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Comparison of factors that may affect the inhibitory efficacy of transgenic RNAi targeting of baculoviral genes in silkworm, *Bombyx mori*

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ABSTRACT

Bombyx mori nucleopolyhedrovirus (BmNPV) is the primary pathogen affecting *B. mori*. This virus could be combated via RNAi of BmNPV genes in transgenic silkworm. However, several factors may affect the resistance of transgenic RNAi silkworm, such as the connection pattern of gene fragments and spacers ("head to head" or "tail to tail"), and the selection of promoters and target genes. In this study, we constructed several transgenic RNAi vectors using different phase genes (*ie-1*, *helicase*, *gp64*, and *vp39*) and promoters (BmNPV IE1 promoter (IE1P), IE1P combined with hr3 enhancer of BmNPV, and *B. mori* A4 promoter (A4P)). Transgenic lines were generated via embryo microinjection using a practical silkworm strain. We analyzed the anti-BmNPV ability, virus gene mRNA level, and BmNPV content of these transgenic larvae. The results showed that "head to head" was better than "tail to tail," IE1P combined with hr3 was better than IE1P and A4P, and an immediate early gene was the best target for RNAi.

1. Introduction

Bombyx mori nucleopolyhedrovirus (BmNPV) is a typical model baculovirus, which is an enveloped virus with a circular double-strand DNA genome that only infects arthropods (Kondo and Maeda, 1991). NPV produces two virion phenotypes during its infection cycle, i.e., an occlusion-derived virus (ODV) that spreads infection among hosts and a budded virus (BV) that transmits infection throughout the host (Keddie et al., 1989; Rahman and Gopinathan, 2004). The polyhedral body, which is a highly symmetrical covalently cross-braced robust lattice (Ji et al., 2010), dissociates after polyhedra are orally ingested by insect larvae and the ODVs are released into the alkaline gastric juice environment. The enveloped virions invade midgut columnar epithelial cells by membrane fusion to cause primary infections (Horton and Burand, 1993; Keddie et al., 1989). Next, the infected epithelial cells produce BVs that initiate a secondary infection (Slack and Arif, 2007). The expression of NPV genes has a temporal pattern with four phases: immediate early (0–4 h post infection, hpi), delayed early (5–7 hpi), late (8–18 hpi) and very late (>18 hpi) (Huh and Weaver, 1990a, b). NPV DNA replication (beginning at 8 hpi) represents the transition from the early stage to the late stage (Rice and Miller, 1986).

The BmNPV genome (T3 strain) contains 128413 bp and 136 genes (Gomi et al., 1999). The NPV *immediate early-1* (*ie-1*) gene, which encodes a 67 kD protein (IE1), is an essential gene for viral DNA replication (Kool et al., 1994; Lu and Miller, 1995; Okano et al., 1999; Taggart et al., 2012) and is a powerful transactivator of early, late, and very late gene expression (Guarino and Summers, 1986; Passarelli and Miller, 1993; Yamada et al., 2002). The NPV *helicase* gene, which encodes a 144 kD protein (DNA Helicase), is an essential gene for NPV replication (Kamita and Maeda, 1993; Kool et al., 1994; Lu and Carstens, 1991; Lu and Miller, 1995) and it is a delayed early gene, which is also involved in baculovirus host range expansion (Kamita and Maeda, 1997). The NPV *gp64* gene, which encodes a 61 kD envelope glycoprotein (GP64), is an essential viral structural protein required for BV production (Monsma et al., 1996; Oomens and Blissard, 1999; Whitford et al., 1989; Zhou and Blissard, 2008) and a late gene with its peak expression at ~12 hpi (Thiem and Miller, 1989; Whitford et al., 1989). The NPV *vp39* gene is an essential late gene (Lu and Miller, 1995; Passarelli and Miller, 1993; Thiem and Miller, 1989), which encodes a 39 kD protein (VP39), and it is a major capsid protein

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whose accumulation is unaffected by temperature (Fan et al., 1996; Thiem and Miller, 1989). BmNPV contains five homologous regions (hrs) (Majima et al., 1993), which are repeated sequences and the origin of viral DNA replication (Kool et al., 1993; Majima et al., 1993). Hrs can increase virus promoter activity (Carson et al., 1991; Guarino et al., 1986; Jiang et al., 2012a). However, the activity of the affected promoter linked to hrs would be enhanced significantly by IE1-mediated activation, which binds to a palindrome in the hrs (Guarino and Dong, 1994; Jiang et al., 2012a; Lin et al., 2010; Nagamine et al., 2005; Pullen and Friesen, 1995; Rodems et al., 1997). BmNPV is the primary pathogen affecting sericulture and it causes severe economic losses. There are very few basic methods for controlling its infectivity.

Knocking down viral genes by RNAi is effective for promoting virus resistance (Carmona et al., 2006; Huelsmann et al., 2006; Murakami et al., 2005), because it can destroy specific mRNAs and lead to target gene knockdown (Fire et al., 1998). Special short hairpin RNAs (shRNA) can effectively inhibit the infection of hepatitis B virus (HBV) (Carmona et al., 2006). The replication of Japanese encephalitis virus (JEV) was also inhibited via transfection with small interference RNA (siRNA) in cell lines and mice (Murakami et al., 2005). RNAi is useful for controlling the proliferation of drug-resistant HIV-1 (human immunodeficiency virus type 1) (Huelsmann et al., 2006). There have been a few reports of resistance to NPV via RNAi (Isobe et al., 2004; Kanginakudru et al., 2007; Valdes et al., 2003). Infection with *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) was suppressed via the introduction of long dsRNAs from *gp64* and *ie-1 in vitro* and *in vivo* (Valdes et al., 2003). Knocking down the *ie-1* gene may enhance the anti-BmNPV capacity of transgenic silkworms (Kanginakudru et al., 2007). However, the survival rate did not increase in a transgenic silkworm, where the *lef-1* gene was targeted via RNAi, after infection with BmNPV, although viral multiplication was decreased partially (Isobe et al., 2004). These studies (Isobe et al., 2004; Kanginakudru et al., 2007) showed that several factors affect the antiviral ability of transgenic silkworm targeting BmNPV genes via RNAi. It has been suggested that these factors include the connection pattern of gene fragments and spacers (“head to head” (5′–5′) or “tail to tail” (3′–3′)), the promoter efficiency, and the target genes efficiency. However, no systematic comparison of these factors has been reported.

