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Inhibition of BmNPV replication in silkworm cells using inducible and regulated artificial microRNA precursors targeting the essential viral gene *lef-11*

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ABSTRACT

Bombyx mori nucleopolyhedrovirus (BmNPV) is a major silkworm pathogen, causing substantial economic losses to the sericulture industry annually. We demonstrate a novel anti-BmNPV system expressing mature artificial microRNAs (amiRNAs) targeting the viral *lef-11* gene. The mature amiRNAs inhibited the *lef-11* gene in silkworm BmN-SWU1 cells. Antiviral assays demonstrated that mature amiRNAs silenced the gene and inhibited BmNPV proliferation efficiently. As constitutive overexpression of mature amiRNAs may induce acute cellular toxicity, we further developed a novel virus-induced amiRNA expression system. The amiRNA cassette is regulated by a baculovirus-induced fusion promoter. This baculovirus-induced RNA interference system is strictly regulated by virus infection, which functions in a negative feedback loop to activate the expression of mature amiRNAs against *lef-11* and subsequently control inhibition of BmNPV replication. Our study advances the use of a regulatable amiRNA cassette as a safe and effective tool for research of basic insect biology and antiviral application.

1. Introduction

The domesticated silkworm (*Bombyx mori*) is a lepidopteran model insect and an important economic insect for silk production and exogenous protein expression (Maeda et al., 1985; Tomita et al., 2003). *B. mori* nucleopolyhedrovirus (BmNPV), which specifically infects the silkworm, is a member of the *Baculoviridae* family and causes diseases that severely impair the sericulture industry. During the infection cycle, BmNPV produces two virion phenotypes: budded viruses (BVs) and occlusion-derived viruses (ODVs). The two virions contain identical genome information and nucleocapsid structure but different viral envelopes, as they are produced at different stages of the virus life cycle. BVs are responsible for systemic infection throughout the host; ODVs are embedded within polyhedral inclusion bodies and mediate vertical transmission between hosts (Rahman and Gopinathan, 2004).

Although baculovirus biology is well studied, there are currently no effective strategies for controlling BmNPV. RNA interference (RNAi) is an evolutionarily conserved mechanism of sequence-specific silencing target gene through small double-stranded RNAs. RNAi has been used as a powerful method for enhancing host resistance to virus infection through knockdown of viral gene expression in animals and plants (Lo et al., 2007; McCaffrey et al., 2003; Qu et al., 2007). The main methods for producing small double-stranded RNAs *in vitro* include synthetic small interfering RNA (siRNA), short-hairpin RNA (shRNA) expression plasmids, and artificial microRNA (amiRNA) expression plasmids. There have been reports of silkworm resistance to BmNPV being enhanced via shRNA constructs (Isobe et al., 2004; Jiang et al., 2012; Kanginakudru et al., 2007; Subbaiah et al., 2013; Zhang et al., 2014). However, to our knowledge, there have been no reports about using amiRNA-based designs to inhibit baculovirus replication in lepidopteran cells specifically.

Compared with synthetic siRNA- and shRNA-based designs, there are several significant advantages to amiRNA-based designs. First, the Pol III promoters U6 and H1, commonly used for expressing shRNAs, are very strong promoters that produce high levels of shRNAs, which cause significant cytotoxicity in host cells.

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Boudreau et al. (2009) proved that amiRNA-based vectors are more amenable to Pol II – mediated transcription than are shRNAs. Expression of tissue-specific or inducible mature amiRNAs allows regulatable expression of mature amiRNAs. Second, coexpression of a fluorescent reporter gene and mature amiRNAs by one promoter can facilitate the tracking of amiRNA expression at the single-cell level (Qiu et al., 2008). Third, multiple amiRNAs can be expressed from the same amiRNA expression vector, which simultaneously knocks down multiple target genes (Liu et al., 2008). Fourth, amiRNA structure, which is similar to that of endogenous miRNAs, avoids induction of the immune response in host cells as compared with shRNA constructs (Bridge et al., 2003).

In this study, we replaced the primary (pri)-miRNA of silkworm endogenous miRNA with a siRNA duplex sequence targeting the BmNPV *lef-11* gene, constructing amiRNA-based anti-BmNPV systems. LEF-11 was recently found to be involved in baculovirus DNA replication and late gene transcription, although the details of its function are not known. Thus, *lef-11* is an appropriate candidate for RNAi-mediated gene silencing (Lin and Blissard, 2002). We demonstrate effective inhibition of luciferase reporter gene expression in all tested insect cell lines. Antiviral and transient assays showed that the mature amiRNAs expressed by two amiRNA-based designs inhibited *lef-11* expression and BmNPV replication, respectively. Further, we combined amiRNA constructs with a baculovirus-induced promoter, establishing a baculovirus-induced RNAi system in stably integrated cells. The inducible anti-BmNPV system in stably integrated silkworm cells inhibited BmNPV proliferation noticeably. As far as we know, this is the first report of a virus-induced RNAi system in lepidopterans. This negative feedback system may be safer than the traditional Pol III – shRNA system against baculovirus, as mature amiRNAs are only expressed following virus infection.

2. Results

2.1. Expression of lef-11 amiRNA from silkworm miRNA-based cassettes

Based on previous reports and the miRNA database miRBase (http://www.mirbase.org/), we selected three endogenous silkworm miRNAs: bmo-mir-92b, bmo-mir-279, and bmo-mir-2764. The selection criteria are as follows: (1) the miRNA must be confirmed to be expressed much higher and wider than others in silkworm); (2) the miRNA structure should be very simple to facilitate nucleotide artificial synthesis; (3) the selected silkworm miRNA does not produce two mature miRNAs from opposite arms of pre-miRNA (Huang et al., 2010; Liu et al., 2009; Yu et al., 2008). The siRNA duplex sequences targeting the BmNPV *lef-11* gene coding sequence and endogenous miRNA flanking sequences were synthesized and cloned into the insect expression plasmid pIZ-DsRed, which is controlled by the *Orgyia pseudotsugata* multi-capsid nucleopolyhedrovirus *OpIE2* promoter (Fig. 1A). The amiRNA cassettes and pDual-Mir-Luc-Reporter-LEF11 plasmid containing a perfect complementary LEF-11 amiRNA target site in the 3′ untranslated region (UTR) of the firefly luciferase gene (Fig. 1B) were cotransfected into silkworm BmN-SWU1 cells; luciferase activities were assayed 48 h post-transfection (p.t.). The pIZ-DsRed plasmid was used as the negative control (no amiRNA expression). We detected expression of the DsRed marker, which indicated the expression of mature amiRNAs (Fig. 1C) under fluorescence microscopy at 48 h post-transfection. Dual luciferase assay data demonstrated that the amiR2764 and amiR279 cassettes downregulated firefly luciferase activity efficiently compared to the DsRed control. Firefly luciferase activity was inhibited by ~80% and ~93% in cells transfected with amiR2764 and amiR279,

