10^6$ BVs per million cells) of wild type AcNPV was layered on the cells, incubated for viral adsorption for an hour. After an hour, the cells were washed once and then replaced with the fresh media. The media from the infected cell-lines were collected at different time points (8- 48 hpi). Plaque assay was carried out from this media to find out the viral replication.

For plaque assay, 0.5 ml of the supernatant containing the virus was diluted in two different ranges. For 8-16 hpi, the media were diluted in the range of 10^1-

10^4. For later stages of infection the range of dilution used was 10^4- 10^8. The dilutions were layered onto a million cells, incubated for an hour and then the media was removed. The cells were then layered with a layer of Sea-Plaque agarose containing the complete medium. Cells were incubated at 26°C until plaques were observed (~ four to five days). For clear visualization of the plaques, neutral red stain was used. The stain was placed on the agarose, incubated for an hour and then excess stain was removed. The plates were returned to the cell culture incubator. Plates were observed every day for about three days, and when plaques were clearly visible, the plates were removed for plaque counting. The plaques were counted manually, and multiplied with appropriate dilution factors to express it as x plaques per ml.

Microscopic observation of infection in polyclonal population

Transformed cells were infected with 5MOI of wtAcNPV in a chamber slide and allowed to infect for 3-4 days. After the specific duration of incubation, the chambers were removed and the attached cells were observed under fluorescence microscope (Nikon Eclipse E600). The number of cells showing polyhedra was noted.

FACS analysis of infection in polyclonal population

The transformed Sf9 cells were seeded at a density of 2×10^5 per well. Control non-transformed cells were also used at the same density. The cells were either infected with 5 MOI of wtAcNPV or mock infected. After 3 or 4 days post infection, the cells were FACS analyzed as follows. The cells were removed gently, washed with 1 X PBS and suspended in the sheath fluid (1x PBS). For identifying the dead components (like membrane, nuclei or other debris), transformed cells were lysed in water by endo-osmosis and vortexed and their FSC and SSC values were noted. Based on this observation, the viable cells were defined by a FSC value between 200 AU and 1000 AU. The number of viable events were counted and partitioned into transformed (high

fluorescence) and non-transformed (auto-fluorescing) regions. The number of events was counted in each region and a plot was generated.

AcNPV-luc infection of polyclonal Sf9 cells

One million polyclonal Sf9 cells were infected with 1MOI of AcNPV-luc for 48-96 hpi. After the scheduled infection period, the cells were harvested, washed twice with 1X PBS and a portion of the cells was incubated with commercially available substrate. After five min. the luminescence were recorded using Turner Design luminometer. The protein concentration of the remaining cells was determined and the luminescence was expressed as AU/mg of protein.

Viral infection in monoclonal population

Due to various reasons, many lines of monoclonal populations were lost in due course. Only two of them could be analyzed for viral assay. Both of them were of low fluorescence emitting populations. The mode of viral infection was same as mentioned earlier. Western blot was carried out using the LEF-3 antibody as mentioned elsewhere.

Transgenic B. mori having pPIG3XP3GFP-FF

Transgenesis

The germline transgenesis of the silkworm eggs was carried out as per the protocol described by Tamura et al. (2000). Briefly, after two hours oviposition, the eggs Nistari marked strain were injected aseptically with a solution containing *pPIG3XP3GFP-FF* and *helper* plasmid at a 1:1 molar ratio, (generation – G0). In the next generation (G1), larvae were screened for the expression of green fluorescent protein in the eyes to confirm successful transgenesis. The transgenics were obtained in third microinjection attempt and reared as normal silkworms described earlier. They where sib-mated for three generations to obtain six homozygous lines. The homozygosity of the transgenics was confirmed by inverse PCR. The six lines of transgenic worms has been obtained were 126A, 126B, 58E, 58C, 93B and 58B2.

Viral assays on transgenics

Larval feeding experiments

For testing the efficacy of the transgenic worms against the virus, the recombinant BmNPV-P10GFP was smeared on to a square piece of mulberry leaf at a concentration of 10^7/ml. One hundred larvae each of five groups viz. NM (control), 126A, 126B, 58C and 58E were used for testing their viral resistance. The silkworm larvae that were out of second and third molt were starved for 12 hours and then fed with the virus smeared mulberry leaf. Each larva was fed with ~5000 (third instar) and ~10000 PIBs (fourth instar) respectively. Only fourth instar larvae were used in the group 58E. Only those larvae that consumed all the virus particles were maintained. The virus fed larvae were subsequently reared on fresh mulberry leaves and observed for symptom of viral infection. The dead larvae were removed immediately to prevent spreading of the secondary viral infection. On fifth day of post-infection, hemolymph was collected from the five randomly picked IV instar larvae by puncturing the ventral side of the silkworm near the second abdominal leg. These larvae were then maintained separately and were not included in the mortality count to avoid any secondary infection.

Intra-hemocoel injection of larvae with BmNPV-P10GFP

For these experiments freshly eclosed fifth instar larvae were used. Two doses of virus 10^7 and 5 X 10^4 free virus (BV)/ml (1:200 times dilution of original stock) were injected. For each dose, 35 larvae were injected. Total volume injected was 12.5 µl, which was equivalent to a viral load of 1.25 X 10^5 and 625 virus/larva for the viral doses of 10^7 and 5 X 10^4, respectively. The progress of symptoms was observed until cocoon formation was started. NM and CSR2 strains were used as controls.

We also tested for viral resistance of freshly released 4^{th} instar larvae with a viral dose of 1000 FV/larva. The percentage mortality was counted based on

the results of the moths emerged from the virus infected larvae. In this case only NM was used as a control.

Pupal injection with BmNPV-P10GFP virus

Five days after the onset of spinning of the cocoons, the pupae were harvested from three transgenic lines (126A, 126B and 58E) and a control non-transgenic line (NM). One hundred each of the pupa was injected with 12.5 µl of BmNPV-P10GFP (1000 BV/pupa). Pupae were maintained at room temperature till the emergence of the moths. As an injection control, pupae were injected with TC-100 media containing kanamycin.

BmNPV resistance in survivors of pupal injection

The transgenic moths that survived and emerged from the pupal injection of virus were crossed among themselves as well as between lines. The crosses were 126A X 126A, 126A X 58E. Fifty larvae that emerged from each these crosses were then fed at the first day of fifth instar with wtBmNPV at a concentration of 2000 PIBs per larva and the mortality was calculated. CSR2 variety was used as a control.

Viral assay in hybrids of transgenic Nistari female and non-transgenic CSR2 male

To study the effect of viral resistance in transgenic and non-transgenic hybrids, a cross between transgenic non-diapause Nistari strain and virus sensitive non-transgenic silkworm strain, CSR2 was made to obtain F_1 hybrids. Though all six lines of transgenics were crossed with CSR2 males, only three crosses were used for the viral assays. F_1 hybrids of control non-transgenic Nistari marked (NM) female and non-transgenic CSR2 were used as a control. The crosses used in viral assays were 126A X CSR2, 126B X CSR2, 58E X CSR2 and NM X CSR2 (control).

Results

pPIGA3GFP-FF plasmid was successfully constructed

The necessary fragments like *ie-1* promoter, *ie-1* gene fragment and poly A signal were PCR amplified and individually cloned into TA vector. The size of each fragment (including the essential restriction sites in primer) is as follows:

ie-1 promoter- 626 bp

ie-1 gene fragment- 466 bp

SV40 poly A signal- 180 bp

They were mobilized to SK+ vector and at each step confirmed by restriction digestion for the appropriate insertion with respect to orientation and sequence. After scrutiny, most of the fragments were mobilized to piggyBac based backbone vector *pPIGA3-GFP*, to obtain *pPIGA3GFP-ie-1-Reverse*. This construct has anti-sense or reverse *ie-1* gene fragment. However, the generation of *pPIGA3GFP-FF* from this vector by simple insertion of the sense (forward) *ie-1* gene fragment proved to be a difficult task. Due to complementary nature of the second *ie-1* fragment, the successful transformation with colonies carrying the construct was not easily obtained. It is believed that the bacteria do not tolerate the flip-flop construct and hence it is excised out by recombination. For preventing the recombination and eventual exclusion of the flip-flop construct, SURE bacterial cells were used. These cells have very low recombination, hence it is the preferred bacterial strain for transforming the difficult inserts that have long inverted repeats. The bacterial colonies having the construct in flip-flop orientation were growing slowly at 37°C as compared to those that did not harbour the construct. Hence, the small colonies were picked up and screened for the presence of the flip-flop vector. Ultimately, a few colonies were identified as potentially harbouring the *pPIGA3GFP-FF* construct. The plasmid was isolated from these colonies and screened for the vector with proper orientation of inserts.

The restriction enzyme *Dra*III gives a specific 847 bp product, if the construct is in 'flip-flop' orientation or 547 bp product, if in head-to-head orientation. Further, *Dra*III – *Sfi* I combination of enzymes gives a characteristic 1105 bp

product only if the construct has 'flip-flop' orientation. The plasmid isolated fulfilled the criterion for presence of the insert in 'flip-flop' orientation (Fig 12). For further confirmation, sequencing was carried out in the region near the 'flip-flop' orientation. A portion of the sequencing of *pPIGA3GFP-FF* that extends in the double stranded flip-flop region is shown in the Fig 13. The sequencing of the *pPIGA3GFP-FF* confirmed the presence of *ie-1* in flip-flop orientation. Interestingly, the region of the DNA that forms loop like form shows poor sequence information. It could be due to the fact that the polymerase enzyme used for sequencing reaction is unable to unentangle the flip-flop region. The sequencing gets murkier at the junction, where flip-flop starts. The vector map of the final construct of *pPIGA3GFP-FF* is shown in the Fig 13.

Successful construction of pPIG3XP3GFP-FF

The vector *pPIGA3GFP-FF* expresses GFP under the silkworm cytoplasmic Actin promoter, which is a nearly universal promoter. The GFP driven by this promoter expresses ubiquitously, hence even unintegrated but vector containing G0 embryos or cells fluoresce, making it difficult for screening the transgenics. One has to maintain all the injected embryos and their first generation offspring for confirmation of germline integration of the construct. To overcome this problem, a new vector was designed by Thomas et al. 2002 with synthetic tissue specific 3XP3 promoter. This synthetic promoter is recognized *in vivo* by *Pax* proteins and the downstream genes are expressed tissue specifically. Hence screening the transgenics larvae becomes easier using 3XP3-GFP as a selection marker. Consequently, the GFP cassette in *pPIGA3GFP-FF* was replaced by 3XP3-GFP marker. For this, the 3XP3-GFP fragment was PCR amplified and cloned into TA vector, from which it was restriction digested and mobilized to *pPIGA3GFP-FF* between *NgoM*IV and *Sfi*I sites (Fig 14). The construct *pPIG3XP3GFP-FF* was again confirmed by

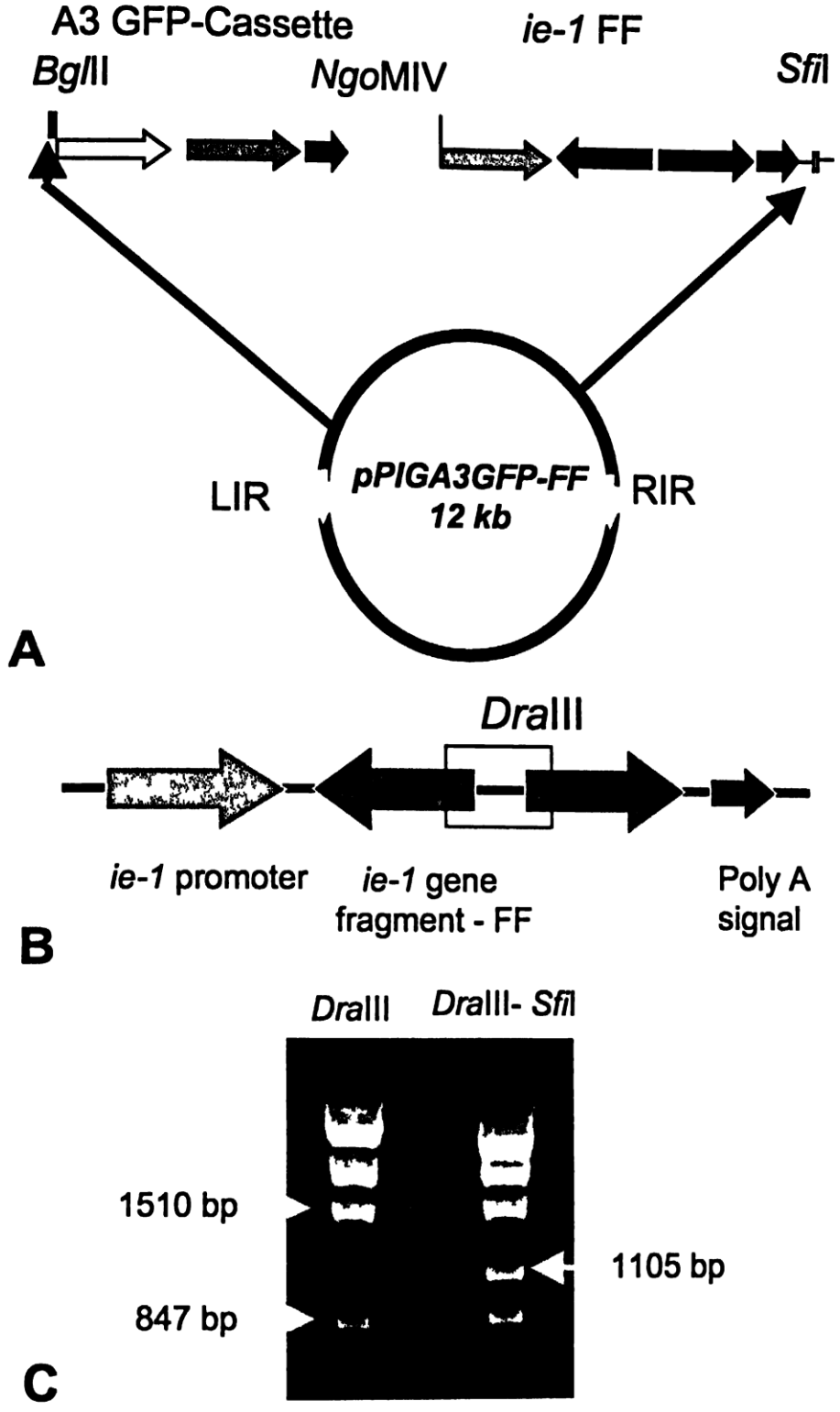


Fig 12 *PiggyBac* based RNAi vector, *pPIGA3GFP-FF* used for obtaining transgenic cell lines. It has A3-GFP as a selection marker gene.