To improve the exploitation of RNAi targeting in BmNPV genes and enhance the resistance of transgenic silkworm, we comprehensively compared the factors that may affect the efficiency of RNAi. In the present study, we compared the different phases of the following target genes: immediate early gene *ie-1*, delayed early gene *helicase*, and the late genes *gp64* and *vp39*. The efficiency of different promoters were compared by controlling the expression of *gp64* dsRNA using a BmNPV IE1 promoter (IE1P), IE1P combined with hr3, and the *B. mori* A4 promoter (A4P). The difference between “head to head” and “tail to tail” was also analyzed by constructing different transgenic RNAi vectors using *ie-1* and *gp64* as target genes. These transgenic RNAi vectors were microinjected into embryos of the practical silkworm strain “932.” These transgenic RNAi lines and nontransgenic lines were investigated in terms of their mortality, the mRNA expression of virus genes, and their BmNPV content. This study is the first comprehensive comparison of the factors that may affect transgenic RNAi targeting of baculovirus genes.

2. Materials and methods

2.1. Insect and virus

Silkworm strain “932” was maintained at the Gene Resource Library of Domesticated Silkworm (Southwest University, Chongqing, China). The wild BmNPV (Guangdong strain, China) was collected as previously described (Jiang et al., 2012a, 2012b).

2.2. Construction of transgenic RNAi vectors

The Genomic DNA of BmNPV OBs was extracted using a MiniBEST viral RNA/DNA Extraction Kit Ver. 3.0 (TaKaRa) and used to amplify the sense fragment and antisense fragment of *ie-1*, *helicase*, *gp64*, and *vp39*. The A3 intron was cloned from the genomic DNA of silkworm strain *Dazao* using the primer A3intron and it was used as a spacer. The termination signal SV40 was amplified from the *piggyBac* [3× p3 EGFP afm] vector using the primer SV40. IE1P was cut from the pBSII-IE1-orf plasmid. The A4P and BmNPV hr3 enhancer were maintained in our laboratory (Jiang et al., 2012a). IE1P, the sense fragment of *ie-1* (IE1-S, amplify with primer IE1-S), A3 intron, the antisense fragment of *ie-1* (IE1-A, amplified using the primer IE1-A), and SV40 were added to the basic transgenic vector *piggyBac* [3× p3 EGFP afm], which has a report gene 3× p3-EGFP-sv40 in which EGFP expression is driven by the 3× p3 promoter and occurs in the compound eyes and nervous tissues of *B. mori* (Jiang et al., 2012a, 2012b; Thomas et al., 2002), to generate the transgenic RNAi vector pb-IE1Pie1T (“tail to tail”). IE1P, IE1-A (amplified using the primer AIE1-A), A3 intron, IE1-S (amplify with primer AIE1-S), and SV40 were added to *piggyBac* [3× p3 EGFP afm] to generate the pb-IE1Pie1H vector (“head to head”) (Fig. 1). IE1P, the sense fragment of *gp64* (GP64-S, amplified using the primer GP64-S), A3 intron, the antisense fragment of *gp64* (GP64-A, amplified using the primer GP64-A), and SV40 were added to *piggyBac* [3× p3 EGFP afm] to generate the pb-IE1Pgp64T vector. Hr3 was added to pb-IE1Pgp64T to generate the pb-HIE1Pgp64T vector. A4P was used instead of IE1P in pb-IE1Pgp64T to construct the pb-A4Pgp64T vector. A4P, GP64-A (amplified using the primer AGP64-A), A3 intron, GP64-S (amplified using the primer AGP64-S), and SV40 were added to *piggyBac* [3× p3 EGFP afm] to generate the pb-A4Pgp64H vector (Fig. 1). A4P, the antisense fragment of *helicase* (Heli-A, amplified using the primer Heli-A), A3 intron, the sense fragment of *helicase* (Heli-S, amplified using the primer Heli-S), and SV40 were added to *piggyBac* [3× p3 EGFP afm] to generate the pb-A4PheliH vector. A4P, the antisense fragment of *vp39* (VP39-A, amplified using the primer VP39-A), A3 intron, the sense fragment of *vp39* (VP39-S, amplified using the primer VP39-S), and SV40 were added to *piggyBac* [3× p3 EGFP afm] to generate the pb-A4Pvp39H vector (Fig. 1). The PCR primers are shown in Supplementary Table 1.

2.3. Transgenic microinjection and screening

The practical silkworm strain 932 is a diapause species, so non-diapause embryos were generated as previously described (Jiang et al., 2012a, 2012b). Embryo microinjection was performed using mixtures of transgenic vector and helper plasmid (Jiang et al., 2012a, 2012b; Tamura et al., 2000; Thomas et al., 2002). The transgenic offspring were generated and screened according to published methods (Jiang et al., 2012a, 2012b; Thomas et al., 2002). Two transgenic lines of pb-IE1Pie1T (named IE1Pie1T-A and IE1-Pie1T-B), two transgenic lines of pb-IE1Pie1H (named IE1Pie1H-A and IE1Pie1H-B), one transgenic line of pb-IE1Pgp64T (named IE1Pgp64T), two transgenic lines of pb-HIE1Pgp64T (named HIE1Pgp64T-A and HIE1Pgp64T-B), one transgenic line of pb-A4Pgp64T (named A4Pgp64T), one transgenic line of pb-A4Pgp64H (named A4Pgp64H), one transgenic line of pb-A4PheliH (named A4PheliH), and two transgenic lines of pb-A4Pvp39H (named A4Pvp39H-A and A4Pvp39H-B) were screened (Supplementary Table 2).