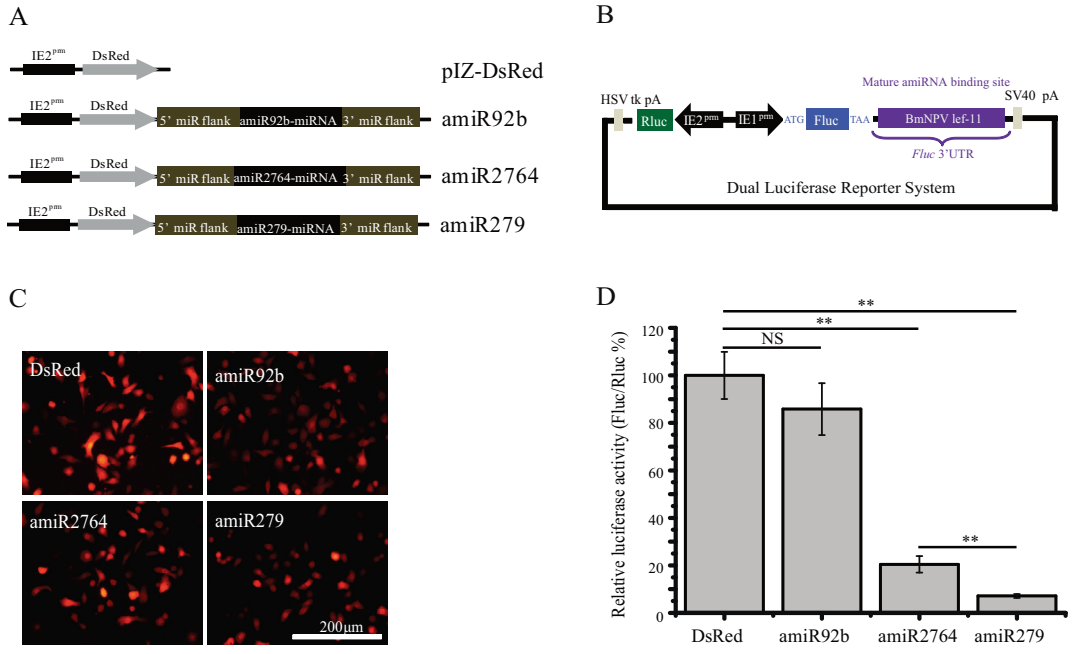

Fig. 1. Construction of amiRNA cassettes. (A) Structure of three amiRNA expression cassettes with 5′ and 3′ miRNA flanking sequences based on the silkworm endogenous miRNAs bmo-mir-92b, bmo-mir-264, and bmo-mir-279. These backbones were inserted into pIZ/V5-His, which contained the OpMNPV IE2 promoter for high-level and constitutive expression of amiRNA. The pIZ-DsRed plasmid was used as the negative control. (B) Schematic depicting the pDual-Mir-Luc-Reporter plasmid (containing a perfect complementary amiRNA target site in the 3′-UTR of the firefly luciferase gene). (C) The pIZ-DsRed control plasmid or amiRNA expression vectors (400 ng) were transfected into BmN-SWU1 cells. Cells producing active mature amiRNAs were marked with DsRed fluorescent reporter protein and observed under fluorescence microscopy at 24 h p.t. (D) Inhibition of luciferase activity by mature amiRNAs expressed by silkworm amiRNA expression cassettes. BmN-SWU1 cells were cotransfected with amiRNA cassettes and LEF-11 luciferase reporter plasmid; luciferase activity was measured 48 h p.t. NS, not significant. Statistically significant differences: $^{**}P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

respectively (Fig. 1D). In contrast, no difference was detected between cells transfected with pIZ-DsRed and amiR92b. These data show that both amiR2764 and amiR279 functioned effectively in BmN-SWU1 cells and that amiR279 exhibited more potent RNAi activity. However, the amiR92b cassette did not express functional mature amiRNAs in BmN-SWU1 cells.

2.2. The amiR2764 and amiR279 cassettes also functioned in other insect cell lines

As RNAi is a highly conserved mechanism among all species, we investigated whether amiR2764 and amiR279 would function in other insect cell lines, namely the *B. mori* – derived BmN4 and BmE-SWU1, *Spodoptera frugiperda* – derived Sf9, *Trichoplusia ni* – derived High Five, *Spodoptera litura* – derived Spli221, and *Drosophila melanogaster* – derived S2 cell lines. Dual luciferase assays were performed following cotransfection of amiRNA cassettes with dual luciferase reporter plasmid in 24-well plates. Both amiRNA-based designs were effective in all studied cell lines. Luciferase activity was inhibited noticeably by at least 50% by amiR2764 and up to 80% by amiR279 (Fig. 2). Our data suggest that the two RNAi vectors not only function broadly in almost all lepidopteran cells, but also in dipteran cells. Our results indicate that amiR2764 and amiR279 are appropriate RNAi platforms for insect research.

2.3. Inhibition of LEF-11 expression in BmNPV-infected BmN-SWU1 cells

We estimated LEF-11 expression levels in cells transfected with amiRNA-based designs and subsequently infected with recombinant BmNPV vBm$^{lef11\text{-}REPMyc}$ through real-time quantitative PCR (RT-qPCR) and western blotting. Cells transfected with pIZ-DsRed and then infected with vBm$^{lef11\text{-}REPMyc}$ (containing a c-Myc tagged LEF-11 controlled by *lef-11* native promoter, unpublished data) were used as the negative control. RT-qPCR showed that cells transfected with amiR2764 degraded *lef-11* mRNA by ~51.6%; amiR279-transfected cells degraded *lef-11* mRNA by ~50.8% as compared to the control. However, there was no change in *lef-11* mRNA transcription in amiR92b-transfected cells as compared with the control (Fig. 3A). LEF-11 expression was determined by western blotting using anti-cMyc antibody at the indicated time points. LEF-11 expression was significantly inhibited in cells transfected with amiR2764 or amiR279 (Fig. 3B).