A. Positions of A3-GFP cassette and *ie-1*FF cassette within *PiggyBac* inverted repeats between *Bgl*II and *Sfi*I site. LIR and RIR indicate left and right inveted repeats respectively.

B. *ie-1*FF cassette with *ie-1* promoter, *ie-1* gene fragment in 'flip-flop' orientation and Poly A signal. Two of the *Dra*III sites that are characteristic feature of 'flip-flop' orientation is shown as a block

C. Confirmation of 'flip-flop' construct by restriction digestion with *Dra*III and *Dra*III and *Sfi*I enzymes. Orange arrow indicates the specific fragments, with expected band size in bp.

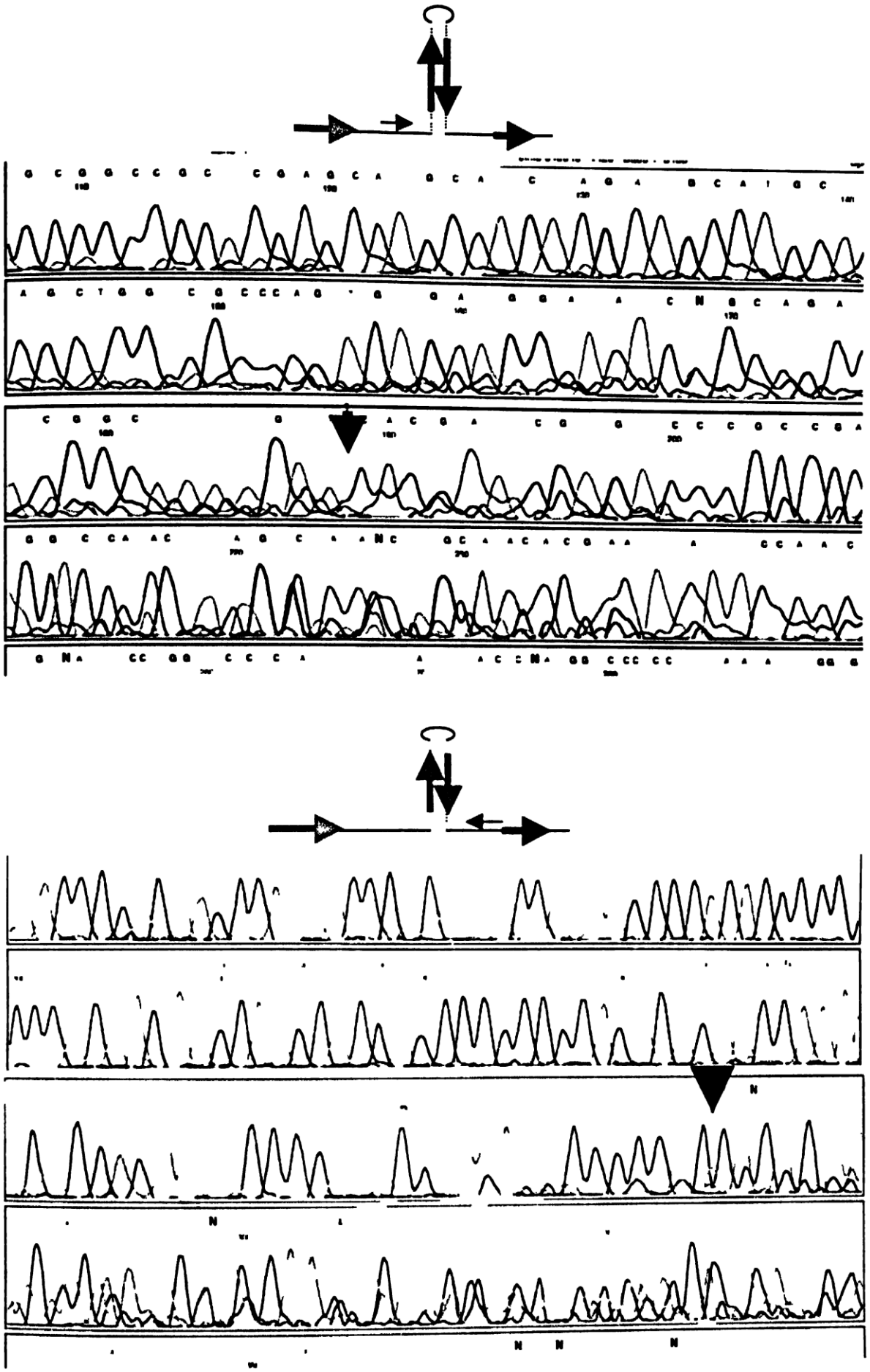


Fig 13. Chromatogram of 5' (upper panel) and 3' (lower panel) sequencing of 'flip-flop' region of *pPIGA3GFP-FF* vector. The colour code of vector is as in Fig 12. Arrow within the phenogram represents the point from where the complimentary region starts. The flip-flop region is seen as a wobble of sequences in the phenogram.

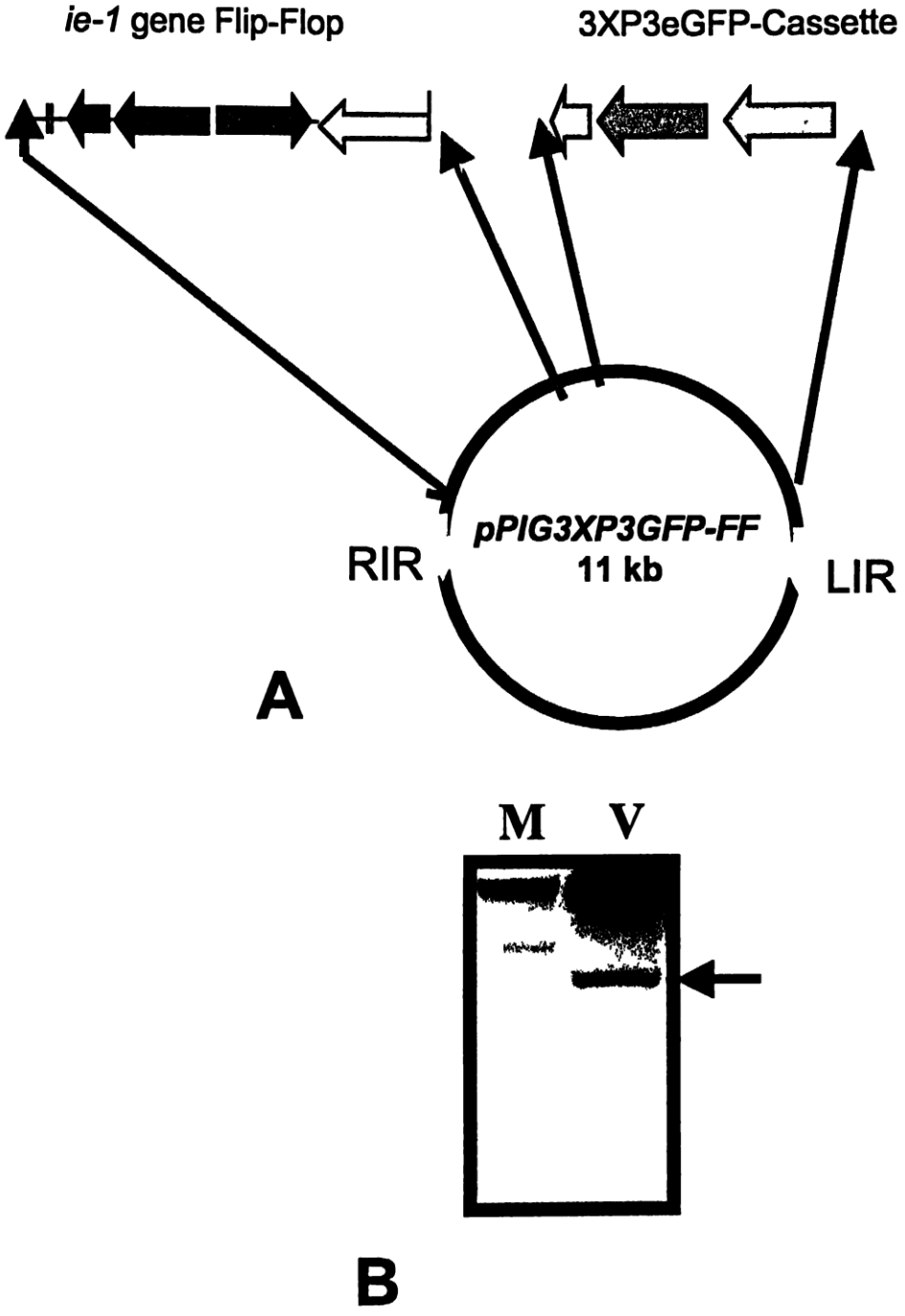


Fig 14 3XP3-GFP based vector for germline transgenesis of the silkworm *B. mori.* The A3GFP cassette of *pPIGA3GFP-FF* vector was replaced by PCR amplified 3XP3-GFP cassette, which has advantage in screening the tranasgenic worms.

A. Graphical representation of *pPIG3XP3GFP-FF*

B. Confirmation of the construct by restriction digestion with *Bgl*II and *Ngo*MIV enzymes. Arrow indicates the release of expected 3XP3GFP band. M – 100 bp marker, V- *pPIG3XP3GFP.*

selective restriction digestion and sequencing. This construct was used for silkworm transgenesis.

In silico modeling of dsie-1 confirms to have a long double-strand nature

To scrutinize whether the RNA produced by *pPIGA3GFP-FF* is having the double stranded nature, we used a computational RNA folding prediction algorithm available online called 'Mfold' (Zuker 2003). Using default parameters, the result showed a high degree of complementarities between sense and anti-sense strands of *ie-1* flip-flop RNA encoded from the *pPIGA3GFP-FF*. The 1991 bp long full-length transcript results in a prominent *dsie-1* duplex, with other minor duplexes (Fig 15). Because of the length of the *dsie-1* RNA, it is expected that a number of siRNA molecules could be produced by action of dicer like enzyme. It is assumed that, in the absence of cytokine based immunity against long stretch of double stranded RNA, the longer stretch of dsRNA is a better inducer of RNAi than smaller fragment of siRNA.

Successful transformation of Sf9 cell line with pPIGA3GFP-FF

Polyclonal population of Sf9 cells with pPIGA3GFP-FF successfully obtained

Exponentially growing Sf9 and BmN cells were transfected with *pPIGA3GFP-FF* or *pPIGA3-GFP*. As a transformed 'negative control' the cells were also transfected without *helper* plasmid. The transfected cells started expressing GFP from second day onwards. The percentage of cells expressing GFP increased as the days passed and by about 6-9 days maximum percentage of GFP expression was noticed (Fig 16). The percentage cells expressing GFP started decreasing from 10th day onwards. In negative controls, where no *helper* plasmid was added, the percentage of cells expressing GFP reached zero by about 16th day, while in those cells where *helper* plasmid was added, there was about 0.5% of the cells expressing GFP. The cells were grown for about a month (about six passages) and at the end of 25th day there was still 0.5% of the cells expressing GFP in 'transformed' culture, where as negative control did not have any fluorescent cells. The negative control cells that contained no