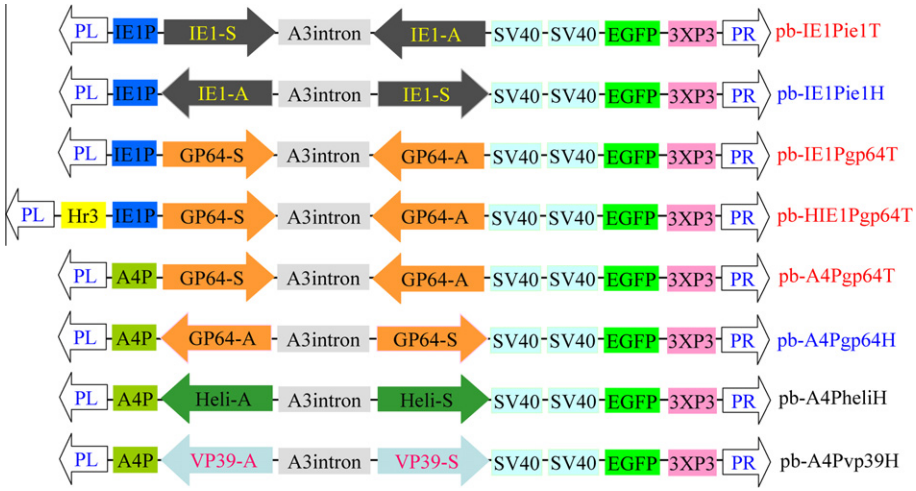

Fig. 1. Schematic of the transgenic RNAi vectors. PL and PR are the left and right terminal inverted repeats of *PiggyBac* [3× p3 EGFP afm], which is a basic transgenic vector containing the report marker 3× p3-EGFP-SV40. Hr3 is an enhancer from BmNPV. IE1P and A4P indicate the IE1 promoter of BmNPV and the A4 promoter of *B. mori*, respectively. IE1-S, GP64-S, Heli-S, VP39-S, and IE1-A, GP64-A, Heli-A, VP39-A represent the sense fragment (S) and the antisense fragment (A) of *ie-1*, *gp64*, *helicase*, and *vp39*, respectively. The arrow direction of gene fragment represents 5′–3′. The A3intron is a spacer cloned from the genomic DNA of *Dazao*. SV40 is the polyadenylation signal.

2.4. Analysis of the insertion sites

Genomic DNA was extracted from these transgenic lines using G1 male moths, which was fully digested with *Hae III* and self-ligated (Jiang et al., 2012a, 2012b). Each ligated product was used for PCR with the transposon-specific primers pBacL and pBacR (Jiang et al., 2012a). The PCR products were cloned and sequenced.

2.5. BmNPV resistance analysis

Each transgenic line was mated with nontransgenic 932 to generate offspring and the resistance of transgenic larvae was investigated via oral infection of wild BmNPV using 3×10^5 occlusion bodies (OB)/larva for newly exuviated third instar larvae, or 10^6 OB/larva for newly exuviated fourth instar larvae. Each transgenic line and nontransgenic 932 was infected three times and each repeat consisted of 70 larvae. Each larva ingested an equal quantity virus, as previously described (Jiang et al., 2012a, 2012b). There were three non-infected repeats, which also included 70 larvae. Mortality was determined daily until the 10th day post-infection (dpi) (Jiang et al., 2012a).

2.6. Analysis of target genes transcripts by qPCR

Total RNA was obtained from the larvae in each transgenic line and nontransgenic 932 at 48 hpi. Each RNA sample extracted from 10 treated larvae was treated with RNase-free DNase I and reverse-transcribed (Jiang et al., 2012a). These cDNA samples were used to detect transcripts of *ie-1* or *gp64* using the primers ie1-QRT and gp64-QRT, respectively. The primer sw22934 for BGIBMGA003186 was used as the reference. The qPCR reaction was performed as previously described (Jiang et al., 2012a).

2.7. qPCR analysis of BmNPV after infection

The total DNA of transgenic and nontransgenic lines was extracted from 10 treated larvae at 48 hpi (Jiang et al., 2012a). Each DNA template was used for qPCR using the primer GP41, while BmGAPDH was used as the control (Bao et al., 2009; Jiang et al., 2012a, 2012b). Each assay was performed in triplicate.

2.8. Investigation of the economic characteristics

The economic characteristics of the transgenic lines were investigated, as previously described (Jiang et al., 2012a). The overall weights of cocoons and cocoon shells were determined for 15 female individuals and 15 male individuals from each line. The cocoon shell rate was the ratio of the cocoon shell weight relative to the overall cocoon weight.

3. Results

3.1. Construction of RNAi vector and screening of transgenic lines

The sense fragments (S) and antisense fragments (A) of *ie-1*, *helicase*, *gp64*, and *vp39* were cloned using genomic DNA from BmNPV. The arrow direction of gene fragment represents 5′–3′. Eight transgenic RNAi vectors were constructed using the transgenic vector *piggyBac* [3× p3 EGFP afm] (Fig. 1). Nondiapause 932 embryos were microinjected with mixtures of the RNAi plasmid and helper vector (Jiang et al., 2012a, 2012b; Tamura et al., 2000; Thomas et al., 2002). The transgenic silkworms were screened according to published methods (Jiang et al., 2012a, 2012b). Supplementary Table 2 shows the results of the transgenic microinjections. Each transgenic line was mated with nontransgenic 932 to produce offspring from each generation.

3.2. Insertion site analysis in the transgenic lines

The insertion sites of each transgenic line were detected via inverse PCR using the transposon-specific primers pBacL and pBacR, the closed left part of the genome sequence of the insertion site and the left part of the insert were amplified using pBacL, whereas the closed right part of the genome sequence of the insertion site and the right part of the insert were amplified using pBacR (Jiang et al., 2012a). After PCR amplification using pBacL and pBacR, only one band was detected in each transgenic line (data not shown). This showed that each transgenic line had only one insertion. The PCR-amplified products were sequenced (Supplementary Table 3). The results of the bioinformatics analysis of the insertion sites are shown in Supplementary 1.