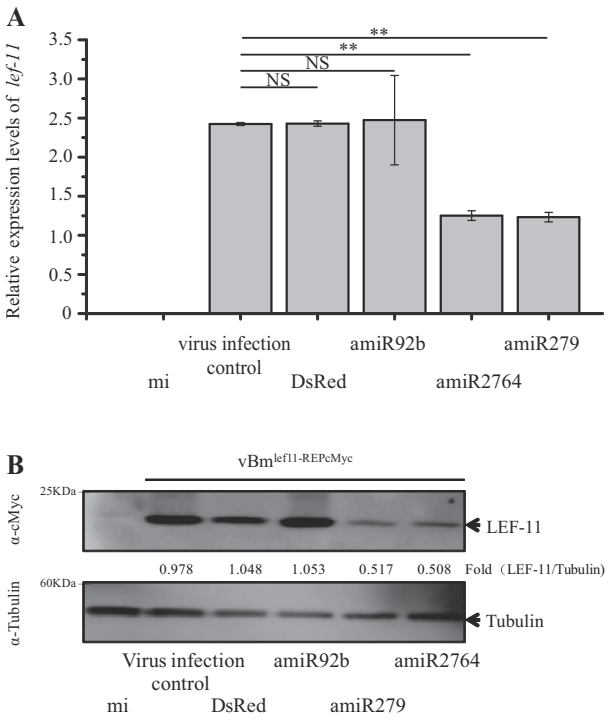

Fig. 3. Silencing of *lef-11* expression in BmNPV-infected BmN-SWU1 cells by amiRNAs. (A) BmN-SWU1 cells expressing amiRNA transiently were cultured in 12-well plates in four replicates; each well was inoculated with recombinant BmNPV vBm$^{lef11\text{-}REPMyc}$ (MOI = 20). At 48 h after BmNPV inoculation, cells and virus were harvested; *lef-11* mRNA expression was determined by qRT-PCR. The silkworm housekeeping gene *sw22934* was used as the internal reference. Three independent experiments were repeated performed in triplicate. The data presented are the means ± SD ($n = 3$). (B) LEF-11 protein levels were analyzed by western blotting using anti-c-Myc and anti-tubulin antibodies. NS, Not significant; mi, mock-infected cells. Statistically significant differences: $^{**}P < 0.01$.

2.4. Effects of amiRNA cassettes on BmNPV replication in silkworm cells

There was functional inhibition of BmNPV following transient transfection by the amiRNA-based designs in BmN-SWU1 cells. BmN-SWU1 cells were transfected with amiRNA cassettes or pIZ-DsRed, and then infected with recombinant BmNPV v39K^{prm}-EGFP (containing a fluorescence marker controlled by a viral late 39K promoter) at 20 multiplicity of infection (MOI). Infected cells were

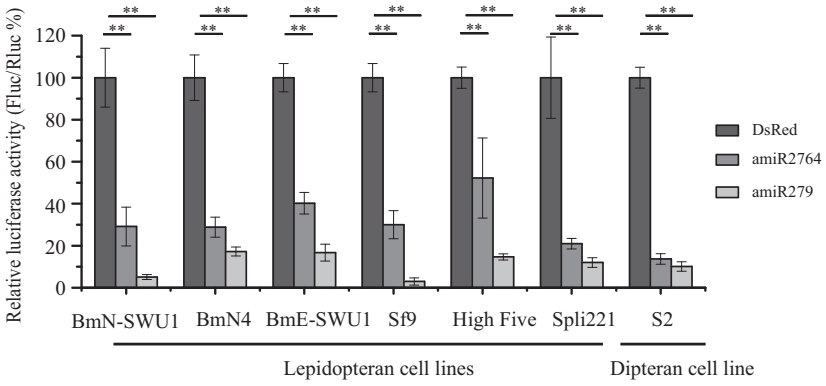

Fig. 2. Detection of RNAi activity of amiRNA cassettes in insect cell lines. Functional expression of mature amiRNAs from amiR2764 and amiR279 cassettes in insect cell lines. Insect cells were cotransfected with amiR2764 or amiR279 and the luciferase reporter plasmid; luciferase activity was measured 48 h p.t. Three independent experiments were repeated performed in triplicate. Statistically significant differences: $^{**}P < 0.01$.

viewed under a fluorescence microscope. Cells transfected with amiR2764 and amiR279 expressing DsRed had low or undetectable enhanced green fluorescent protein (EGFP) signals. In contrast, DsRed-negative cells had strong EGFP signals compared to cells transfected with pIZ-DsRed or the amiR92b construct (Fig. 4A). We counted and analyzed the proportion of baculovirus-infected cells (EGFP-positive) in amiRNA-expressing cells (DsRed-positive) (Fig. 4B). The amiR2764 or amiR279 constructs decreased virus infection efficiency by ~90% and ~99%, respectively as compared to the DsRed control plasmid (Fig. 4B). However, the amiR92b cassette had no effect on BmNPV replication.

The effects of the amiRNA designs on viral protein expression were detected by western blotting. We selected three proteins as detection targets: GP64 (BmNPV BV envelope protein necessary for pH-dependent membrane fusion during viral entry), P35 (caspase inhibitor that blocks virus-induced apoptosis), and EGFP (controlled by the BmNPV late phase promoter 39K). The amiR2764 and amiR279 cassettes significantly reduced the amount of baculovirus EGFP. However, both amiR2764 and amiR279 had minimal or undetectable inhibition on baculovirus GP64 and P35 synthesis (Fig. 4C). This result is consistent with that of Lin and Blissard, who reported that ablation of viral LEF-11 completely inhibited late and very late transcription but had no effect on baculovirus early gene expression (Lin and Blissard, 2002). Inhibition of viral replication was further confirmed by detection of viral DNA accumulation and viral protein expression in cells transfected with the amiRNA-based designs. The qRT-PCR quantification revealed an obvious decrease of BmNPV DNA accumulation in amiR2764- or amiR279-transfected cells compared with untransfected cells. However, no such effect was observed in cells transfected with pIZ-DsRed or amiR92b (Fig. 4D). Together, these results demonstrate that mature amiRNAs from miR2764- or miR279-based designs could inhibit BmNPV replication in BmN-SWU1 cells.