*ie-1*FF region of *pPIGA3GFP-FF*

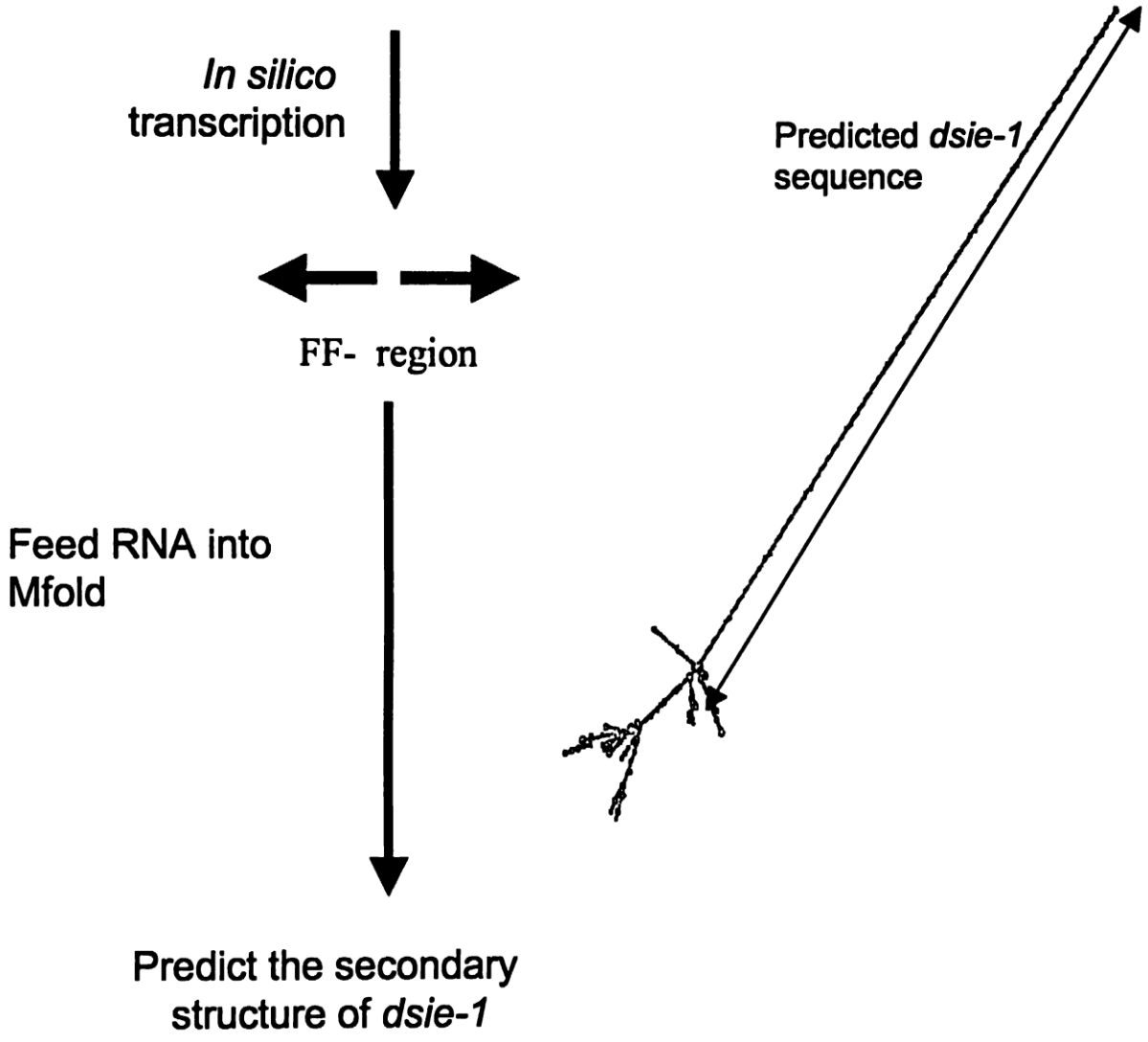


Fig 15 *In silico* modelling of the 'flip-flop' region of *pPIGA3GFP-FF* vector using the online tool 'mfold'. The *in vivo* transcription of *dsie-1* is expected to produce a long stretch of double strand RNA.

A. Procedure of data feeding

B. Long stretch of *dsie*-1 along with a ds region of junk DNA of vector. Total length of the ds region~ 1 kb in length and is shown with an arrow.

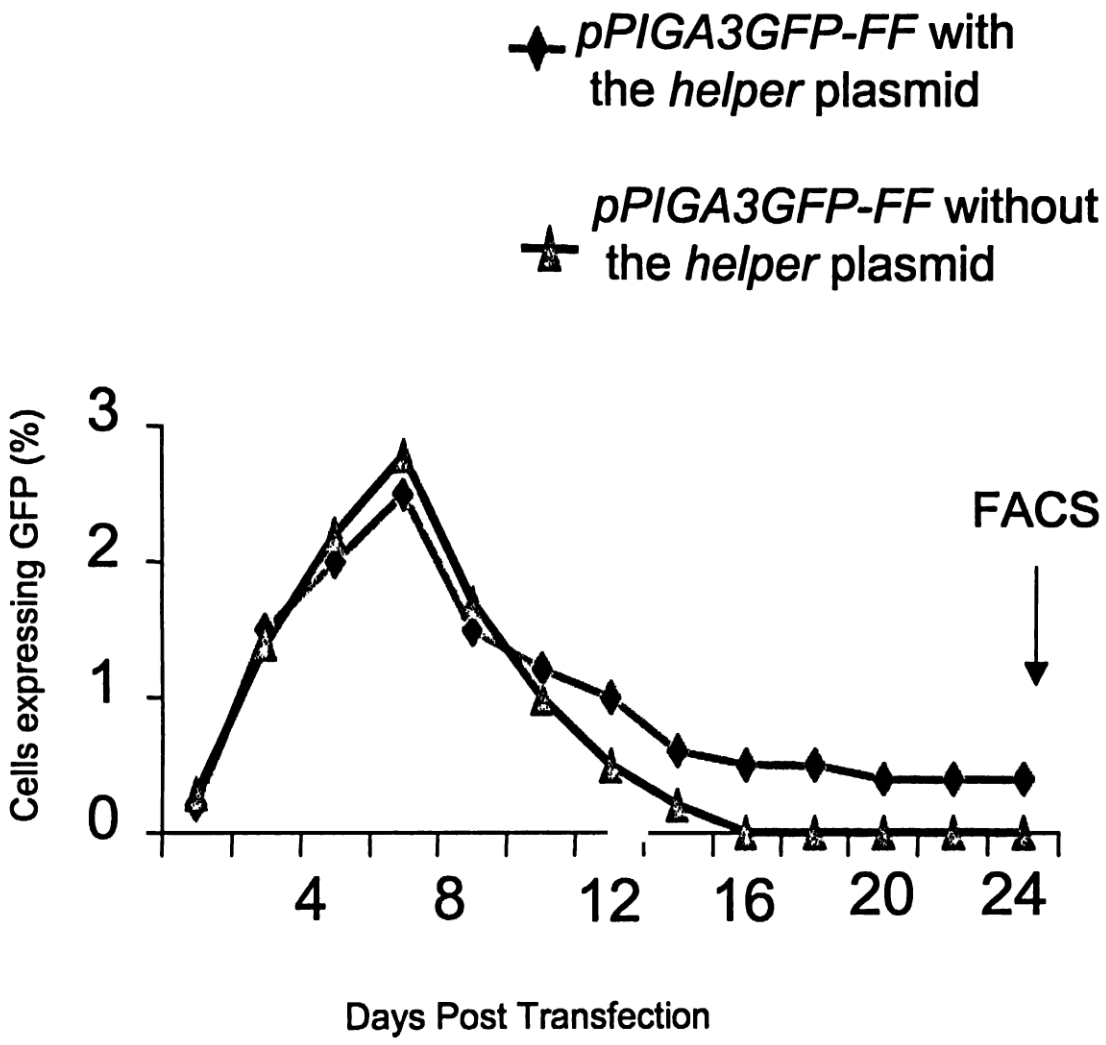


Fig 16 Green fluorescence decay of the Sf9 cells that have been transfected with *pPIGA3GFP-FF* and with or without *helper* plasmid. The percentage of fluorescing cells rapidly increased by 4th day and gradually decreases by 14th day. Only the cells that have permanently transformed continued to fluoresce even after passing through three to four passages. After the stabilization of the fluorescence, FACS was carried out to select only the permanently transformed cells.

helper plasmid were discarded. The 'transformed cells' were then FACS selected. However, the BmN cells did not survive the harsh treatment of FACS selection and hence no transformed lines could be obtained. The fluorescent transformed Sf9 cell-lines were polyclonal, and were used in viral assay as a polyclonal population. Even after sorting, we noticed that not all sorted cells were showing the fluorescence. This could be due to various reasons. The transgene might have integrated in the 'silent' locus of the genome, the event, which was considered as fluorescent by the FACS machine may not have been a really fluorescent (border-line events) event, but still got sorted, or there might have been endogenous removal of the transgene. The cells that expressed only GFP (transformed *pPIGA3-GFP*- negative control which does not produce *dsie-1*) were also obtained in a similar fashion.

A single monoclonal line of Sf9 was obtained from the polyclonal population

Monoclonal populations of cells were obtained by limit dilution method, wherein the cells were seeded to a dilution to get about 4-5 cells per 35mm culture dish. They were allowed to grow in conditioned media. The cells formed a patch like appearance with daughter cells surrounding the original cell. These cells were allowed to divide for about 7-8 generations, by which time there were about hundred cells in each cluster. These cells were removed using a pipette tip and reseeded to obtain second seeding, again to obtain second sub-culture. The cells isolated from these second cultures were tested for fluorescence. The colonies without fluorescence were discarded, since they do not carry the *pPIGA3-GFP-FF* construct. Only fluorescing cells were maintained. A total of six-cell populations was obtained.

AcNPV resistance was observed in transformed Sf9 cell line

Reduced plaque formation in transformed lines

Transformed Sf9 cell-line having the *pPIGA3GFP-FF* was tested for its viral resistance potency. The cell-line having *pPIGA3GFP* was used as a negative control. For viral resistance assay, the cell lines were infected with 5 MOI of

wtAcNPV for 21-48 hpi. The supernatant media containing the BV was collected at 21 – 48 hpi and plaque assay was carried out. The result of plaque assay is shown in Fig 17. The transformed cells showed significantly decreased budded viral release than the control cells ($P < 0.05$). These results provide evidence for suppression of viral proliferation.

FACS analysis shows selective advantage of transformed cells

If there is a selective advantage of the transformed cells in abrogating the viral proliferation, then in the polyclonal population the number of transformed (that is GFP expressing) cells should increase once the cells start lysing during viral infection (Fig 18). FACS analysis of the infected cells confirmed the selective advantage of the GFP expressing transformed cells, where the percentage of GFP expressing cells increased from 24 to 38 after viral infection by third day post infection (Fig 19A) suggesting that the transformed cells were protected against virus infection.

Microscopic observation shows non-transformed cells were preferred for ODV production in the polyclonal population

To see the formation of the polyhedra in AcNPV infected polyclonal Sf9 cells, the cells were observed under fluorescent microscope. The polyhedra were found more predominantly in non-transformed cells than in transformed cells on fourth day post infection (Fig 19B). However, by fifth day the number of cells having ODVs increased and there was no difference in the number of ODVs between transformed and non-transformed cells. Beyond this time almost all the cells were lysed irrespective of the presence of transgene.

Recombinant AcNPV expressing luciferase under polyhedrin promoter confirms viral resistance in polyclonal population during early phase of infection

To reconfirm the viral resistance in transformed lines, a recombinant AcNPV expressing luciferase under polyhedrin promoter (AcNPV-luc) was used instead of wt AcNPV. The polyclonal Sf9 cells were infected with AcNPV-luc for 48–96 hpi. The luciferase activity was measured as an indicator of viral

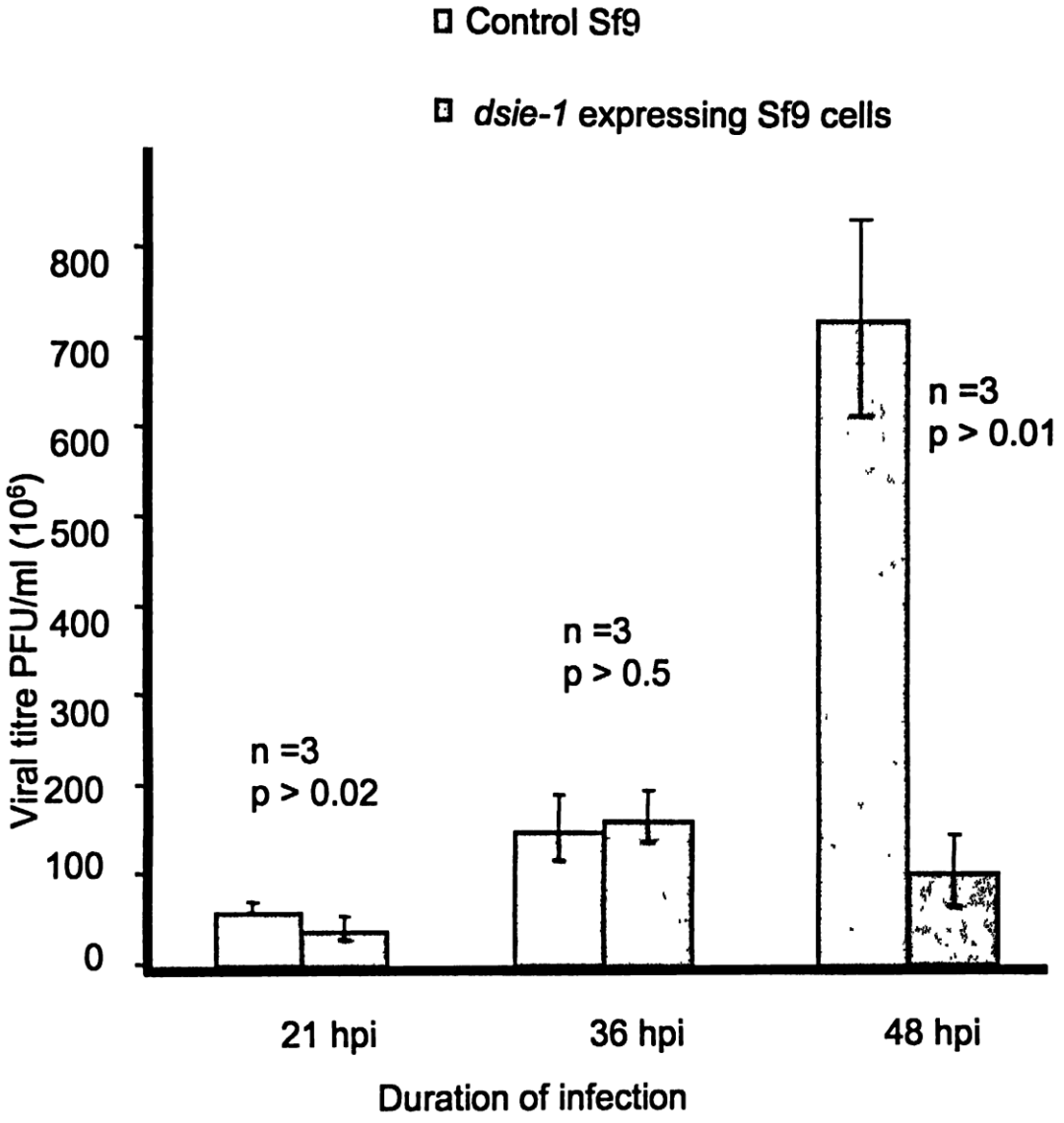


Fig 17 Plaque assay on polyclonal transformed Sf9 cells. The viral titre is expressed as plaque forming unit (PFU) per ml of the culture media and represents the free virus. Significantly reduced plaque formation was noticed in transformed cells at 48 hpi.