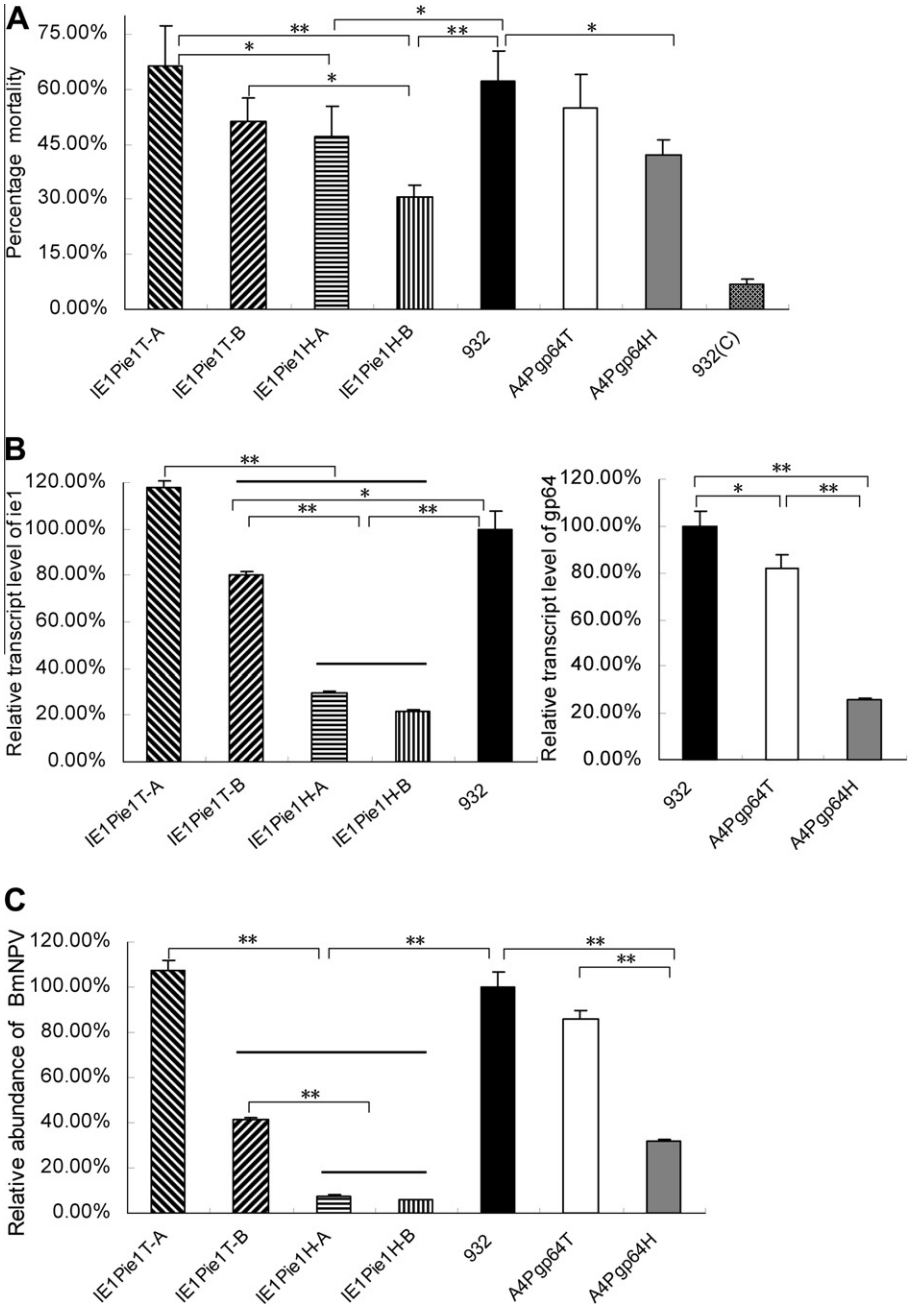

Fig. 2. Detection of different connections between gene fragments and spacers. (A) The mortality statistics. Transgenic lines IE1Pie1T-A, IE1Pie1T-B, IE1Pie1H-A, IE1Pie1H-B, A4Pgp64T, A4Pgp64H, and nontransgenic 932 were infected orally with BmNPV using 3×10^5 OB/larva for newly exuviated third instar larvae. Each larva ingested the same quantity of OBs. The mortality of each line is an average of three infection replicates. The mortality was recorded daily until 10 dpi and the accumulative mortality up to 10 dpi is shown for each line. 932(C) was the non-infected control. (B) The mRNA expression of the target genes. cDNA of IE1Pie1T-A, IE1Pie1T-B, IE1Pie1H-A, IE1Pie1H-B, A4Pgp64T, A4Pgp64H, and 932 were extracted from ten treated larvae at 48 hpi. The *ie-1* gene of IE1Pie1T-A, IE1Pie1T-B, IE1Pie1H-A, IE1Pie1H-B, and 932 were detected using the primer ie1-QRT, while the *gp64* gene in A4Pgp64T, A4Pgp64H, and 932 was tested using the primer gp64-QRT. The mRNA content of the BmNPV gene in 932 was set to 100% and the values of the transgenic lines were normalized against it. (C) Analysis of the BmNPV content. The total DNA was extracted from ten treated larvae at 48 hpi. The accumulated virus DNA level was analyzed using qPCR with the primer GP41. The average level of 932 was set at 100% and the values of the transgenic lines were standardized against it. Bars represent the standard deviations. Statistically significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$.

3.3. Comparison of the connections between gene fragments and spacers

The connection pattern of the gene fragments and spacers contains "head to head" (5′–5′) and "tail to tail" (3′–3′) links. To determine the best option, we constructed the vectors pb-IE1Pie1T and pb-IE1Pie1H (Fig. 1), where the dsRNA of *ie1* was driven by IE1P in "tail to tail" and "head to head" conformations, respectively. The corresponding transgenic lines were IE1Pie1T-A, IE1Pie1T-B, IE1-Pie1H-A, and IE1Pie1H-B. In the vectors pb-A4Pgp64T and pb-A4Pgp64H (Fig. 1), the dsRNA of *gp64* was driven by A4P using "tail to tail" and "head to head" conformations, respectively. The corresponding transgenic lines A4Pgp64T and A4Pgp64H were generated.