2.5. Establishment and characterization of transgenic silkworm cells expressing amiRNAs against baculovirus lef-11 gene

To better quantify the effects of amiRNAs on viral multiplication, we generated transgenic silkworm cells for stable amiRNA expression based on the commercial stable expression vector pIZ/V5-His (Invitrogen) by multiple copy integration of the vector according to the manufacturer manual. The amiR279 cassette was selected for the stable integration assay because its inhibition of baculovirus replication was more efficient compared to that by the amiR2764 cassette. The amiR279 cassette was transfected into BmN-SWU1 cells and the cell culture selected with Zeocin antibiotic (400 μg/mL). After 3-month selection, DsRed-positive cells were further purified by flow sorting (Fig. S1). Stable integration

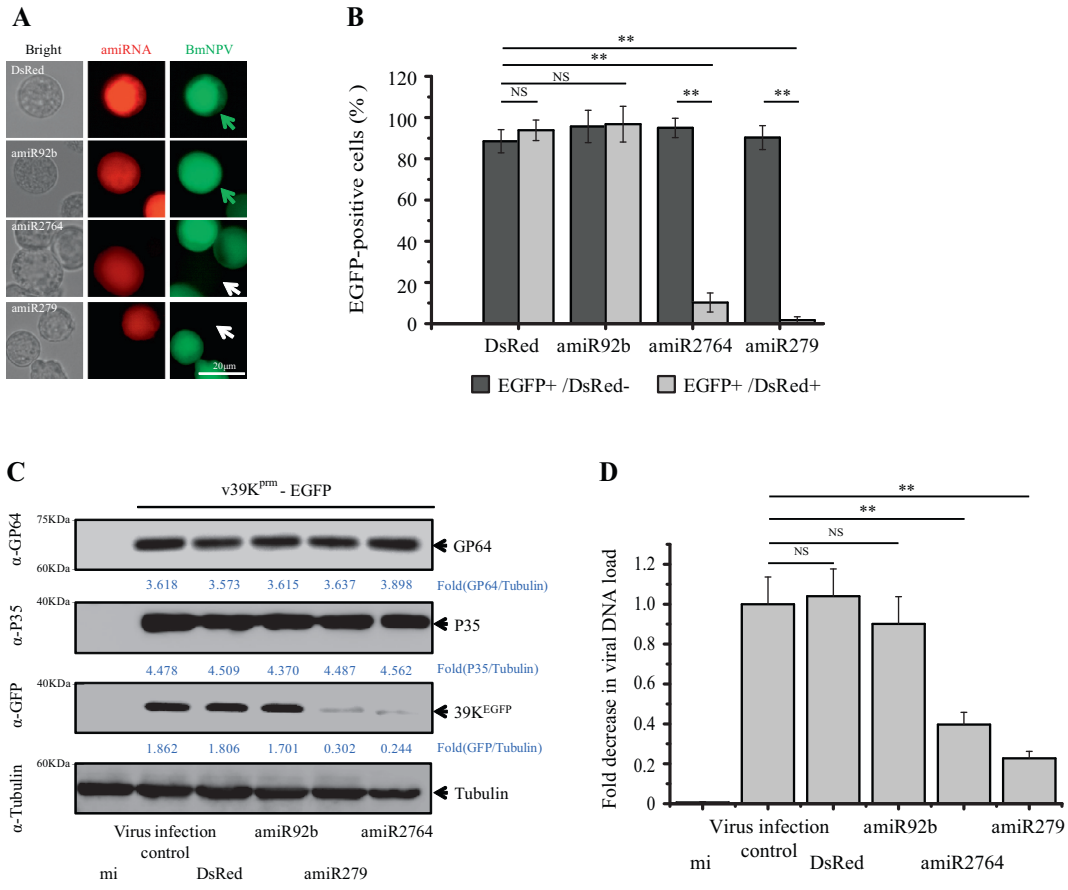

Fig. 4. Inhibitory effects on BmNPV replication after *lef-11* silencing by transient amiRNA transfection. (A) Cells transfected with amiRNA cassettes were infected with v39K^{prm}-EGFP virus at 20 MOI. Infected cells were examined under an Olympus TE-2000 fluorescence microscope at 72 h p.i. DsRed-positive (red) and EGFP-positive (green) cells denote amiRNA expression and BmNPV infection, respectively. (B) BmNPV infection rates (EGFP-positive, EGFP+) in amiRNA-expressing cells (DsRed-positive, DsRed+) and in amiRNA-non-expressing cells (DsRed-negative, DsRed−) were counted by selecting five different fluorescent fields. Three independent experiments were carried out in triplicate. The data presented are the means ± SD ($n = 3$). (C) Western blots of reduced expression of viral late gene (BmNPV 39K promoter driving EGFP) in amiRNA-transfected cells. Tubulin was used as the loading control. (D) Total cellular DNA was extracted from BmN-SWU1 cells at the indicated times after BmNPV infection and underwent qRT-PCR analysis using GP41 DNA primers. Relative copy numbers were calculated using *B. mori* GADPH DNA as the internal control. Samples from each time point were tested in triplicate; the mean value was used for analysis relative to BmNPV genomic copies. NS, Not significant; mi, mock-infected cells. Statistically significant differences: $^{**}P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cells underwent further antiviral assay. At 48 h p.i., the infection efficiency of BmNPV in amiRNA stably integrated cells was detected and analyzed. Stable cells had little resistance to baculovirus (Fig. 5A). Compared to transient transfection, the infection efficiency in stable cells was ~98%; it was only ~1% in amiR279 transient expression cells (Fig. 5B). Subsequent dual luciferase assay demonstrated that stable integration of the amiR279 construct resulted in the loss of the *lef-11* mRNA targeting capability (Fig. 5C). To determine the reasons behind this, we detected the level of mature amiRNAs in stable integration cells by poly (A) tail RT-qPCR. The expression level of mature amiRNAs in stable integration cells was very low compared to that of transient expression cells (Fig. 5D). We believe that the constitutive and high-level expression of amiRNAs in the cells may have oversaturated the cellular native miRNA pathway and caused global miRNA inhibition and cell death. Consequently, the obtained stable cell clones lost the ability to express functional mature amiRNAs.

2.6. Development of baculovirus-induced system for controlling amiRNA expression

To overcome the disadvantages of constitutive amiRNA expression, we constructed a regulatable amiRNA expression system. In this system, amiR279 was controlled by a baculovirus-induced promoter and enhancer that enabled adjustable control and high-level amiRNA expression (Fig. 6A). We constructed the Inducible amiR279 cassette by replacing the OpIE2 promoter of the amiR279 cassette with a homologous region 3 (hr3)-39K fusion promoter (Fig. S2). Promoter activity assay demonstrated that the hr3-39K fusion promoter had no transcriptional ability in BmNPV-uninfected cells, but exhibited >50-fold enhanced transcription activity in BmNPV-infected cells. To determine whether the regulatable amiRNA expression system functioned in silkworm cells, the Inducible amiR279 cassette was transfected into BmN-SWU1 cells. Transfected cells were infected with v39K^{prm}-EGFP and examined for DsRed expression. Fluorescence microscopy analysis showed that DsRed was expressed only in baculovirus-infected cells (Fig. 6B). Luciferase activity assays showed that transient expression of the Inducible amiR279 cassette did not decrease firefly luciferase activity in BmNPV-uninfected cells as compared to the pIZ-DsRed control. However, firefly luciferase activity decreased by ~50% in BmNPV-infected cells as compared with BmNPV-uninfected cells (Fig. 6C). The findings indicated that baculovirus specifically induced functional mature amiRNA expression in cells transfected with the Inducible amiR279 cassette.