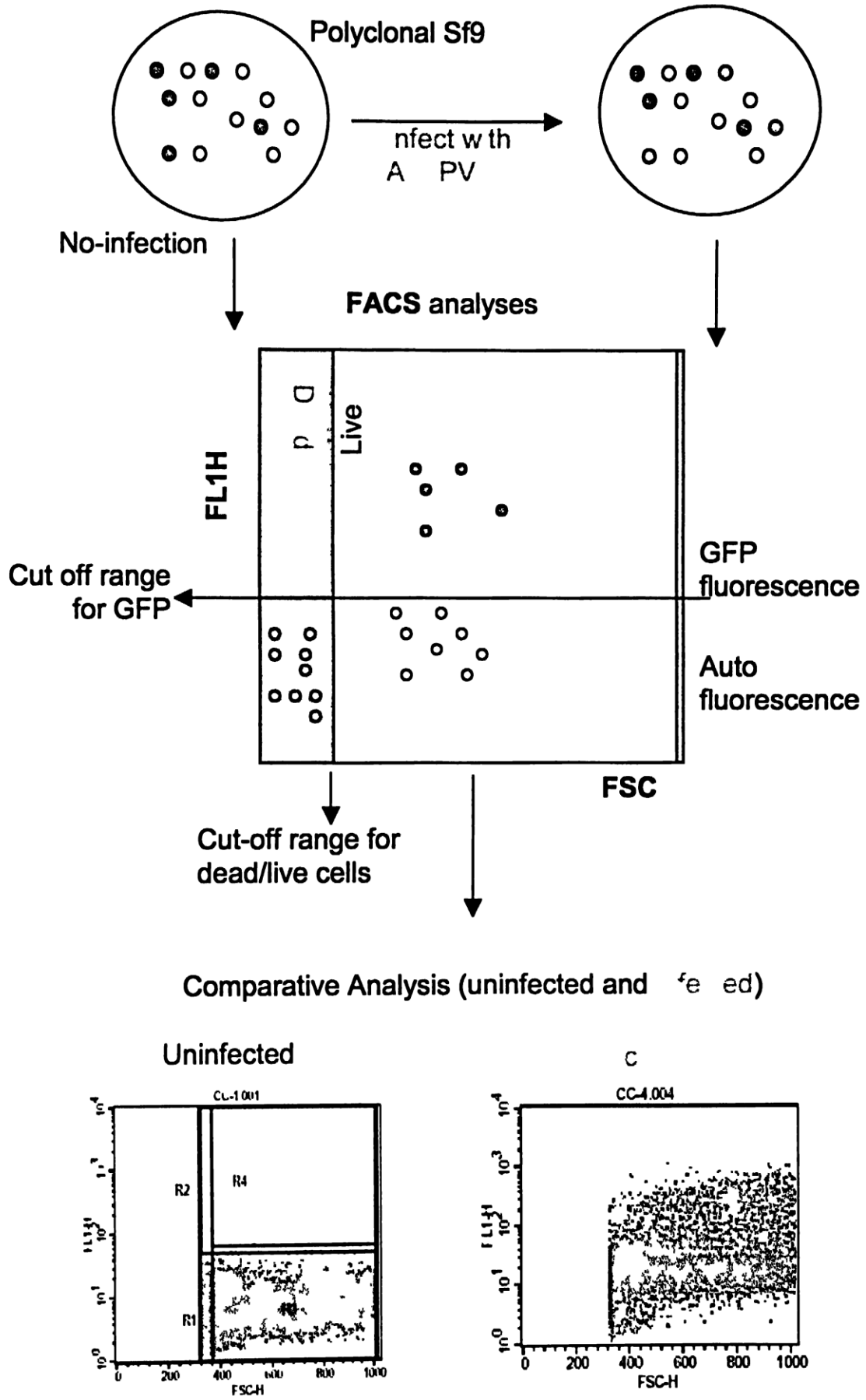


Fig 18 FACS analyses showing selective advantage of *dsie-1* expressing transformed Sf9 cells. Cells were either mock infected or infected with AcNPV and channelized to dead and live cell zones. The dead cells are indicated as red coloured, non-fluorescing cells as yellow and GFP positive cells as green circles. The lowest panel shows actual FACS histogram in which each red dot represents an individual cell. Cells were counted and represnented in Fig 19.

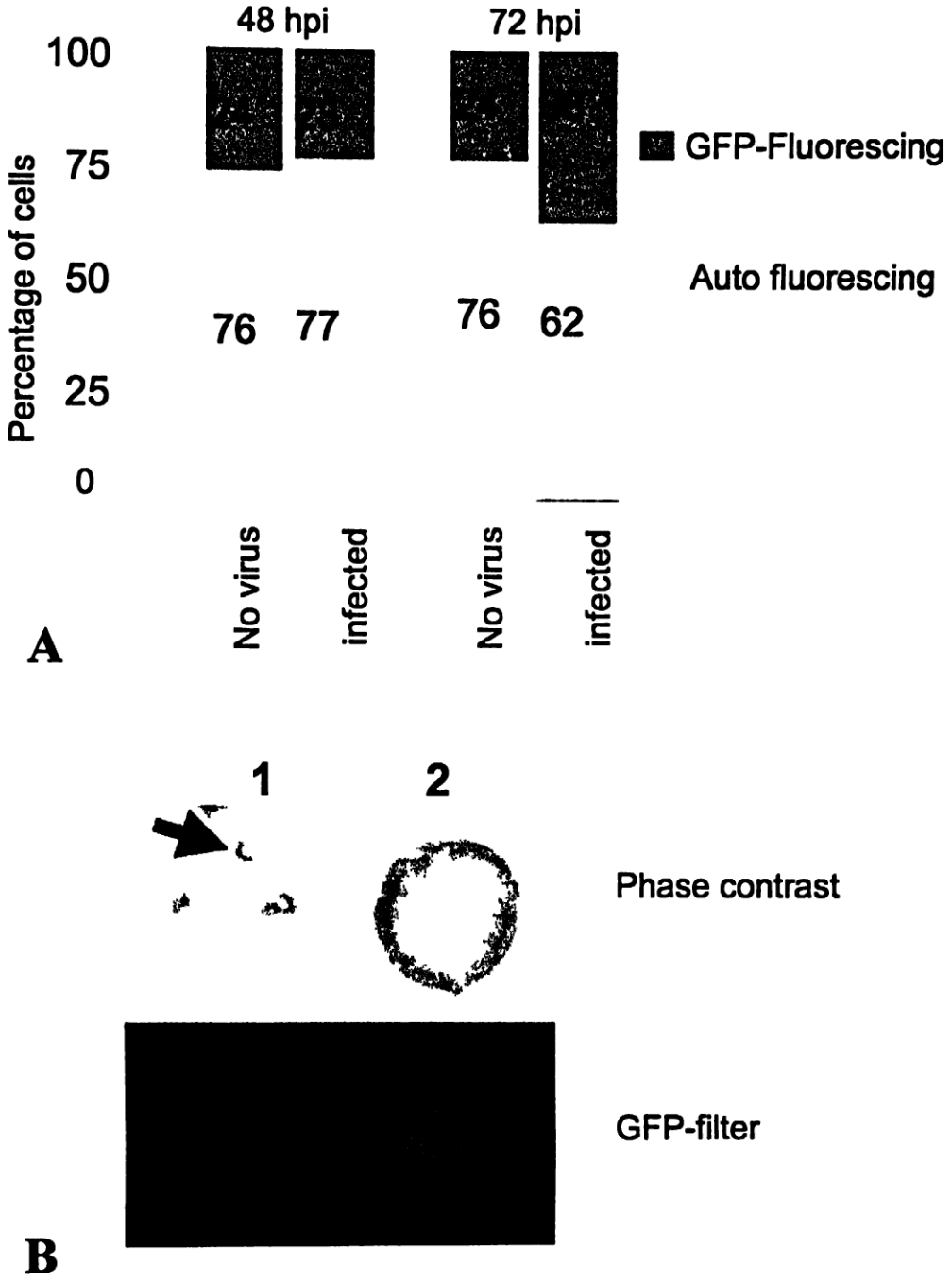


Fig 19 Selective advantage of transformed cells in a polyclonal population of Sf9 cells against AcNPV infection.

A. FACS analysis. The bars indicate the percentage of live cells in polyclonal population. At 72 hpi, the percentage of fluorescing cells increased from 24 to 38 suggesting selective advantage of the transgenic fluorescing cells

B. Microscopic analysis of polyclonal Sf9 cells. The cells were infected with wtAcNPV and microscopic observation was carried out on the third day of post infection. Polyhedra could be observed predominantly in non-fluorescing cells (indicated with an arrow) than in fluorescing cells. 1 – A non fluorescent cell, 2 – Fluorescent cell.

infection. The luciferase activity was significantly lower in transformed cells than the control cells up to 72 hpi. However, by 96 hpi, there was no difference between the control and transformed cell-lines, which suggests the initial protection of cells and subsequent recovery of virus (Fig 20).

Monoclonal population tested did not show efficient viral blockage

Two monoclonal populations of transformed cells were obtained by limit dilution method. Both of them showed a very low fluorescence under fluorescent microscope. When viral infection was carried out on one of these lines, we did not observe any inhibition in viral proliferation (Fig 21). In subsequent passage, these cells could not be maintained and were lost. They could not be recovered from the liquid nitrogen stock.

Germline transgenesis and viral assays on transgenic silkworms

Successful germline transgenesis of the silkworm with pPIG3XP3GFP-FF achieved to obtain six homozygous lines of the transgenics

By piggyBac transposon mediated transgenesis, six homozygote transgenic silkworm lines of Nistari Marked (NM) strain having the construct *pPIG3XP3GFP-FF* that can produce *dsie-1 in vivo* were obtained. These transgenic lines produced green fluorescence in the moth eyes (Fig 22). From 1000 injected eggs, 618 G0 first instar larvae were reared on mulberry leaves (61% of hatching). Around 500 fertile G0 moths were obtained, mated and the G1 offspring were screened for GFP expression in the stemmata. Six transgenic individuals were obtained from the 618 matings (percentage of transformed G0 moths: 1.1%). On the basis of the intensity of the GFP in eyes driven by 3 X P3 promoter, six lines were selected. They were sib-mated to obtain six homozygous lines of transgenics. These six homozygote lines were named as 126A, 126B, 58C, 58E, 93B and 58B2.

BmNPV feeding experiments showed reduced mortality in transgenics

We carried out *per os* BmNPV infection of the transgenic lines 126A, 126B, 58E and 58C and control NM silkworms with 5000 PIBs per larva of 3rd instar.

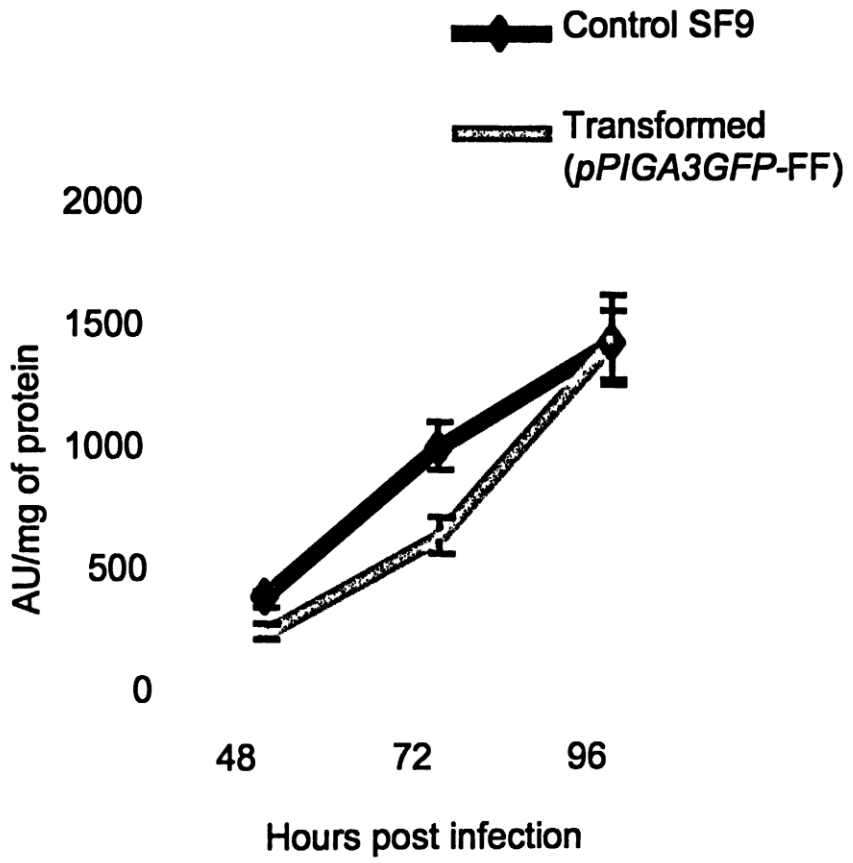


Fig 20 Demonstration of inhibition of AcNPV infection by luminometric assay in transformed Sf9 cell lines. Polyclonal and control Sf9 cells were infected with recombinant AcNPV-*luciferace* virus. Infection was followed by measuring the luminescence. Results confirm the initial inhibition and subsequent recovery of the viral proliferation.