After oral route with BmNPV, the mortality rates of IE1Pie1T-A, IE1Pie1T-B, IE1Pie1H-A, IE1Pie1H-B, A4Pgp64T, A4Pgp64H, and 932 were 66%, 51%, 47%, 310%, 55%, 42%, and 62%, respectively, where 932(C) was used as the non-infected control (Fig. 2A). This

suggested that the resistance of IE1Pie1H-B was significantly higher than that of IE1Pie1T-B, while the resistance of A4Pgp64H was higher than that of A4Pgp64T. At 48 hpi, the mRNA expression of target gene in each line was detected. The mRNA content of 932 was set as 100% and the values of the transgenic lines were standardized against this value. qPCR showed that the mRNA expression levels of *ie-1* in IE1Pie1T-A, IE1Pie1T-B, IE1Pie1H-A, and IE1Pie1H-B were 118%, 80%, 29%, and 21% relative to that of 932, respectively, while the mRNA expression levels of *gp64* in A4Pgp64T and A4Pgp64H were 82% and 26% relative to that of 932, respectively (Fig. 2B). These results indicated that the viral mRNA contents in IE1Pie1H-B and A4Pgp64H were significantly lower than those in IE1Pie1T-B and A4Pgp64T, respectively. The accumulated BmNPV DNA number in each line was also assayed via qPCR at 48 hpi (Jiang et al., 2012a, 2012b), which in IE1Pie1T-A, IE1Pie1T-B, IE1Pie1H-A, IE1Pie1H-B, A4Pgp64T, and A4Pgp64H were 107%, 41%, 8%, 6%, 86%, and 32% relative to that of 932, respectively (Fig. 2C). It revealed that the BmNPV contents of IE1-Pie1H-B and A4Pgp64H were significantly lower than those of IE1-Pie1T-B and A4Pgp64T, respectively.

After comparing the antiviral ability (Figs. 2A, S1A and S2A), the mRNA expression of target gene (Fig. 2B), and the virus levels (Fig. 2C), we concluded that pb-IE1Pie1H was better than pb-IE1-Pie1T while pb-A4Pgp64H was better than pb-A4Pgp64T. These results showed that the connection pattern "head to head" was better than the "tail to tail" configuration.

3.4. Analysis of different promoters

We constructed three vectors to determine the ideal promoter for silencing virus genes, i.e., pb-IE1Pgp64T, pb-HIE1Pgp64T, and pb-A4Pgp64T (Fig. 1), in which *gp64* dsRNA was driven by IE1P, IE1P combined with hr3, and A4P, respectively. The corresponding transgenic lines IE1Pgp64T, HIE1Pgp64T-A, HIE1Pgp64T-B, and A4Pgp64T were detected.

The results of resistance analysis showed that the mortality rates of IE1Pgp64T, HIE1Pgp64T-A, HIE1Pgp64T-B, A4Pgp64T and nontransgenic 932 were 77%, 53%, 45%, 55%, and 62%, respectively (Fig. 3A), it suggested that HIE1Pgp64T-B significantly decreased the mortality. HIE1Pgp64T-A, HIE1Pgp64T-B, A4Pgp64T could significantly reduce the *gp64* mRNA expression, which in IE1Pgp64T, HIE1Pgp64T-A, HIE1Pgp64T-B, and A4Pgp64T was 112%, 44%, 41%, and 82% relative to that in 932, respectively (Fig. 3B). At 48 hpi, the accumulated virus DNA levels in IE1Pgp64T, HIE1Pgp64T-A, HIE1Pgp64T-B, and A4Pgp64T were 124%, 70%, 60%, and 86% relative to that in 932 (Fig. 3C), which indicated that HIE1Pgp64T-A and HIE1Pgp64T-B could significantly inhibit BmNPV proliferation.

Based on the resistance results (Figs. 3A, S1B and S2B), the *gp64* mRNA expression levels (Fig. 3B), and the BmNPV contents (Fig. 3C), we found that IE1P combined with hr3 was better than IE1P and A4P, while A4P was better than IE1P.

3.5. Determination of different phase target genes

The expression of BmNPV has a temporal pattern (Huh and Weaver, 1990a, 1990b). To investigate the effects of different phase genes, we selected the immediate early gene *ie-1*, delayed early gene *helicase*, the late genes *gp64* and *vp39*, as target genes. The corresponding transgenic lines IE1Pie1H-B, A4PheliH, A4Pgp64H, and A4Pvp39H-B were analyzed.

The mortality rates of IE1Pie1H-B, A4PheliH, A4Pgp64H, A4Pvp39H-B, and 932 were 31%, 34%, 42%, 42%, and 62%, respectively (Fig. 4A), which revealed that the antiviral capacity was significantly enhanced in A4PheliH, A4Pgp64H, and A4Pvp39H-B, but especially in IE1Pie1H-B. The four transgenic lines could significantly decrease the virus mRNA expression and inhibit viral multiplication. qPCR results showed that the *ie-1* mRNA content of IE1Pie1H-B, A4PheliH, A4Pgp64H, and A4Pvp39H-B was 21%, 41%, 31%, and 59% relative to that of 932, respectively (Fig. 4B), while