2.7. Negative feedback inhibition of BmNPV by virus-induced amiRNA expression

We generated an Inducible amiR279 cassette–stably integrated cell line; positive transgenic cell clones were confirmed by reverse PCR and sequencing (data not shown). The expression and processing of mature amiRNA was detected in baculovirus-infected cells but not in mock-infected cells (Fig. 7A). We evaluated the antiviral

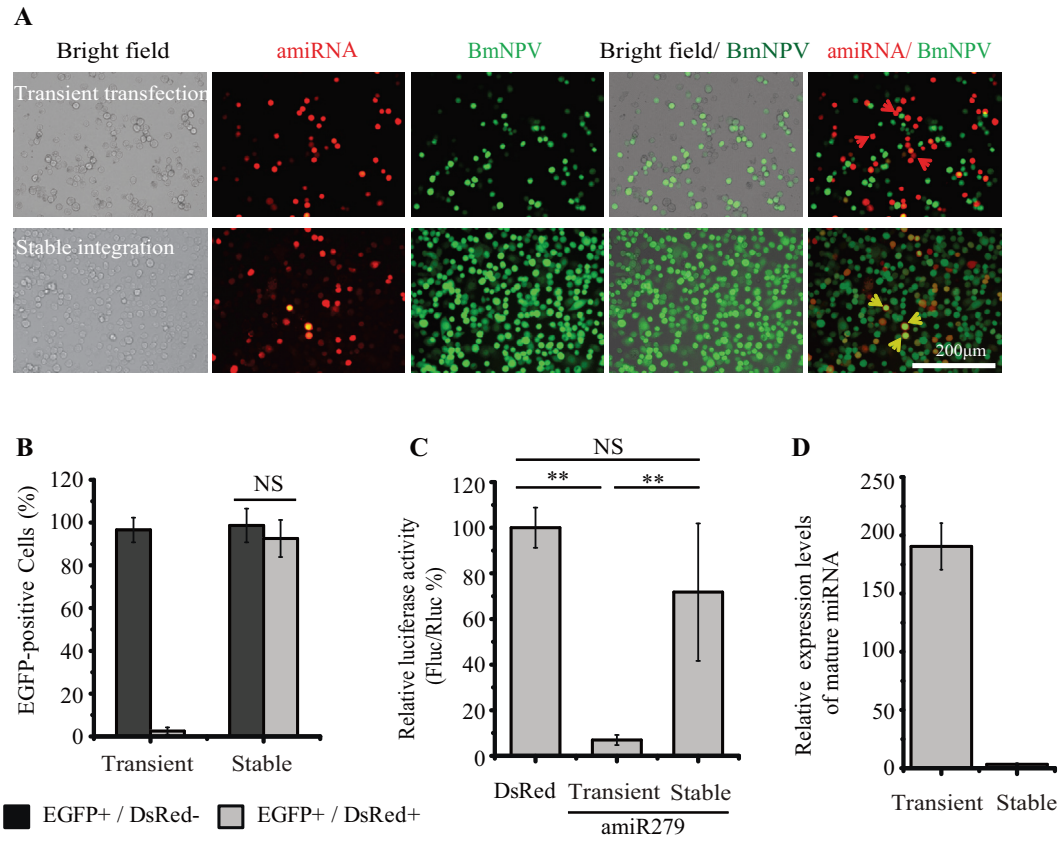

Fig. 5. Inhibitory effects of amiRNAs on BmNPV infection in stable integration cells. (A) Cells transiently transfected with amiR279 or the stable integrated cell line were infected with v39K^{prm}-EGFP virus at 20 MOI. Infected cells were examined under an Olympus TE-2000 fluorescence microscope at 72 h p.i. (B) BmNPV infection rates (EGFP-positive, EGFP+) in amiRNA-expressing cells (DsRed-positive, DsRed+) and in amiRNA–non-expressing cells (DsRed-negative, DsRed−) were counted by selecting five different fluorescent fields. Three independent experiments were carried out in triplicate. The data presented are the means ± SD (n = 3). (C) Inhibition of luciferase activity. BmN-SWU1 cells were cotransfected with amiRNA cassettes or nothing, and LEF-11 luciferase reporter plasmid; luciferase activity was measured at 48 h p.i. (D) Detection of amiRNA expression using poly (A) tail RT-qPCR in amiR279-transiently transfected cells or stably integrated cells. NS, Not significant. Statistically significant differences: $^{**}P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