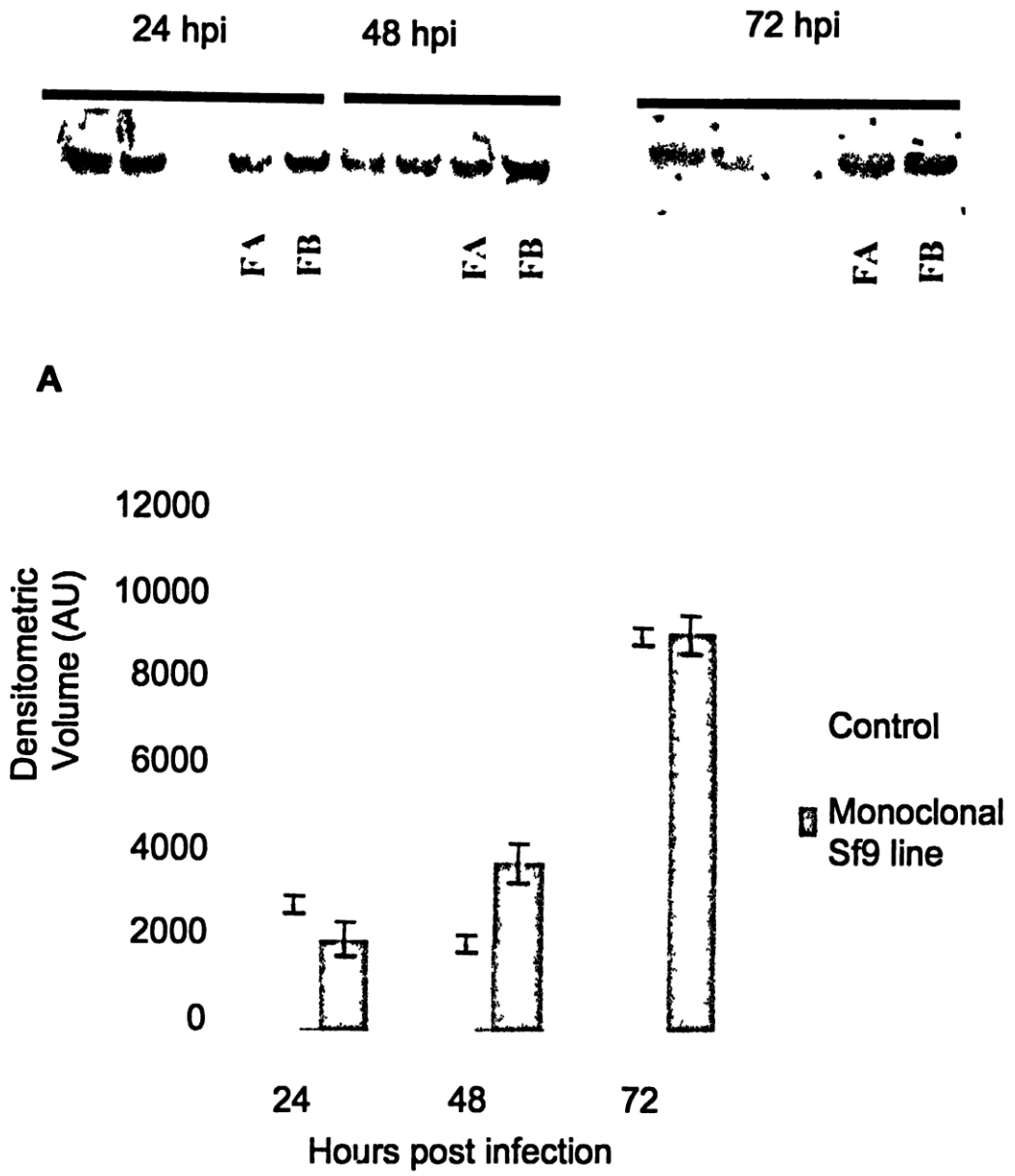


Fig 21 Western blot analysis of a monoclonal Sf9 cell line (3f) using anti-Lef-3 antibody. Result indicates that there was no viral inhibition in this particular monoclonal line. C= Control, F= Monoclonal line – 3f, A & B = Replicates

A. Western blot of Lef-3 protein using anti-Lef-3 antibody.

B. Histogram of density of the Lef--3 western blot bands.

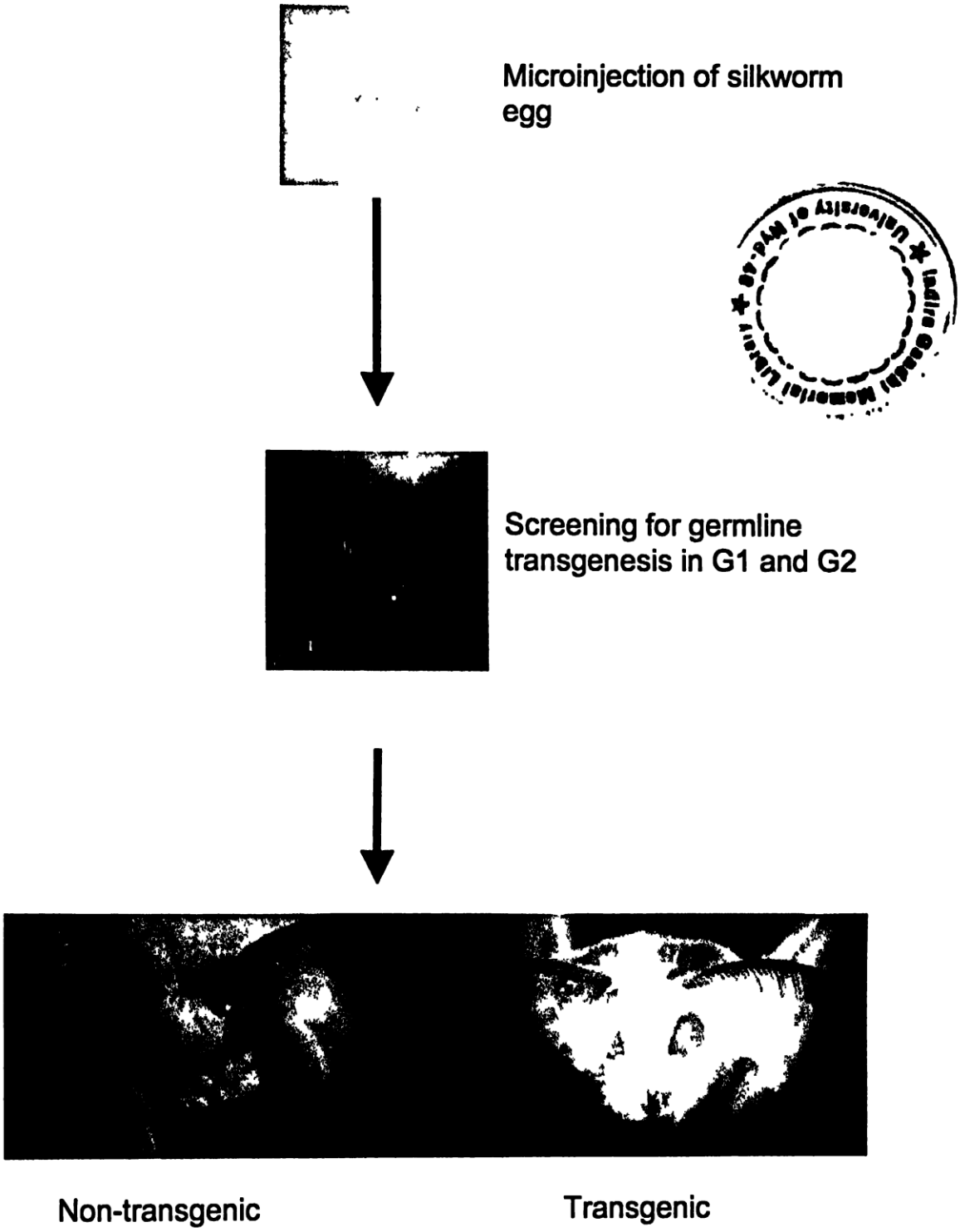


Fig 22 Transgenic silkmoths showing green fluorescence in the eyes under fluorescent light (Right). Control moth does not show any fluorescence in the eyes (left). Transgenics were obtained by microinjection of *pPIG3XP3GFP-FF* and *helper* plasmids to embryos of Nistari strain within six hours oviposition. By controlled breeding, six homozygote lines of transgenic silkworms have been obtained.

Since this viral dose is slightly more than the LD_{50} for Nistari strain (LD_{50} ~ 4000 PIBs/larva at third instar) we used this dosage. One hundred healthy larvae of each line were fed with PIBs and only those larvae that consumed all the viral particles were retained and reared on fresh mulberry leaves till the survived larvae reached moth stage. The experiments were also carried out on 4^{th} instar larvae with a viral dose of 10^4 PIBs per larva. The results of both the experiments are shown in Fig 23. The silkworm mortality was followed until the surviving larvae formed cocoons and the subsequent emergence of moths. The percentage mortality in NM, 126A, 126B and 58C were 89, 17, 44 and 10, respectively for 3^{rd} instar group while it was 85, 10, 13 and 42 respectively for the 4^{th} instar group. These results indicate that the transgenic silkworms, especially the lines 126A and 126B are protected against the BmNPV infection. To confirm the reduced viral proliferation, the hemolymph of five infected larvae were pooled together. The number of polyhedra (PIBs) in hemolymph of the BmNPV-P10GFP fed IV-Instar larvae were counted and expressed as PIBs/ml of hemolymph (Fig 23) and is tabulated below.

Table 5: The number of PIBs observed in the hemolymph of BmNPV-P10GFP fed fourth instar larvae

Line	PIBs in hemolymph ($X 10^4$/ml)
NM	2.8
126A	0
126B	0.01
58E	> 3000

Hybrids of transgenic female and non-transgenic male also showed viral resistance

To test whether the protective effect of transgenic lines could be observed in hybrids of transgenic and non-transgenic lines, we crossed transgenic Nistari females with non-transgenic BmNPV sensitive CSR2 variety of silkworm. The

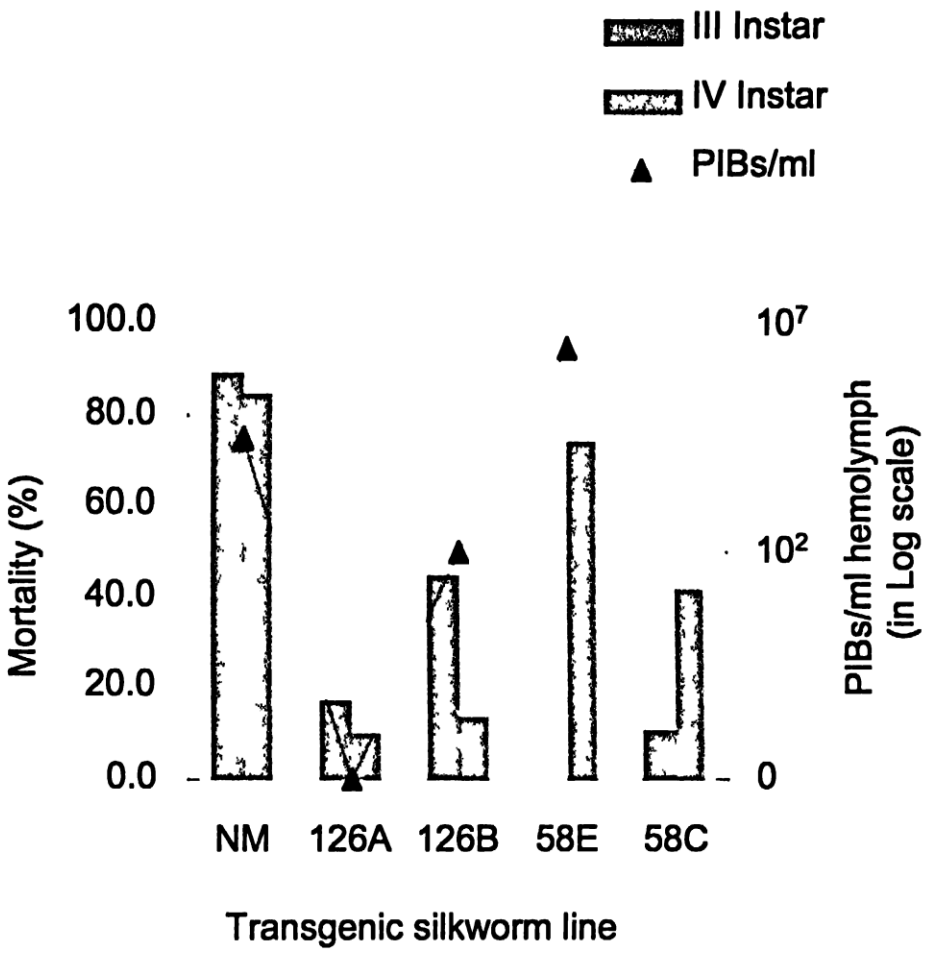


Fig 23 BmNPV resistance in four homozygous transgenic and one non-trangenic control (NM) lines of the silkworm. The third (blue) and fourth (brown) instar larvae were fed with BmNPVP10-GFP at a dose of 5000 and 10000 PIBs/larva respectively. The mortality was counted based on the number of moths survived. The black line indicates the number of PIBs/ml of hemolymph of fourth instar larvae. Line 58E was not tested in third instar stage.

hybrid silkworms were tested in fifth instar day one stage for baculoviral resistance by *per os* inoculation of the BmNPV –P10GFP virus at a dose of 42000 PIBs per larva. Since we observed the hybrid vigour in these hybrid lines, we used a very high dose of virus for *per os* experiment. The results are summarized in Table 6, which indicate the protection of the hybrids particularly of 126A and 126B against very high dose of BmNPV infection.