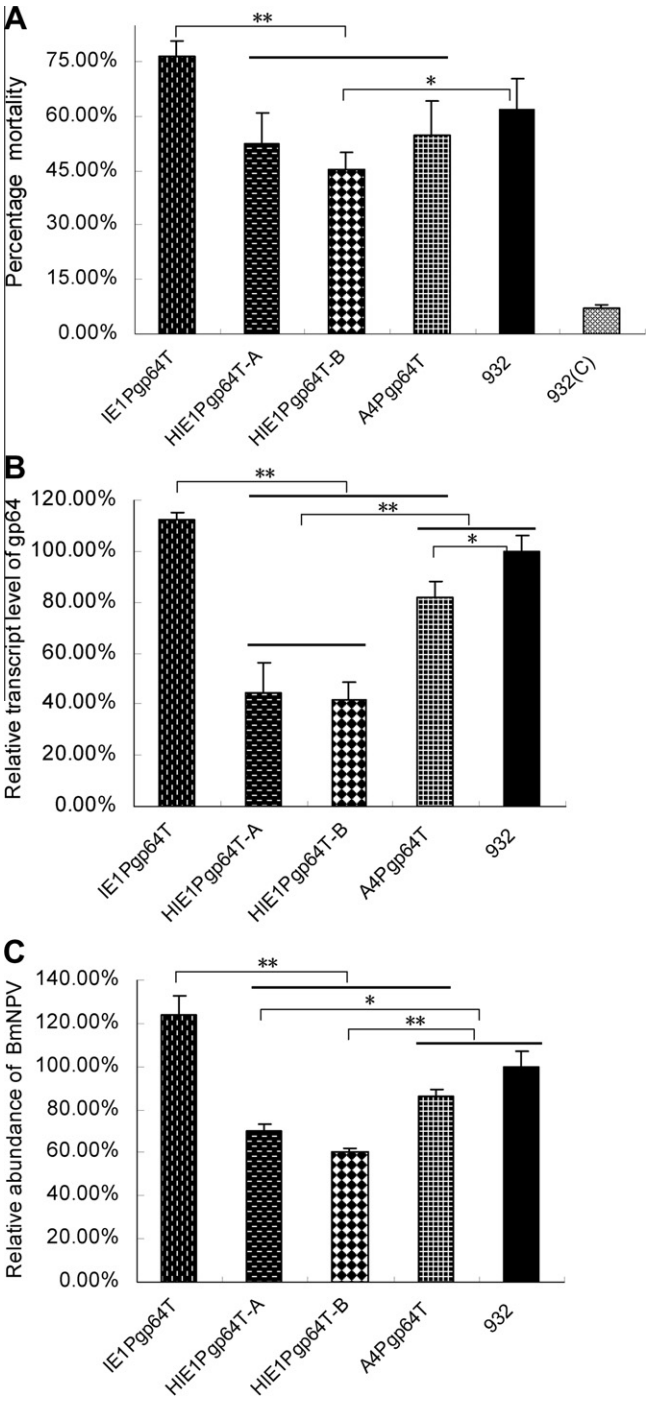

Fig. 3. Comparison of different promoters. (A) Resistance analysis. IE1Pgp64T, HIE1Pgp64T-A, HIE1Pgp64T-B, A4Pgp64T, and 932 were infected with BmNPV *per os* using third instar larvae. The mortality of each line is an average of triplicate infection replicates. The mortality was accumulated up to 10 dpi. (B) mRNA expression of *gp64*. Ten treated E1Pgp64T, HIE1Pgp64T-A, HIE1Pgp64T-B, A4Pgp64T, and 932 larvae were used to extract cDNA at 48 hpi, respectively. qPCR was carried out using the primer gp64-QRT. (C) Detection of the virus level. The total DNA was obtained from ten treated larvae at 48 hpi. The DNA content of BmNPV was determined by qPCR. In the qPCR results (B, C), the average level for 932 was set to 100% and the values of the transgenic lines were normalized against it. Bars represent the standard deviations. Statistically significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$.

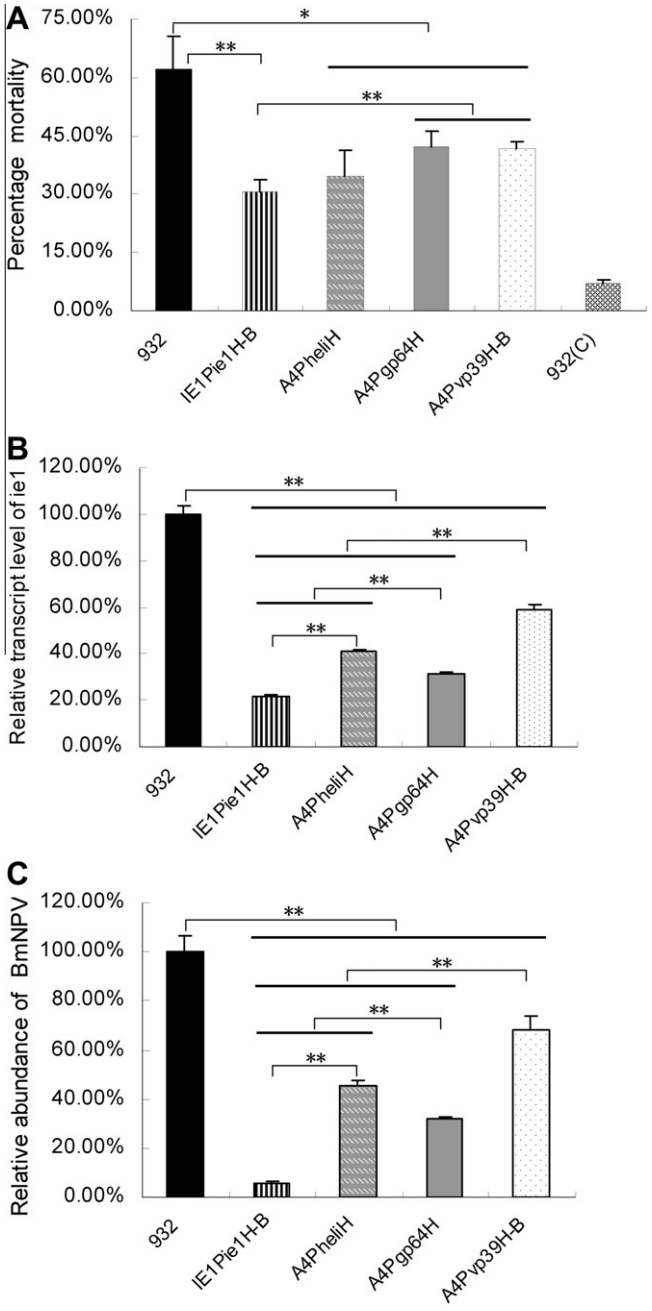

Fig. 4. Analysis of different phase target genes. (A) Antiviral capacity analysis. IE1Pie1H-B, A4PheliH, A4Pgp64H, A4Pvp39H-B, and 932 were infected orally with BmNPV using third instar larvae. Each line shows the triplicate infection result, and the mortality was analyzed until 10 dpi. (B) *ie-1* mRNA level. (C) BmNPV content. The cDNA and total DNA from ten treated larvae in each line were extracted at 48 hpi and used to detect *ie-1* mRNA levels (B) and virus (C), respectively. The average amount of 932 was set to 100% and the values of the transgenic lines were standardized against it. Bars represent the standard deviations. Statistically significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$.

the accumulated BmNPV content in IE1Pie1H-B, A4PheliH, A4Pgp64H, and A4Pvp39H-B was 6%, 45%, 32%, and 68% relative to that in 932, respectively (Fig. 4C).