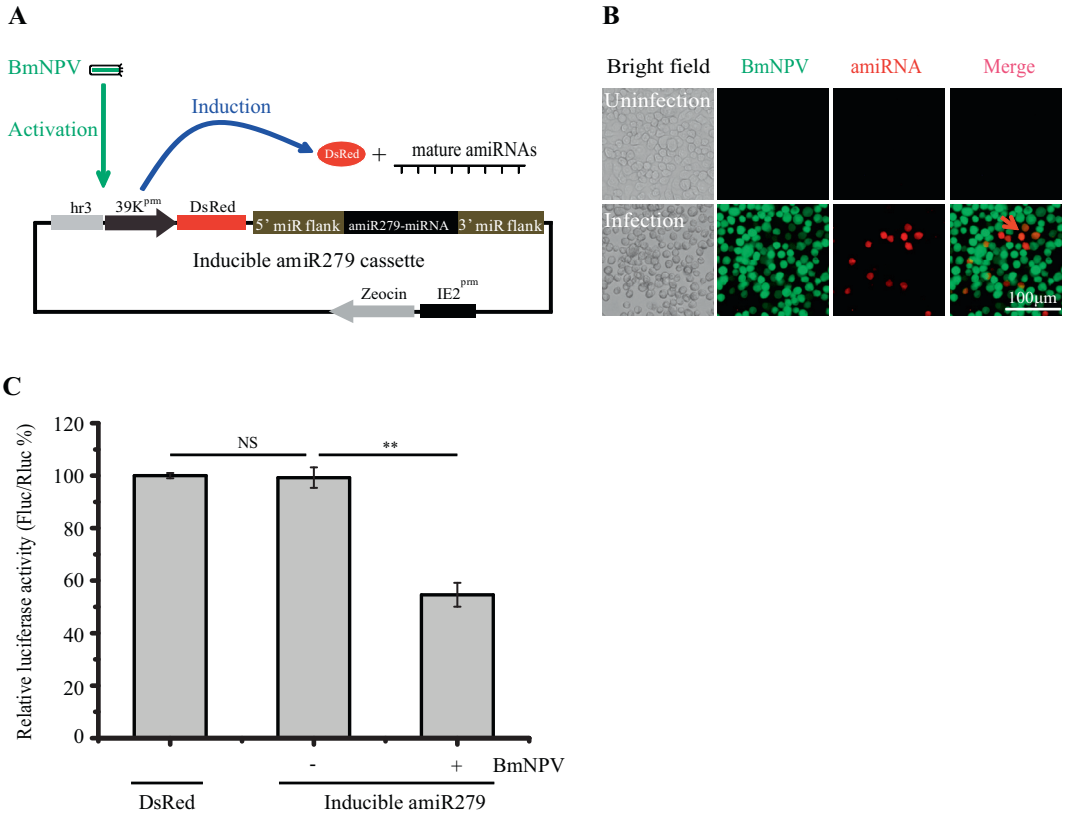

Fig. 6. Baculovirus-induced system for controlling amiRNA expression. (A) Schematic representation of the amiR279 inducible expression cassette cloned into pIZ/V5-His. (B) Fluorescence microscopy analysis demonstrating amiRNA (DsRed-marked) expression only in BmNPV-infected cells. The data presented are the mean of at least three independent experiments. (C) Inhibition of luciferase activity by *lef-11* amiRNA expressed by the Inducible amiR279 cassette. BmN-SWU1 cells were cotransfected with 400 ng Inducible amiR279 and 100 ng pDual-Mir-Luc-Reporter plasmid (5:1). At 24 h p.t., transfected cells were mock- or BmNPV-infected with v39K^{prm}-EGFP at 20 MOI. Luciferase activity was measured 36 h after BmNPV infection. NS, not significant. Statistically significant differences: $^{**}P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activity of the transgenic cell line against BmNPV by western blotting and viral titration assay. Transgenic cells were infected with v39K^{prm}-EGFP (MOI = 20). Cells were harvested for western blotting and cell supernatants were collected for 50% tissue culture infectious dose ($TCID_{50}$) titration (Wu et al., 2010). As expected, EGFP (driven by the baculovirus 39K promoter) was significantly inhibited in Inducible amiR279 – integrated cells as compared to mock-treated BmN-SWU1 cells (Fig. 7B). In the $TCID_{50}$ titration assay, the viral titer was decreased by 3.25 log $TCID_{50}$ in the Inducible amiR279 cell clone at 24 h p.i. as compared to the 4.38 log $TCID_{50}$ in mock-infected cells. A similar inhibition effect on BmNPV infection was detected up to 120 h p.i. (Fig. 7C). The mature amiRNAs expressed in the Inducible amiR279 transgenic cells inhibited viral replication significantly.

3. Discussion

The silkworm is a typical lepidopteran model and is economically important to the silk industry. However, it is usually susceptible to BmNPV infection, which can lead to significant economic loss. Several studies have shown that shRNA-based designs inhibited baculovirus replication efficiently (Kanginakudru et al., 2007; Subbaiah et al., 2013; Valdes et al., 2003). In this study, we used amiRNA-based designs constructed according to silkworm endogenous miRNA to inhibit BmNPV replication. We first screened three silkworm endogenous miRNAs: bmo-mir-279, bmo-mir-92b, and bmo-mir-2764. We further constructed three amiRNA expression cassettes targeting the BmNPV *lef-11* gene based on the backbones of the above endogenous miRNAs (Fig. 1A). Compared with conventional shRNA-based designs, our amiRNA-based designs enhanced RNAi activity by 80% in BmN-SWU1 cells (data not shown). In addition, a noteworthy advantage of miRNA-based designs is their ability to co-express mature miRNAs and a fluorescent marker, which permitted the observation of miRNA expression at the single-cell level (Fig. 4A).

We compared the inhibitory efficiency of the three amiRNA cassettes for the same target mRNA. In a luciferase activity assay, the amiR2764 and amiR279 cassettes exhibited high RNAi activity in BmN-SWU1 cells, while the amiR92b cassette did not (Fig. 1D). As all amiRNAs expressed from these three cassettes bind to the same site on the *lef-11* gene, and the cassettes differ merely in terms of their miRNA flanking sequences, we infer that the flanking sequences are important for amiRNA processing. This finding is similar to that in the report stating that flanking non-structured RNA sequences were required for efficient procession of pri-miRNA hairpins (Zeng and Cullen, 2005).

Many proteins are required in the miRNA transcription, cleavage, and maturation process. For example, Drosha and XPO-1 are involved in the primary miRNA processing process (Bussing et al., 2010; Lee et al., 2003); Exportin-5 is related to pre-miRNA export from the nucleus to the cytoplasm (Yi et al., 2003); Dicer and R3D1-L cleave pre-miRNAs and catalyze mature miRNAs (Jiang et al., 2005). AmiR2764 and amiR279 functioned in all studied insect cell lines, suggesting that these miRNA biogenesis molecules are highly conserved in all insects. However, the amiRNA cassettes displayed different RNAi efficiency in different insect cell lines