Table 6: Percentage mortality by *per os* inoculation of BmNPV-P10GFP in fifth instar larvae of the hybrids of transgenic female with non-transgenic CSR2 male.

Cross	n	Mortality (%)	No. of Pupa formed
NM x CSR2	35	24 (68%)	11
126A x CSR2	35	8 (22%)	27
58E x CSR2	35	16 (45%)	19
126B x CSR2	35	13 (37%)	22
93B x CSR2	35	21 (60%)	14

Intra-hemocoel injection of BmNPV-P10GFP to IV and V instar confirmed refractoriness of transgenic silkworms to BmNPV infection

When the viral load of 1000 BV/larva was injected to the silkworms of fourth instar, mortality was high in controls than in transgenics (Table 7).

Table 7: Percentage mortality by intra-hemocoelic injection of BmNPV-P10GFP in fourth instar larvae

Line	n	Mortality	% Mortality
NM	50	50	100
126A	50	28	56
126B	50	30	60
58E	50	45	90

When virus was injected at a dose of 125000 per larva of fifth instar, all the worms irrespective of the group died resulting in 100% mortality even in transgenics. However, when the viral load was 625 BV per larva, the onset and severity of symptoms was slow in transgenic worms than in controls. No viral infection was observed in water injected controls (Fig 24). Thus intra-hemocoelic injection of free virus confirms the pattern of virus resistance as seen by *per os* infection.

Reduced mortality observed in BmNPV injected transgenic pupae

Hundred each of transgenic pupae as well as control NM pupae were injected with a viral dose of 1000 BV/larva. The percentage of survival of these virus-injected silkmoths was counted based on the emergence of the moths. The percentage survival was 16, 3, 1 in 126A, 126B and 58E groups respectively, while no moths emerged from the control non-transgenic NM. All control water injected pupae emerged without any mortality. The result also supports the previous observation that the transgenics are resistant to baculovirus infection.

The transgenic hybrid showed pronounced resistance to BmNPV

The moths that emerged from the survivors of the pupal injection (above section) were crossbred and the offspring were tested for viral resistance. The cross evaluated was 126Ax 58E. The results indicate that the percentage mortality was predominant in control CSR2 than in transgenics. The mortality was only 5 percent in the transgenics hybrid of 126Ax 58E as compared to 25 and 100% in 126AX126A and CSR2. These results suggest heterotic effect of transgenic lines for viral resistance (Fig 25).

Discussion

For having heritable RNAi mediated viral suppression, we cloned *ie-1* promoter and 470 bp fragment of N-terminus of *ie-1* gene in opposite

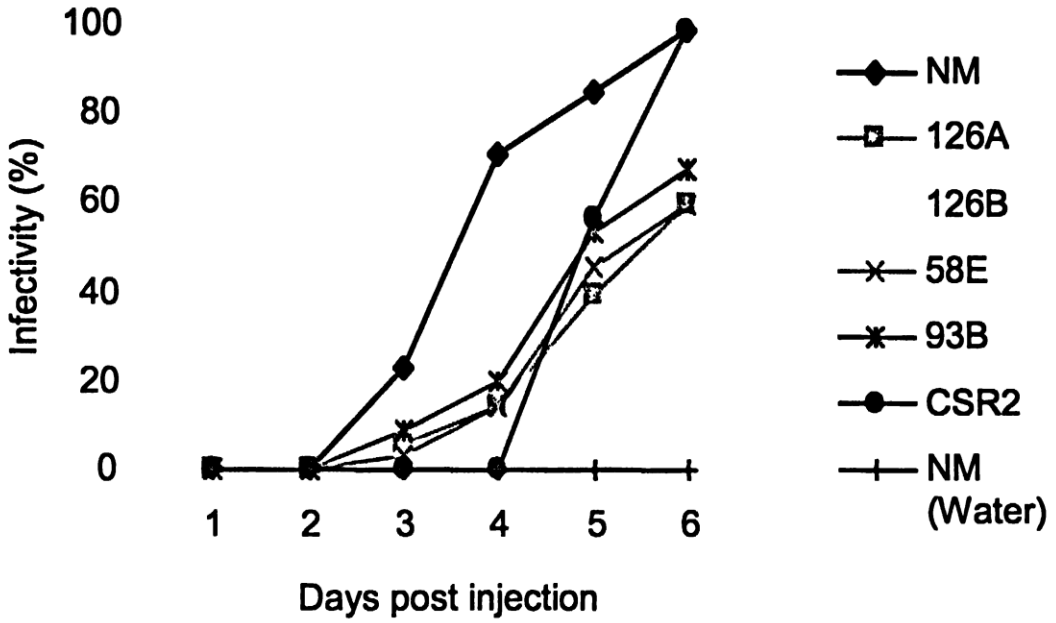


Fig 24 Progression of viral symptoms in V Instar larvae injected with BmNPV-P10GFP virus. Four different lines of the transgenic silkworms and NM and CSR2 non-transgenic controls were injected with a viral dose of 625 (all except NM) or 350 (NM) BV/larva. Results indicate the slow progression of viral infection in transgenics than in non-transgenic silkworms. Water was injected to NM as an injection control to rule out the mortality due to injury during injection.

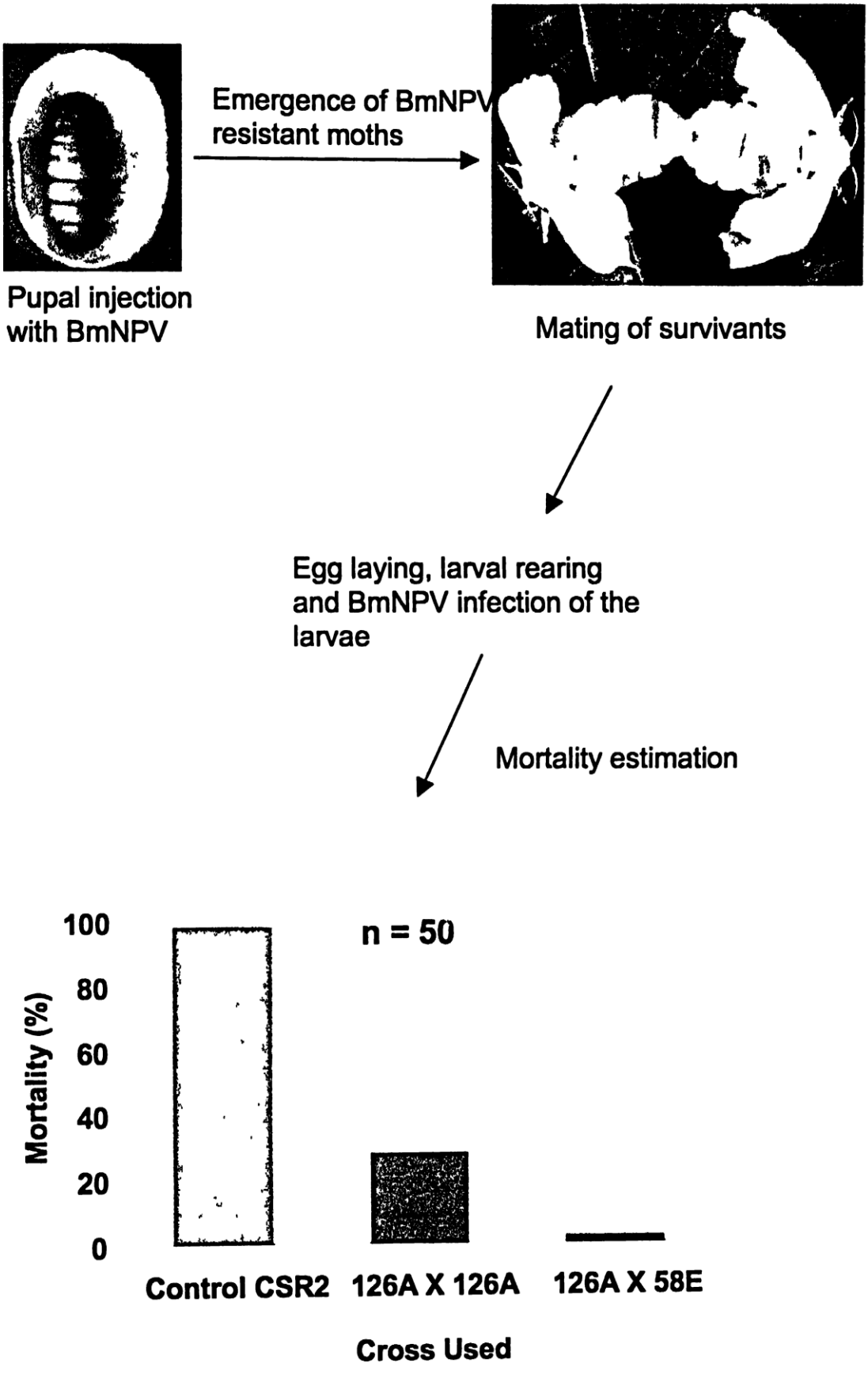


Fig 25. Demonstration of heterotic effect in transgenic hybrid for viral resistance.

orientation in the piggyBac transposon based vector *pPIGA3GFP* to obtain the plasmid *pPIGA3GFP-FF*. This plasmid construct could be used to permanently transform the cell-lines and silkworms. By using this plasmid we obtained a polyclonal Sf9 cell population with ~69 % of the cells expressing GFP. With number of passages, however, we noticed the reduction in the percentage of transformed cells in this polyclonal population. After about 150 passages, the percentage of transformed cells has now been reduced to about 24. Our results by using transformed Sf9 cells show that the AcNPV proliferation is reduced in initial phase of infection, but again virus recovers to a level similar to that of control non-transformed cells. Plaque assay, western blot and recombinant AcNPV with luciferase support these observations. We assume that the non-transformed population of Sf9 cells may dilute the resistance induced by the transformed cells. When we tested two low fluorescing transformed monoclonal populations, we did not see any improvement in inhibition of viral proliferation. This could be due to the 'positional effect'. Since the fluorescence of these lines was very low, we could assume that the transgene might have got lodged in relatively 'silent zone' of the chromatin, hence it may not be producing the *dsie-1* in sufficient quantity to suppress viral proliferation.

Carrying out the plaque assay is a time consuming process with difficulty in reproducibility. Hence, this test was later replaced with western blot experiments. Again we used total protein instead of any specific house keeping protein as a loading control for the reason mentioned in chapter II.

Though we attempted to obtain a transformed BmN cell line, they did not recover after the rigorous treatment of FACS. Hence we continued to use Sf9 cells for most of the experiments.

In the final phase of our work, we obtained six homozygote transgenic silkworms by using the piggyBac transposon based germline transgenesis. We used the vector that expresses the selection marker 3XP3-GFP and *dsie-1*. We

tested their anti-viral activity by intra-hemocoel injection of BmNPV-P10GFP as well as *per os* inoculation of the virus. Based on initial screening, we used four lines of transgenic lines, 126A, 126B, 58E and 58C in most of the experiments. A non-transgenic Nistari marked (NM) was used as a control.

We observed that the transgenic silkworms were infact resistant to BmNPV infection irrespective of the route of viral administration *i.e. per os* or intra-hemocoelic injection. As we tested viral resistance in three important developmental stages of silkworm, *viz*, third-, fourth- and fifth instar larvae, we did observe stage specific differences in the extent of mortality. While the mortality was lower in orally fed group of fourth instar larvae except for 58C than fifth instar larvae, intra hemocoelic injection did not show such variations. This is probably because of the fact that the natural port of entry of baculovirus is midgut and that midgut offers primary defence against viral entry. This could also be inferred from our observation that the injection of virus to pupae did not yield significant survivability as we noticed more than 80% mortality. This could also mean that free viruses probably were less prone to RNAi mediated viral suppression than virus derived from PIBs. Our result indicated that the efficiency of the transgenic lines to impart viral resistance was 126A > 126B > 58E ~ 58C. Other lines showed more or less similar viral resistance as 58E, hence they were not tested extensively.