Compared with A4PheliH, A4Pgp64H, and A4Pvp39H-B, the resistance of IE1Pie1H-B was highest (Figs. 4A, S1C and S2C), while the mRNA content of *ie-1* (Fig. 4B) and the accumulated virus content (Fig. 4C) of IE1Pie1H-B were the lowest. These results showed that the immediate early gene was the best target gene for RNAi.

3.6. Economic characteristics of the transgenic lines

To determine whether the economic characteristics of the transgenic RNAi lines were affected, we measured the overall cocoon weights and cocoon shell rates in the transgenic lines (Jiang et al., 2012a). There were no differences in the economic characteristics of the transgenic lines and nontransgenic 932 (Fig. 5). The cocoon weights after infection of BmNPV were not lower than that of normal feeding in these transgenic silkworms (Fig. S3). These results suggested that expressing the dsRNA of the BmNPV gene did not affect the economic characteristics of transgenic silkworms.

4. Discussion

BmNPV is one of the most serious pathogens affecting *B. mori*, which is a typical model of lepidopteran (Duan et al., 2010; Xia et al., 2004, 2007, 2009), and it causes severe economic losses during sericulture. RNAi is a powerful tool for host defense against viral infection (Carmona et al., 2006; Fire et al., 1998; Huelsmann et al., 2006; Murakami et al., 2005). A previous report showed that silencing the *lef-1* gene did not reduce mortality after BmNPV infection of transgenic silkworm (Isobe et al., 2004), whereas targeting of the *ie-1* gene enhanced the antiviral ability capacity of transgenic RNAi silkworms (Kanginakudru et al., 2007). These results showed that several factors may affect the resistance of transgenic hosts via RNAi of pathogen genes. In this study, we systematically compared the factors that may affect the anti-BmNPV capacity via the RNAi of virus genes in transgenic silkworms.

The transgenic RNAi vector included sense fragments and antisense fragments of target genes and spacers. The RNAi efficiency can be increased by using an intron to splice inverted-repeat fragments (Smith et al., 2000). In this study, we used the A3 intron cloned from the genomic DNA of *Dazao* as a spacer (Fig. 1). It was assumed that the endogenous intron spacer would have no harmful effects in transgenic silkworms. The transgenic RNAi vectors pb-IE1Pie1T, pb-IE1Pie1H, pb-A4Pgp64T, and pb-A4Pgp64H (Fig. 1) were constructed to compare the connection patterns of gene fragments and spacers. Our results showed that "head to head" was better than the "tail to tail" configuration. In transgenic lines with the "head to head" pattern, the mRNA level of the target gene was significantly lower than that with "tail to tail" (Fig. 2B), which was reflected in the fewer virus content (Fig. 2C) and higher resistance to infection (Figs. 2A, S1A and S2A). Ni et al. (2009) reported similar results in *Drosophila*, where eight individual endogenous genes were selected for knocking down by RNAi in transgenic *Drosophila* using "head to head" and "tail to tail." The phenotypes generated with the "head to head" pattern were stronger in five cases whereas the "tail to tail" pattern resulted in stronger phenotypes in only one case. The results reported by Ni et al. (2009) and the current study suggest that "head to head" is a better pattern for knocking down endogenous or exogenous genes in transgenic RNAi hosts.

Promoter selection is also very important for the efficiency of transgenic RNAi. The RNAi efficiency differed considerably when gene fragments were driven by different promoters as shown in previous studies (Boden et al., 2003; Henriksen et al., 2007; Kawasaki and Taira, 2003; Makinen et al., 2006; Weiwei et al., 2009). In the current study, we tested IE1P, IE1P combined with hr3, and A4P, which were used to drive *gp64* dsRNA in the vectors pb-IE1Pgp64T, pb-HIE1Pgp64T, and pb-A4Pgp64T, respectively (Fig. 1). The *gp64* mRNA content of HIE1Pgp64T-B was significantly lower than that of IE1Pgp64T and A4Pgp64T (Fig. 3B). Enhancers can significantly increase the RNAi efficiency (Weiwei et al.,

