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In vivo RNA interference of *BmNHR96* enhances the resistance of transgenic silkworm to BmNPV

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ABSTRACT

We previously identified a nuclear hormone receptor gene, *BmNHR96*, which promotes *Bombyx mori* nucleopolyhedrovirus (BmNPV) entry into silkworm cells. In an attempt to create an antiviral silkworm strain for better silk production, we used RNAi to downregulate *BmNHR96* in silkworm larvae. We screened the resulting *BmNHR96*-RNAi silkworm strain (*NHR*) and also explored the antiviral mechanism *in vivo*. We found that the survival rate of the *NHR* strain was higher than that of the *Dazao* strain, when silkworm larvae were infected with BmNPV via oral ODV infection and BV injection. More importantly, the economic characteristics (silk yield) of the transgenic line remained unchanged. These findings reveal that RNAi of *BmNHR96* could be an effective way to enhance the tolerance of *B. mori* to BmNPV infection.

1. Introduction

The silkworm is a typical lepidopteran model insect and has high economic value in terms of silk production for farmers in many developing countries, such as China, India, Brazil, Vietnam and Ethiopia [1]. However, several types of pathogens such as viruses, fungi and bacteria cause massive economic losses every year in the sericulture industry. Among these pathogens, the *Bombyx mori* nucleopolyhedrovirus (BmNPV) is the most predominant threat to sericulture in almost all countries, but there are no effective prevention and control strategies in place to mitigate its infection [2,3]. Therefore, there is an urgent need to cultivate new silkworm strains that are resistant to BmNPV infection.

BmNPV is a member of the Baculoviridae family and has a circular double-stranded DNA genome [4]. This virus has two virion phenotypes during its infectious life cycle: occlusion-derived virus (ODV) and budded virus (BV), with ODV being responsible for lateral transmission between individuals and BV being responsible for the spread of the infection throughout the host [5,6]. ODV particles are packaged and protected in the viral polyhedral body, which is a highly symmetrical and covalently cross-linked robust lattice [6]. BmNPV invades silkworm larvae mostly via oral infection. Once the polyhedral bodies are ingested by the host larvae *per os*, a primary infection is initiated after dissociation of the polyhedral bodies, and ODV particles are released into the alkaline environment of the midgut [7–9]. Subsequently, ODV particles infect the midgut epithelial cells of silkworm larvae, and at about 24 h after its infection, release BV particles into the body cavity to complete the primary infection phase [7–9]. Then, BV particles spread through the host via the tracheal system to start the secondary infection phase. At the late stage of infection, progeny ODV particles are occluded in polyhedral bodies and are released into the natural environment after the death and decomposition of the host [7–9]. A new round of infection starts when these ODV particles are ingested by another silkworm.

Understanding the mechanisms of interaction between BmNPV and its host will help breed antiviral silkworm strains for the sericulture industry. Some of the genes involved in BmNPV infection, including *BmAtlastin-n*, *BmNOX*, *Bmlipase-1*, *BmREEPa*, *caspase-1* and *serine protease*, have been identified [10–14]. The function of

Abbreviations: BmNHR96, *Bombyx mori* nuclear hormone receptors 96; BmNPV, *Bombyx mori* Nucleopolyhedrovirus; ODV, occlusion-derived virus; BV, budded virus; qRT-PCR, quantitative real time-PCR.

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these genes during BmNPV infection of silkworms can be effectively determined by creating transgenic lines with better traits, such as disease resistance, using advanced transgenic technologies. Pathogen resistance can be enhanced in plants by downregulating the expression of essential viral genes or host genes that facilitate the invasion of pathogens in transgenic hosts by RNAi [15,16]. Mohamed et al. showed that in rice plants, RNAi of the *S7-2* or *S8* gene, each of which are essential for the infection of the rice black-streaked dwarf virus (RBSDV), effectively induced high resistance to the RBSD disease [17]. Similarly, in animals, over-expression of endogenous or exogenous antiviral genes via transgenic technology has increased disease resistance [18]. Transgenic technology has already been used in silkworms to strengthen their resistance to viruses. The anti-BmNPV capacity of silkworm has been enhanced through over-expression of BmAtlastin-n [10] or by simultaneously targeting several essential BmNPV genes, thus conferring greater protection against BmNPV [19].

In our previous study, we reported a nuclear hormone receptor gene, *BmNHR96* (*B. mori nuclear hormone receptor 96*), which plays an active role in BV invasion [2]. We showed that the entry of BV particles in *BmNHR96*-overexpressing BmN-SWU1 cells was significantly higher than in control cells, indicating that *BmNHR96* could enhance BV entry into BmN-SWU1 cells [2]. In the present study, we downregulated the expression of *BmNHR96* using RNAi and investigated the resulting changes in anti-BmNPV activity in silkworms. We further compared the economic characteristics of the transgenic and non-transgenic lines. Our findings provide a foundation for breeding silkworms with excellent antiviral capacity and illustrate the mechanisms of BmNPV infection.