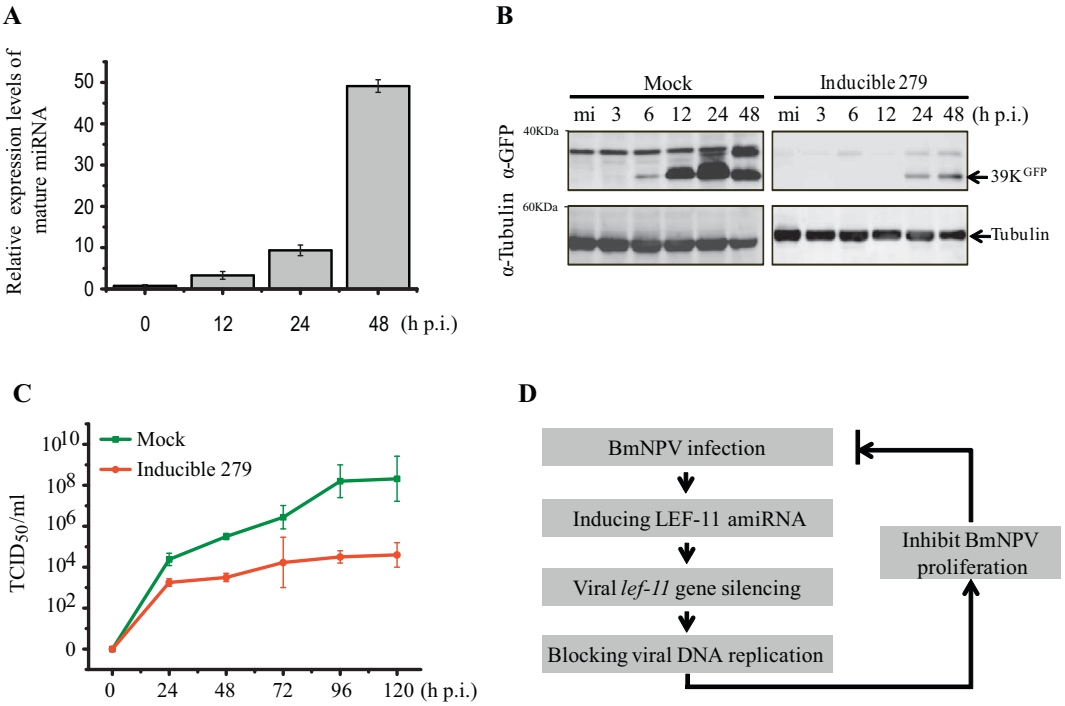

Fig. 7. Inhibition of baculovirus replication by negative feedback system. (A) Detection of amiRNA expression using poly (A) tail RT-qPCR in Inducible amiRNA–stably integrated cells. (B) Western blots of transgenic cells lysed at the indicated hours after v39K^{prm}-EGFP or mi infection and analyzed by immunoblotting using anti-EGFP antibody. (C) Virus growth curves determined by $TCID_{50}$ end point dilution assays. Cells were infected with v39K^{prm}-EGFP at 20 MOI; supernatants were harvested at the selected time points for the titer assay. Each data point was determined from the mean of three independent transfections or infections; error bars denote standard deviations. (D) Schematic representation of anti-BmNPV feedback strategy. mi, mock-infected cells.

(Fig. 2). It has been demonstrated that Exportin-5 and Argonaute 2 were limiting factors for miRNA biogenesis (Vickers et al., 2007). Moreover, Exportin-5 or Argonaute 2 overexpression significantly enhanced the efficiency of miRNA-mediated RNAi. Hence, we speculate that the different expression level of some molecules involved in miRNA biogenesis in different insect cell lines might be the major reason for the difference observed in the RNAi activity of the amiRNA cassettes in different cells (Diederichs et al., 2008; Yi et al., 2005).

Overexpression of siRNAs from shRNA-based designs induced acute cytotoxicity, while siRNAs from miRNA-based designs did not (Grimm et al., 2006; McBride et al., 2008). However, we demonstrated that siRNA duplex from the amiR279 cassette might have oversaturated the cellular endogenous miRNA pathways and caused high toxicity in BmN-SWU1 cells. These damaged cells were typically killed during the antibiotic selection. Consequently, the eventual amiR279 cassette–stably integrated clones could not express functional mature amiRNA and lost the ability to inhibit BmNPV proliferation.

These results raise concerns about the safety of using amiRNA-based designs as potential antivirus tools. To address this issue, we constructed an inducible anti-BmNPV amiRNA system named Inducible amiR279, in which an amiRNA coding sequence was inserted downstream of a baculovirus-induced promoter and hr3 enhancer (Fig. 5A). The baculovirus 39K gene is a delayed-early gene that has no transcriptional activity in uninfected cells and highly inducible activity at 3 h after baculovirus infection (Xue et al., 2012). It was reported that baculovirus hr3 is an efficient enhancer for activating the transcription of many promoters through interaction with baculovirus IE1 protein (Lu et al., 1997). Our strategy was based on a combined promoter in which the enhancer hr3 is fused to the BmNPV 39K promoter.

The hr3-39K fusion promoter was induced in BmNPV-infected cells, while its activity was undetectable in uninfected cells (Fig. S2). Controlled by the hr3-39K promoter, the Inducible amiR279 system was activated by BmNPV infection, which functioned in a negative feedback loop to induce transcription of amiRNAs directed against BmNPV LEF-11. The viral load closely regulated the level of amiRNA expression (Fig. 6C). To our knowledge, there have been no previous reports of a baculovirus-induced amiRNA expression construct that utilized negative feedback to inhibit virus replication. To investigate the potential of this system in anti-BmNPV research, we established the Inducible amiR279–stably integrated cell line. Antiviral assays demonstrated that the transgenic cells had enhanced anti-BmNPV capacity as compared with mock-treated BmN-SWU1 cells. Therefore, our system can be used as an efficient and safe anti-BmNPV strategy that can be inserted into the silkworm genome to obtain transgenic silkworms with high resistance to BmNPV.

In conclusion, we explored a novel regulatable anti-BmNPV system activated by BmNPV infection. This system will be a safe and effective tool for breeding high-resistance transgenic silkworms without affecting their growth and development.

4. Materials and methods

4.1. Cell cultures and baculovirus

B. mori–derived BmE-SWU1 (Pan et al., 2007), BmN-SWU1 (Pan et al., 2010), and BmN4 (a gift from Dr. Dao-Jun Chen); *S. frugiperda* – derived Sf9; *T. ni* – derived High Five (Sf9 and High Five were purchased from Invitrogen); and *S. litura* – derived Spli221 cell lines (a gift from Prof. Yang Cao) were cultured at 27 °C in TC-100 medium (United States Biological) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (200 U/mL), and streptomycin (200 U/mL). The *D. melanogaster* cell line S2 (a gift from Prof. Hong-Juan Cui) was maintained at 27 °C in Schneider's Drosophila

medium (Life Technology) containing 10% FBS. Recombinant BmNPV (v39K^{prm}-EGFP) containing an EGFP marker gene under the control of the 39K promoter was constructed from a bMON7214 bacmid, which contains the BmNPV genome (Shen and Chen, 2012). The vBm$^{lef11\text{-}REPMyc}$, which inserts one copy of a c-Myc–tagged *lef-11* gene controlled by its native promoter into the *polh* locus was constructed in the same manner. All viruses were plaque-purified and confirmed by analyses of PCR-amplified genomic segments. They were propagated in silkworm BmN-SWU1 cells. Viral titers were determined by end-point dilution assay.