Chapter IV

Epilogue

The face of truth is covered with a golden disc. Unveil it, nourisher, for one whose duty is to see the truth – Ishavasya Upanishad (2000 BC)

RNAi is fast becoming an effective tool as a gene-knockdown strategy for the benefit of mankind, be it curing the human diseases like cancer or be it shutting down the pathogen proliferation. One can envisage using RNAi to knockdown the essential genes of a pathogen that afflicts considerable damage to economic insects like the domesticated silkworm, *B. mori*. Since BmNPV infection is one of the major *B. mori* diseases, targeting BmNPV and eventual elimination of infection can be a key strategy in improvement of silk yield. Development of baculoviral resistant strains of the silkworm could improve the cocoon output and hence the financial status of the poor farmers who depend on the sericulture for their livelihood. The possibility of introduction of genes to knockdown viral replication is augmented by successful germline transgenesis of the silkworm by piggyBac transposon based vectors and availability of complete genome sequence information and gene functions of BmNPV. With improvement in silkworm transgenesis and in understanding the baculoviral biology, we can now introduce genes that can help silkworm fight against viral replication.

However, we should not be ignorant of the fact that the fight for existence drives the virus to evade all such mechanisms that are designed to eliminate its existence. Baculovirus and its hosts may not be any exception to show this evolutionary trend. Both of them have developed mechanism to counter the strategy that threatens to eliminate them. During infection of the permissive host by AcNPV, the levels of host actin, histone, and heat shock 70 (hsp70) RNAs are reduced substantially (Ooi and Miller, 1988). As virus takes over the host machinery during early phase of infection, most of the cellular transcription/translation machinery is shutdown to facilitate the viral proliferation. The early gene products like *ie2* prevent cell-cycle progression, arresting the cells in S or G2/M phases (Braunagel et al. 1998, Saito et al. 2002). In response to the viral attack, the host activates protective measures like apoptosis. The importance of apoptosis in preventing viral spreading and

reducing *in vivo* infectivity of a baculovirus is well studied (Clem and Miller, 1993, Hasnain et al. 2003). The transcription and translation of 'early' class of baculoviral genes like *ie-1* induces this protective self-limiting process. However, the baculovirus counter this offense by producing *P35,* an anti-apoptotic protein that restricts the early 'premature' apoptosis of the cells, thus helping the viral proliferation to occur. Ultimately, one of the mechanisms gets an upper hand resulting in either the establishment of infection or elimination of the virus.

With an intention of obtaining transgenic silkworm strains that are resistant to BmNPV replication, we initiated this study to observe the efficacy of RNAi mediated baculoviral suppression. We approached the objective by targeting essential baculoviral gene *ie-1* in both transient transfection studies (discussed in chapter II) and heritable transgenic studies (Discussed in Chapter III). We studied the RNAi mediated baculoviral resistance *in culturo* in two lepidopteran cell-lines, namely BmN and Sf9 as well *in vivo* in *B. mori.* By transiently transfecting *dsie-1* and quantifying the GP64 protein load, we observed that there was an initial suppression of viral proliferation in both BmN and Sf9 cell-lines, but with progression of infection viral proliferation recovered to a level similar to controls. The results of viral suppression and its subsequent recovery to control levels was confirmed by independent experiments like FACS, microscopic observation and RT-PCR and were consistent in BmN and Sf9 cell lines. The results were independent of higher amount of *dsie-1* in Sf9 cells and prior activation of RNAi machinery by *dsie-1* was necessary for inducing the viral resistance. We also obtained a transformed Sf9 cell-line that has a construct to produce *dsie*-1 with GFP as a selection marker. The transformed Sf9 lines expressing *dsie-1* also showed initial suppression of viral proliferation and subsequent recovery. The results were confirmed by plaque assay as well as by using a recombinant AcNPV expressing luciferase. The microscopic and FACS analysis of the polyclonal Sf9 cell lines confirmed the initial protection

of the transformed cell lines. The polyhedra were slowly formed in GFP expressing transformed lines than in non-expressing lines in the polyclonal population. Similarly, by fourth day post infection the percentage of GFP expressing cells increased in the polyclonal population, confirming the selective advantage of the transformed cells. However beyond this time point, the cell lysis was observed even in the transformed cell lines, reaffirming the recovery of the normal viral proliferation in transformed lines at very late stages of infection.

During our study we also observed that BmN cells are not easily amenable and more refractory for transfection studies than Sf9 cells. Though we attempted to obtain a transformed BmN cell line, they did not recover after the rigorous treatment of FACS. Hence we continued to use Sf9 cells for most of the experiments. BmN cells were refractory to exogenous nucleic acids uptake and showed poor transfection efficiency compared to Sf9 cells. The low level of anti-viral activity shown was probably due to the cells that 'accepted' the dsRNA, the percentage of which was very low. BmN cells show very low efficiency with most of the commercial transfection reagent as well as $CaCl_2$ method. The viral titre observed could be the reflection of those cells that have 'not-accepted' the dsRNA. Further, they are not hardy cells, easily get 'tired' and failed to recover after the tough treatment met with the FACSorting. Hence in the present study we could not recover any of the transformed lines after sorting. The best approach in the future studies for obtaining the transformed lines is to use less severe selection procedures, like antibiotic resistance as a marker gene.

When we challenged the silkworm larvae of CSR2 strain that had been injected with *dsie-1*, we observed that the larvae were protected against BmNPV infection at a low dose of BmNPV (400 PIBs/larva), but the mortality was not significantly altered in *dsie-1* injected worms when the viral load was high (4×10^5 PIBs/larva). These results confirm that the protection induced by *dsie-1*

was of temporary nature and virus could over come the RNAi induced suppression of IE-1 especially when the viral load was very high.

The observed partial inhibition of viral protein expressions during early phase of infection suggested the initial suppression of viral proliferation by targeting essential genes like *ie-1*. However, transient studies indicated that leaky expression of IE-1 is sufficient to establish the subsequent viral proliferation suggesting the inadequacy of *dsie-1* in complete removal of IE-1. Also, it could be due the dilution effect, since *dsie-1* (or siRNA) is unexpected to be transmitted to all the daughter cells during cell proliferation. Hence the new cells generated are unprotected in the absence of RNAi.

There has been an initial report suggesting the successful suppression of AcNPV proliferation in Sf9 cell lines and *Spodoptera frugiperda* (Valdes et al. 2003) by RNAi mediated targeting of IE-1. However, we observed a moderate suppression by baculoviral proliferation only at early stages of infection, but not at later stages of infection *in culturo*. The complete lysis of even the monoclonal transformed cells after 120 hpi suggests the inefficiency of viral blockage while using *ie-1* promoter. Similarly, the *dsie*-1 could not protect the silkworms when the viral dose was very high. Isobe et al. (2004) reported similar observations by targeting *lef-1* genes. They observed moderate viral inhibition but not complete protection in the silkworm that constitutively expressed dsRNA against *lef-1*.

While dilution effect could account for the absence of viral suppression at later stages of infection, the reason for failure of transformed cell lines to completely protect against baculovirus needs to be ascertained. One reason could be due to inactivation of 'endogenous' *ie-1* promoter used in the present study to produce the *dsie-1* at later stages of infection, since it is known that integrated *ie-1* promoter is transcriptionally inactivated at later stages of infection (Jarvis 1993). However, there is no established protocol to demonstrate the production of dsRNA. As soon as dsRNA is produced it will

be acted upon by Dicer, and hence will be chopped off to ~ 21-22 nt fragments. These small fragments are difficult to be identified especially when their expression is very low as in the case of a weak promoter like *ie-1* promoter. Hence it could not be demonstrated whether the *ie-1* promoter is functional sufficiently and gets down regulated. The other reason could be due to insufficiency of *dsie-1* in complete blockage of *ie-1* expression. It is likely that the lepidopteran system may lack the 'amplification' signal of RNAi hence there was not sufficient amount of *dsie-1* or corresponding siRNA. Hence the amount of siRNA may not be in sufficient quantity to block the proliferating virus. Targeting GFP expression itself is being explored for demonstration of RNAi, where-in *dsGFP* is produced along with viral targets.

At least two transgenic lines of the silkworms were found to be refractory to viral proliferation. These two lines are 126A and 126B. Further, 58E also showed viral resistance albeit less efficiently than 126 A and 126B lines. We observed that the viral suppression was indicated by reduced mortality in the transgenics and also confirmed with the observation that number of PIBs in hemolymph of transgenics was less than in control.

The difference between the performances of the lines probably could be attributed to "position effect". The integration site of transgene is known to influence the expression of the transgene. Due to various reasons, we could not complete analyzing the copy number of the inserts, chromosomal localization and other aspects of the site of integration of the transgenes. In future such studies could be helpful, especially the relationship between the copy number and the effect of the transgene in inducing anti-viral defence.

We also observed the different scenario with different route of infection as well as different stages of infection. Profound anti-viral effect was observed when ODV was inoculated through *per os* route than when administered through intra-hemocoelic injection of free virus. Such dissimilarity may be attributed to the differences in the efficiency of viral spreading when

administered by different routes. Further, it may also be due to the difference in the efficiency of the promoter in eliciting anti-viral response. Since the natural 'port of entry' of virus is midgut, it is likely that the *ie-1* promoter derived from the virus used in the present study is more active in midgut cells than in other cells. However, this highly speculative argument needs to be verified with further experiments. Similarly, our results indicate that the stage of inoculation could also influence the severity of infection. The mortality was marginally reduced when virus was fed in fourth instar than in third instar. We speculate that it could be due to accumulation of more siRNA as the larval development proceeds. Further, it could also be due to the difference in the efficiency of promoter. Again, these observation needs to be confirmed by further experiments. Similarly, the difference observed between the cell-line and the transgenic moth could potentially be due to the efficiency of the promoter. Alternatively, the 'poly clonal' nature of the cell-line might explain the viral recovery and complete lysis of the cell in later stages of infection that is attributed to viral proliferation in non-transformed cells. The increased titre of the virus could hamper the immunity induced by *dsie-1*. The quantity of *dsie-1* probably was insufficient to block viral proliferation when viral load was very high. This is infact confirmed by *dsie-1* injected worms that are susceptible to viral infection when the virus load is very high. In this regard, even transgenic lines showed susceptibility when the higher viral load was administered.

CSR2 strain shows a unique feature during viral infection. At initial stages of infection the larvae look healthy, but at later stage of infection, symptoms are revealed instantly and in a day or two from this point, almost all the larvae succumb to infection. This is in quite contrast to Nistari strain, which shows the symptoms of infection from second day onwards. All BmNPV-P10GFP infected larvae show a unique greenish-yellow discoloration of the skin/cuticle at later stages of infection (3^{rd}-7^{th} day post-infection) and they wander a lot. For improving the anti-viral effect, it will be interesting to see the effect of

multigene targeting wherein different essential baculoviral genes could be targeted simultaneously. We are continuing our research in this direction. Use of a better universal promoters like heat shock protein and cytoplasmic actin needs to be explored as the drivers of double strand of target RNA. However, further studies are needed to confirm the long-term efficacy of RNAi for using as an anti-pathogen tool. Since viruses have developed the pathways to suppress the RNAi, it is necessary to strike a cautionary note while using RNAi as a tool. Evidences are emerging from different studies, which indicate the failure of RNAi strategies to gene knockdown due to ineffective delivery of dsRNA, activation of immune pathways in mammals as well as successful combating of RNAi machinery by the virus. It has been reported that many viruses including invertebrate viruses suppress the RNAi pathway by expressing inhibitors of RNAi pathways (Riu et al. 2004). Even though it is highly speculative, we are prompted to think about the possibility of such an inhibitor of RNAi in the baculovirus and *in silico* studies could help us in identifying such inhibitors in baculoviral genome, present if any. It is essential to know such inhibitors for improved efficiency of RNAi in combating baculoviral infection.

In conclusion, we have shown here that the RNAi mediated baculoviral resistance is possible in the lepidoptera, with particular emphasis to the domesticated silkworm, *B. mori*. The transgenic strains of the silkworms having construct for RNAi mediated BmNPV resistance could successfully be produced by piggyBac mediated germline transgenesis. We conclude that further studies in this direction could enlighten us to use RNAi as a tool for baculoviral inhibition of infection.

A final word

I have made an attempt to evaluate the efficiency of RNAi in silkworm and to obtain transgenic silkworm strains that inhibit the viral proliferation. Though my attempts to get a completely resistant strain have not richly paid, I

personally believe that this attempt is a first step towards achieving the final goal of obtaining a better strain of the silkworm having BmNPV resistance. A number of caveats still remain and a few more robust experimental approaches are needed to be addressed to answer a number of observations reported here. Further studies need to be carried out to strengthen the viewpoints that I have presented here. A number of factors determine the success of this goal. In the present context of RNAi based strategy, the viral gene to be targeted, the choice of promoter, and the efficiency of the RNAi machinery all contribute to the success. Further, success of transgenic worms *vis-à-vis* wild type worms in their natural niches also determine the outcome of the strain in the long run. The transfer of the transgenes needs to be carried out to the commercially successful strains so as to benefit the sericulturists. Before the release of a viral resistant transgenic strain, it is essential to find whether the transovarial transmission of BmNPV is more in resistant strain, whether the transgenic resistant strain harbours replicative, but non-detrimental viral particles and whether the fecundity of transgenic strains is anyway compromised. Further studies on long standing implications of transgenics and their maintenance need to be tested, which all together constitute another research topic of interest.

The evolution continues with fight for existence and survival of the fittest.

Chapter V

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Kelava ballavarinda kalthu kelavam malpavarinda kandu maththe halavam thane swathaha maadi thili enda saravjna – (In Kannada language meaning - Learn somethings from those who know; Watch somethings from those who do; Learn manythings by self experience - Sarvajna - Kannada poet – 17th Century, As in Wikepidia online dictionary).