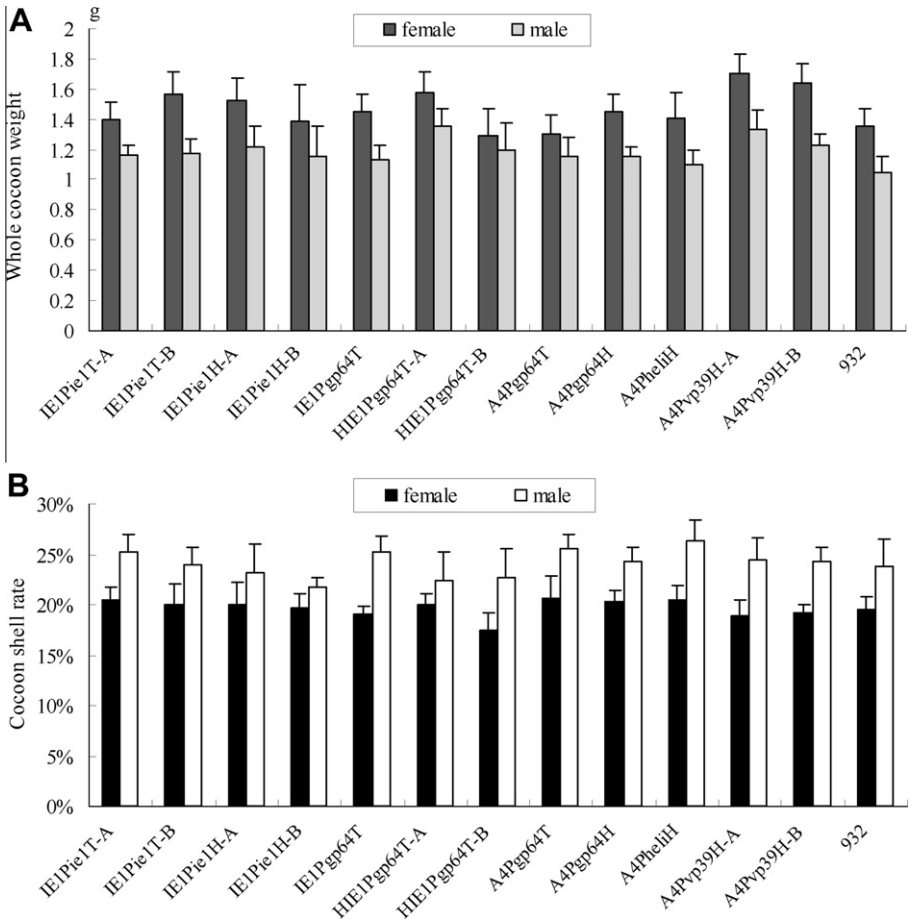

Fig. 5. Economic characteristic analysis. We randomly selected 15 cocoons from female individuals and 15 cocoons from male individuals in each line. The weights of overall cocoons and cocoon shells were measured separately. The cocoon shell rate was the ratio of the cocoon shell weight relative to the overall cocoon weight. Each value was an average of 15 replicate measures. Bars represent the standard deviations.

2009). Our previous study demonstrated that the promoter activity can be enhanced significantly by BmNPV infection, if there is an upstream hr3 (Jiang et al., 2012a). Thus, we assumed that the significantly increased *gp64* dsRNA level resulted in decreased *gp64* mRNA level in HIE1Pgp64T-B after infection with BmNPV. The *gp64* mRNA expression level (Fig. 3B), the amount of BmNPV(Fig. 3C), and the mortality rate (Figs. 3A, S1B and S2B) with HIE1Pgp64T-B were the lowest among IE1Pgp64T, HIE1Pgp64T-B, and A4Pgp64T, which showed that IE1P combined with hr3 was the best of all the promoters tested.

Antiviral RNAi is affected by the target gene selection (Kneidinger et al., 2012; Leonard and Schaffer, 2005; Shah and Schaffer, 2011; von Laer et al., 2006). Previous studies have suggested that targeting early viral genes is essential for therapeutic efficacy (Leonard and Schaffer, 2005; Shah and Schaffer, 2011; von Laer et al., 2006). NPV has immediate early, delayed early, late, and very late genes (Huh and Weaver, 1990a, b). The multiplication of AcMNPV was inhibited by the introduction of transient dsRNA, while the virus quantity in cells transfected with the dsRNA of the immediate early gene *ie-1* was lower than that with the dsRNA of the late gene *gp64* (Valdes et al., 2003). Our results showed that silencing genes in the immediate early, delayed early, or late phase could enhance the antiviral capacity of transgenic RNAi silkworm, although the immediate early gene was the best target gene for RNAi (Figs. 4, S1C and S2C). It presumed that the other essential immediate early genes, such as *pe38* (Krappa and Knebel-Morsdorf, 1991) were also good targets for suppressing viral replication. A similar study of human adenovirus showed that silencing the early and middle genes had a greater effect on inhibiting adenovirus proliferation than silencing the late genes (Kneidinger et al., 2012).

Antivirus is a worldwide problem and hot research area. In the previous reports, many studies were done in cell lines (Henriksen et al., 2007; Kneidinger et al., 2012; Kretova et al., 2012; Ramirez-Carvajal and Long, 2012; Weiwei et al., 2009). It has been suggested that the results may be closer to the actual situation if using host individuals. In this study, the factors that may affect transgenic RNAi vector construction were analyzed in the individual larvae of model *B. mori*. The different insertion site in different transgenic line is a variable that might have an effect on the results, which will be resolved by the breakthrough of site specific integration in silkworm. The antiviral capacity was enhanced (Fig. 4) while the economic characteristics did not change (Figs. 5 and S3) in our transgenic RNAi silkworms, which suggested that these transgenic silkworms could be used for sericulture. Knocking down several target genes simultaneously by RNAi is more effective than targeting only one gene (Kretova et al., 2012; Leonard and Schaffer, 2005; Liu et al., 2008; Ramirez-Carvajal and Long, 2012; Shah and Schaffer, 2011). Recently, Subbaiah et al. generated a transgenic silkworm targeting multiple BmNPV genes by using A3 promoter, which exhibited a higher resistance to that targeting a virus gene (Subbaiah et al., 2012). We are also constructing a new transgenic RNAi vector, in which multiple essential immediate early genes from BmNPV will be targeted simultaneously under the control of IE1P combined with hr3 using a "head to head" pattern.

Overexpression of antiviral genes and the silencing of viral genes in transgenic hosts are effective antiviral strategies. In our

previous studies, we reported that the anti-BmNPV capacity can be improved by overexpressing endogenous *Bmlipase-1* (Jiang et al., 2012b) or exogenous *hycu-ep32* (Jiang et al., 2012a) in transgenic silkworm. The results of Kanginakudru et al. (2007) and the current study demonstrated that knocking down the expression of BmNPV genes could enhance the resistance of transgenic silkworm. Thus, it is likely that a combination of these methods could create a more resistant silkworm strain.

In conclusion, we systematically compared the factors that might affect the therapeutic efficacy of transgenic hosts by RNAi targeting of viral genes in the model insect *B. mori*. This report could pave the way for antiviral research in animals and humans, and it also provides a reference for gene function studies using RNAi.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.12.020.

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