2. Methods and materials

2.1. Silkworm strain and virus

The *Dazao* strain of silkworm was maintained at the Silkworm Gene Bank (Southwest University, Chongqing, China). The BVs and ODVs were collected from cells and silkworm larvae, respectively, as described previously [10,20].

2.2. Vector construction

We synthesized the miRNA (GenScript, Nanjing, China) and constructed the pIZ-DsRed-*BmNHR96*-RNAi vector, as previously described [10,20]. Then, a transgenic vector was constructed by first amplifying the *BmNHR96*-RNAi fragments from pIZ-DsRed-*BmNHR96*-RNAi using primers 1F and 1R [20] and ligating them into the piggyBac vector [3 × P3 eGFP AFM].

2.3. Microinjection and screening

A mixture containing the transgenic vector, piggyBac [3 × P3-eGFP, OpIE2-*BmNHR96*-RNAi] (400 ng/μL), and the helper vector, pHA3PIG (400 ng/μL), was injected via microinjection (Eppendorf, Hamburg, Germany) into the *Dazao* embryos within 4 h of oviposition. Then, the G0 silkworms were maintained at 25 °C, inbred and backcrossed to yield G1 silkworms. *BmNHR96*-RNAi transgenic silkworms were identified based on DsRed expression in the whole body and eGFP-positive eyes by visualization with a fluorescent stereomicroscope (Olympus, Tokyo, Japan).

2.4. Analysis of the insertion site

Genomic DNA was extracted from *BmNHR96*-RNAi transgenic silkworms and digested with *Hae* III (Takara, Dalian, China) for 16 h at 37 °C and then self-ligated with solution I (Takara). The products were then used as templates for inverse PCR with primers specific for the transposon, pBacL (2F and 2R) and pBacR (3F and 3R), as described in a previous study [21]. Next, the PCR fragments were cloned into the pMD19-T vector (Takara) and sequenced, followed by sequence comparison with the genome sequence in silkDB (http://www.silkdb.org/silkdb/) (see Table 1).

2.5. Oral infection and injection of BmNPV

For oral infection, day 1 4th-instar larvae of the *BmNHR96*-RNAi and *Dazao* strains were individually fed fresh mulberry leaves, cut into 1-cm diameter round pieces and smeared with 1×10^6 ODV particles, as previously described [10,20]. Larvae that consumed the whole mulberry leaf were collected for survival rate analysis. For the injection experiment, 1×10^6 BV particles were injected into the body cavity of day 1 4th-instar larvae of the *BmNHR96*-RNAi and *Dazao* strains using a capillary tube. Further analysis was performed only on successfully-injected larvae that showed no leakage of the injected solution [10,13].

2.6. Survival analysis

The *NHR* and *Dazao* larvae in the oral infection and injection groups were divided into 3 groups with 100 larvae in each group. For each silkworm strain, 3 groups of uninfected larvae were also set aside as controls. All larvae were reared under the same conditions, and the survival rate was analyzed from the time of infection until 10 days post-infection.

2.7. Quantitative real time-PCR (qRT-PCR)

Total RNA was extracted from the control and infected larvae of the *Dazao* and *NHR* strains at 24 and 48 h post-infection using the Total RNA Kit II (OMEGA, Norcross, USA) and reverse transcribed into cDNA. cDNA template from 8 larvae per group was used for qRT-PCR reactions. The genes and their respective primers for qRT-PCR included *BmNHR96* (4F and 4R), *BGIBMGA010281* (5F and 5R),

Table 1
Primers used in this study.

Primer	Sequence
1F	TTGGCGCGCCGGATCATGATGATAAACAATGTAT
1R	TTGGCGCGCCTGTTCTTTCCTGCGTTATCCCCTG
2F	ATCAGTGACACTTACCGCATTGACA
2R	TGACGAGCTTGTTGGTGAGGATTCT
3F	TACGCATGATTATCTTTAACGTA
3R	GTACTGTCATCTGATGTACCAGG
4F	AATGTTTTGCCGTCGGTATG
4R	TCGGTTCTCTTCTATTTTTCGC
5F	GGTGCCTACGAAGGTGGGG
5R	GCTAAATGAGGTTTGCGGAA
6F	TTGTCCTTGCCCTGTTGTAGTAA
6R	TTTTGGTAATGTTGCGTTGTTCT
7F	CGAGACGGCTGCACAAAA
7R	TGCCCAAAAGAAACCCACA
8F	CACCATCGTGGAGACGGACTAC
8R	ACCTCGCACTGCTGCCTGA
9F	CTAATGCCCGTGGGTATGG
9R	TTGATGAGGTGGCTGTTGC
10F	GACACGAATTTTAGACGCCATT
10R	CGATTCTTCCAGCCCGTTT
11F	CGGTGTTGTTGGATACATTGAG
11R	GCTCATCCTGCCATTTCTTACT
12F	CGTAGTAGTAGTAATCGCCGC
12R	AGTCGAGTCGCGTCGCTTT
13F	CATTCCGCGTCCCTGTTGCTAAT
13R	GCTGCCTCCTTGACCTTTTGC

Underline sequence present Asc I restriction enzyme cutting site.

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