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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).