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B) Some Useful web sites, online-tools and softwares used for research purpose

For collection of references, abstracts and other research materials, most useful site is http://www.ncbi.nlm.nih.gov

Sequence homology search is at http://www.ncbi.nlm.nih.gov/BLAST/

The Universal Virus Database, ICTVdB, by International Committee on Taxonomy of Viruses at http://www.ncbi.nlm.nih.gov/ICTVdb/index.htm

A useful guide for protocols at http://www.protocol-online.org/

Multiple Sequence Alignment by using the software ClustalW at http://clustalw.genome.jp/

B. mori EST database at http://www.ab.a.u-tokyo.ac.jp/silkbase/

The microRNA Registry at http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml

Mfold is available at http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html

SilkSatDb developed by our lab is available at http://www.cdfd.org.in/silksatdb

For silkworm genome sequence Silkworm Knowledge base, available at http://silkworm.genomics.org.cn/

Genome sequence of silkworm from Japanese group is available at http://sgp.dna.affrc.go.jp/KAIKO/

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Chapter VI

Summary

Endaro Mahaanubhaavulu Andarikee Maa Vandanaalu (In Telugu language meaning – I will bow to all those great people - Thyagaraja - A Tamil and Telugu Poet 1767 (?) – 1848, As in Wikepidia online dictionary)

Baculoviridae is one of the fourteen major families of invertebrate viruses that infect arthropods, primarily insects. This family consists of two genera, Granulosis virus (GV) with twelve species and nuclear polyhedrosis virus (NPV) with 24 species. The baculoviruses are unusual in that they produce two types of virions, the occlusion-derived virus (ODV) and the budded virus (BV or free virus). ODVs (also known as Polyhedral Inclusion Bodies -PIBs) are enclosed within a sheath of protective cover that protects the virus in hostile environment and they cause host-to-host infection. The BV initiates the intra-individual infection and is unstable in open environment. Most baculoviruses are host-specific and are thus named accordingly. *Autographa californica* multiple nuclear polyhedrosis virus (AcNPV) is considered as the type species of NPVs and is one of the well-studied invertebrate viruses. Another well known species of baculovirus, *Bombyx mori* nuclear polyhedrosis virus (BmNPV), is a major pathogen of the domesticated silkworm, *Bombyx mori* L. This important lepoidopteran host is a unique organism having both scientific and economic significance. Due to availability of a number of strains, mutants and genetic markers, *B. mori* has emerged as a molecular model for genetic and genomic analyses. It is also a major contributor to the sericulture, accounting for more than 90% of the global silk out put. The economic viability of the sericulture industry largely depends on the silkworm strains that are resistant to various diseases and high silk yielding property. Among the parasites that infect silkworm, BmNPV causes heavy mortality resulting in cocoon crop losses to an extent of 40-60% annually. Thus the development of silkworm strains that are refractory to baculoviral infection is crucial for enhancing the silk production and improving the standard of living of thousands of farmers.

Life cycle of baculovirus starts when caterpillars eat polyhedra contaminated mulberry leaves. Once inside the gut, polyhedral cover is dissolved by extreme alkaline pH (about 10) of the midgut digestive juice, releasing virions. Virions thus released enter midgut cells and viral genes start transcribing in a cascade

fashion. The early genes are transcribed from the early promoters by α-Amanitin sensitive host RNA polymerase II, followed by the expression of other late expression factors (LEFs) and very late genes. While eighteen of the LEFs contribute to the expression from late promoters, six of them (*immediate early-1*, *lef-1*, *lef-2*, *lef-3*, *p143* and *p35*) are essential for viral replication. The *ie-1* gene is one such essential immediate early gene, the size of which in BmNPV is 1.75 kb. It has been hypothesized that the 67 kDa IE-1 protein oligomerizes within the cytosol and subsequently enters the nucleus, where it gets involved with the viral DNA replication factories to function as modulator of viral gene expression. Because of its important role in viral replication, knocking-down or blocking the expression of *ie-1* transcript could prove to be an effective strategy for inhibiting the virus proliferation. In this regard, RNA interference (RNAi) could come handy to achieve the objective of suppressing the baculoviral proliferation.

RNAi is a recently discovered mechanism of mRNA degradation by homologous double-stranded RNA (dsRNA). This mechanism is now believed to be an ancient anti-viral strategy, part of which is also utilized for gene regulation process. The RNAi mechanism can be categorized into three steps, initiator, effector and maintenance. In the initiator step, the Dicer enzyme or its homologue cleaves any dsRNA into smaller ~21-22 nucleotide fragments. These 'small interfering RNA' (siRNA) then become the co-substrate for an enzyme complex called 'RISC' (RNA induced silencing complex). In the effector step, RISC, by complementary base pairing between siRNA and homologous mRNA, destroys the latter resulting in gene silencing. The signals of RNAi are spread to the whole organism in the maintenance step. Researchers now exploit this gene silencing phenomenon as a technique for anti-viral and anti-parasite strategy.

In silkworm, we hypothesized that RNAi mediated gene knockdown of *ie-1* gene should lead to suppression of BmNPV in transgenic cell culture and in

transgenic silkworms that express double stranded RNA against *ie-1* gene. In this regard, the objective of the present study was set to analyze the feasibility of RNAi mediated baculoviral resistance using *in culture* and *in vivo* conditions. We approached the study of RNAi mediated resistance against baculoviral proliferation by both transient transfection and heritable integration of a transgene, expressing *dsie-1*.

To test the efficacy of RNAi in inhibiting the baculoviral infection, we initiated the cell culture study in two lepidopteran cell lines, namely, BmN (from *B. mori*) and Sf9 (from *Spodoptera frugiperda*). We analysed the viral proliferation by checking the expression of a viral coat protein, GP64. BmN cells were incubated with *dsie-1* for a day followed by infection with wtBmNPV. The viral proliferation was monitored for a duration of 24 –96 hpi. The western blots using anti-GP64 antibody indicated a 50% reduction in the GP64 protein as compared to the mock transfection at 24 hpi. However, the GP64 protein level in *dsie-1* treated cells reached that of controls by 96 hpi. The observations were further confirmed by RT-PCR, microscopic and FACS analyses. Further, we found that the prior activation of RNAi machinery was necessary for RNAi mediated viral suppression. This was confirmed by the observation that the reduction in viral proliferation occurred only when *dsie-1* was transfected prior to the viral challenge and not when *dsie-1* transfection and viral infection were carried out simultaneously. In addition, the lysate from the *dsie-1* treated cells cleaved anti-sense *ie-1* (*asie-1*) RNA, while the lysate from control cells could not cleave the *asie-1*, confirming the need for prior activation of RNAi machinery. Following the transfection of Sf9 cells with different amounts of *dsie-1*, we found that the amount of dsRNA that could suppress the virus maximally was 1 μg for half a million cells, and increased amounts of *dsie-1* beyond this did not increased the viral inhibition.

To test the efficiency of the injection of *dsie-1* in eliciting anti-viral activity in silkworms, a diapause strain (CSR2) of the silkworm susceptible to baculoviral

infection (LD_{50} ~400 PIB/larva) was used. Buffer control or *dsie-1* (25 µg/larva) was injected into the hemocoel of the fifth instar larva (n = 30) and 24 hours later each of the larvae was fed with either 400 PIBs (10^4 PIBs/ml) or with 400000 PIBs (10^7 PIBs/ml) of wt BmNPV. The larvae were maintained till the moths emerged and mortality was noted based on the number of moths emerged. No mortality was observed in *dsie-1* injected larvae fed with a lower dose of virus (10^4 PIBs/ml), while control buffer injected worms showed 20% mortality. However, at a higher dose of virus (10^7 PIBs/ml), there was 60% mortality in both control and *dsie-1* treated groups suggesting the protection rendered by injection of *dsie-1* was efficient only when the viral load was low.

A piggyBac transposon based vector has been successfully used by the French research group for germline transgenesis of the silkworms. In the present study we used this vector backbone and cloned 620 bp of *ie-1* promoter and 470 bp fragment of N-terminus of *ie-1* gene in opposite orientation to obtain the plasmid *pPIGA3GFP-FF*. For *in culturo* transgenesis of the lepidopteran cell-line, Sf9 cells were transfected with this vector along with the helper plasmid encoding piggyBac transposase. Once the GFP expression was stabilized (after 6 passages), we selected the transgenic Sf9 population by FACS. The polyclonal Sf9 cells thus obtained were a heterogeneous population with ~69% of the cells expressing GFP.

Transgenic Sf9 cell-line having the *pPIGA3GFP-FF* was tested for its viral resistance potency. The cell-line having *pPIGA3GFP* was used as a negative control. For viral resistance assay, cell lines were infected with 5 multiplicity of infection (MOI) of wtAcNPV. The supernatant media containing the BV was collected at regular interval of hpi and plaque assay was carried out. The release of BV was slow in transgenic cells at about 36 to 48 hpi when compared to the control cells, indicating the suppression of viral proliferation at this stage.

The polyclonal population of transgenic cells infected with a wtAcNPV showed a distinct pattern of infection in which the GFP expressing (*dsie-1*

positive cells) showed reduced PIBs than the non-fluorescing cells. However, on increasing the duration of infection, the GFP expressing cells also started showing the polyhedra and by fifth day of infection all most all the surviving harboured polyhedra and started lysing. The inhibition of viral infection at early stages was also confirmed by using a recombinant AcNPV that expressed luciferase gene under polyhedrin promoter.

To find the survivability of transgenic cells in the presence of virus, we infected the polyclonal transgenic cells with AcNPV and followed the progression of infection by FACS analysis. On third day of infection, the percentage of fluorescing transgenic live cells increased from 24 to 38 while the non-fluorescing cells decreased. However by fourth and fifth day of infection there was an excessive cell death leading to obscure FACS analysis.

We also carried out viral suppression assay by measuring LEF-1 and IE-1 production in a monoclonal population of transgenic SF9 cells using respective antibody. However this cell line did not show any significant viral suppression. It could be because of a very low level of expression of *dsie-1* transgene possibly due to positional effect.

With the number of passages, we noticed the reduction in the percentage of transgenic cells in the polyclonal population. After about 150 passages, the percentage of transgenic cells was reduced from 69 to 24.

In the final phase of our work, we constructed a piggyBac transposon based vector having the selection marker *3XP3-GFP* and the DNA for expressing *dsie-1 (pPIG3XP3GFP-FF).* Since the expression of GFP is limited to only a few organs in the transgenics, 3XP3-GFP selection marker helps for easy screening of the transgenic silkworms at early stages of development. By co-injecting *pPIG3XP3-GFP-FF* and helper plasmid to freshly laid eggs of Nistari strain, we obtained several lines of transgenic silkworms. By controlled breeding, we obtained six homozygous lines of transgenic worms, which we

tested for their anti-viral activity using a recombinant GFP expressing BmNPV, BmNPV-P10GFP.

For intra-hemocoelic injection experiments, we used 4 groups of larvae, Nistari (NM) non-transgenic-control and 3 transgenic lines 126A, 126B and 58E. Thirty-five 5th Instar larvae of day one were injected with 625 BV/larvae in each group. No significant mortality was observed in any of the groups until seventh day pi. However, the symptom of viral infection was more evident in the control group than in the transgenic silkworms. By sixth day all control larvae were found to be infected whereas in transgenic larvae ~60-75% were infected. Furthermore, when fourth instar larvae were injected with 1000 BV per larva, we observed 100, 56, 60 and 90% mortality in NM, 126A, 126B and 58C lines respectively.

We tested the *per os* inhibition of viral infection in the three transgenic lines (126A, 126B and 58C) and control silkworms. One hundred each of 3rd and 4th Instar larvae were fed with 5000 and 10000 PIBs per larva respectively and only those worms that consumed all the viral particles were selected and maintained. The results indicated that the percentage mortality was 89, 17.6, 44.7 and 10.5 for control, 126A, 126B and 58C respectively in 3rd Instar larvae, while it was 85, 10.4, 13.6 and 42.3 respectively for 4th Instar larvae. These results indicate that the transgenic silkworms, especially the lines 126A and 126B are protected against the BmNPV infection.

To test whether the protective effect of transgenic lines could be observed in the hybrids, mating was done between non-transgenic BmNPV sensitive variety (CSR2) of male silkworm and the three lines of transgenic females worms (126A, 126B and 58E). The hybrid silkworms were fed with BmNPV-p10-GFP virus. This virus can form polyhedra and fluoresces in the infected worms. We fed the hybrid larvae of fifth instar with the virus at a dose of 420000 PIBs per larva. The percentage mortality of 68, 22, 45, 37 and 60 was observed in the hybrids of control X CSR2, 126A X CSR2, 58E X CSR2, 126B

X CSR2 and 93B X CSR2 respectively. The resistance of the hybrids 126A X CSR2 and 126B X CSR2 to baculovirus was quite apparent, suggesting that the RNAi successfully prevents the viral proliferation.

In conclusion, we have shown here that (a) the RNAi mediated baculoviral resistance is possible in lepidoptera, with particular emphasis to the domesticated silkworm, *B. mori* and (b) Transgenic strains of the silkworms having transgene for RNAi mediated viral inhibition of infection could successfully be produced by piggyBac mediated germline transgenesis. We also conclude that further optimization by using a better promoter and having multiple gene targets for RNAi could enhance the anti-viral effect in the silkworms.