4.2. Plasmid construction

The DsRed reporter protein gene from the pDsRed2-1 plasmid (Clontech Laboratories) was cloned into the pIZ/V5-His vector to generate pIZ-DsRed. The pIZ-DsRed plasmid was used to express DsRed protein and mature amiRNAs constitutively. The baculovirus-induced promoter 39K (provisional Chinese Patent No. 201010231957.9, Pan et al.) with the enhancer hr3 was cloned into pIZ-DsRed to replace the *OpIE2* promoter. The resulting phr3-39K-DsRed plasmid was used as the vector backbone for baculovirus-induced amiRNA expression. Based on miRBase, we selected three silkworm endogenous miRNAs: bmo-mir-92b, bmo-mir-279, and bmo-mir-2764 as the backbones for the amiRNA construction and expression. Briefly, the natural siRNA duplex of endogenous miRNA was replaced with artificial synthetic siRNA duplex sequences targeting the BmNPV *lef-11* gene. AmiRNA sequences with flanking sequences were synthesized and cloned into a pUC57-simple vector by GenScript (Nanjing, China). The inserts were released using AscI and SacII digestion and cloned into pIZ-DsRed or phr3-39K-DsRed. The anti-BmNPV amiRNA sequences are listed in Table 1. To investigate the effect of mature amiRNAs on target gene expression, an RNAi reporter plasmid was created by inserting a ~500-bp *lef-11* target sequence into the multiple cloning site of a pDual-Mir-Luc-Reporter plasmid (constructed and conserved in our laboratory). All plasmids were confirmed by sequencing.

4.3. Dual luciferase reporter assays

The amiRNA expression plasmids and dual luciferase expression plasmids (pDual-Mir-Luc-Reporter-lef11) were cotransfected into BmN-SWU1 cells. Cells (1×10^5/well) were seeded in 24-well plates (Corning). After 24 h, cells were cotransfected with 400 ng amiRNA expression plasmid and 100 ng pDual-Mir-Luc-Reporter plasmid (5:1). Cells were harvested 48 h p.t.; luciferase activities were measured using a GloMax-Multi Detection System (Promega) with a Dual-Glo luciferase assay kit (Promega). The results were calculated as the relative luciferase activity (i.e., Fluc/Rluc). Assays were performed in triplicate.

4.4. Real-time quantitative PCR

Total RNA from each sample was extracted 48 h p.i. as described above. Genomic DNA was removed using RQ1 RNase-Free DNase (Promega). Reverse transcription was carried out using MLV Reverse Transcriptase (Promega). The complementary DNA (cDNA) template (1 μL) underwent real-time PCR using specific primers for BmNPV *lef-11* (F: 5′-CCCAACAGCCTGGCAATTC-3′; R: 5′-TCAACACGCTCAAGCACACAA-3′). The silkworm housekeeping gene sw22934 was used as the internal control (F: 5′-AACACCCCGTCCTGCTCACTG-3′; R: 5′-GGGCGAGACGTGTGATTTCCT-3′). RT-qPCR was performed using a StepOnePlus™ Real-Time System (Applied Biosystems). Amplification was carried out in 15-μL reaction mixtures containing 1 μL cDNA, 0.5 mM of each primer, and 2× SYBR Select Master Mix (Life Technology) in each well of a 96-well plate. The reaction procedure was 94 °C for 10 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 40 s. Melting curve analysis was performed to confirm specific amplification. Each expression assay was repeated thrice. Student's *t*-test was used to evaluate statistical significance ($P < 0.01$).

4.5. Western blotting

At 48 h p.i., cells were washed twice with phosphate-buffered saline (PBS) and lysed with 100 μL immunoprecipitation lysis buffer (Beyotime, Beijing, China) that contained 1% phenylmethylsulfonyl fluoride protease inhibitors (Beyotime). After 30 min on ice, lysates were spun at 12,000×*g* for 15 min and the supernatants were collected. The supernatant proteins then underwent concentration measurement using a BCA Protein Assay Kit (Beyotime). Proteins were resolved with 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and underwent immunoblot analysis with rabbit α-cMyc (Abcam), mouse α-GP64 (Abcam), rabbit α-P35 (Imgenex), mouse α-GFP (Beyotime), and mouse α-tubulin antibodies (Beyotime). The tubulin band indicated the total protein

Table 1
Complete sequences of aritificial miRNA constructs. (LEF-11 pre-miRNA sequences are in blue. *Asc*I and *Sac*II restriction enzyme sites are underlined.)

	Sequence (5' to 3')
Artificial pre-mir92b	GGCGCGCCCGCGCGATCCCGTTAGCTGGCCGGTCGGTCGTTCACGCGGTGGCCACGCCCATGCTGCACA TACGTGTCCCGTTTGTGTTTCATATCAACGACACGATGTCCAACATGTCTGTGGCCACAGCTCTACGTGA ACCAACTCAACATTACGGTCTCCAGAAGGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCC GCGCGATCCCGTTAGCTGGCCGGTCGGTCGTTCACGCGGTGGCCACGCCAAGGCGTCTCTTACATCACT GGTTTGTGTTTCATATCATTGTGATGAAGACACACCTTACTGTGGCCACAGCTCTACGTGAACCAACTCA ACATTACGGTCTCCAGAAGAAAACCGCGG
Artificial pre-mir2764	GGCGCGCCCAGCTACAACGCACCCCATAAGGAGCCCTTGGTCTACTGTCAGTTCTTCTACATGTTGGACA TCGTGTCGCCTTGATTCGAATTATACGACACGATGTCCAACATGTAGAATTATCTGAACTACTACGGACG GACTTCAATCTTCAAATGGTAATTTTTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCCAG CTACAACGCACCCCATAAGGAGCCCTTGGTCTACTGTCAGTTCTTCTTAAGGTGTGTCTTCATCACACGTT GATTCGAATTATTTGTGATGAAGACACACCTTAAGAATTATCTGAACTACTACGGACGGACTTCAATCTT CAAATGGTAATTTTTCCGCGG
Artificial pre-mir279	GGCGCGCCCGGCGTAGTAATCAGCGGAGACGTCAATTTCTTTCGATCGTGTTTTTTGAGCGTAGTATGTT TATTTACACCATATACGCTCAAACGACACGATCCATGGAAGTTGCGAGGTTCGTCGCCGAGAGCACGAA CCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCTAGTAATCAGCGGAGACGTCAATTTCTTTCGTAAGGT GCATCTTCATCACGAATGTTTATTTACACCATTGTGATGAAGACACACCTTACCATGGAAGTTGCGAGGT TCGTCGCCGAGACCGCGG

loading level. Horseradish peroxidase–conjugated goat anti-mouse or goat anti-mouse immunoglobulin G (Beyotime) was used as the secondary antibody. Protein bands were visualized using a Lumi-Light PLUS Western Blotting Kit (Roche) in a Clinx chemiluminescence imaging system. The intensity of bands was quantified using Image J software. Each band density was first normalized by dividing it by the density of the Tubulin band in the same lane and expressed as fold change.

4.6. Virus infection assay

BmN-SWU1 cells were first infected with v39K^{prm}-EGFP for 1 h at 4 °C at 20 MOI. Infected cells were washed three times with normal medium. Then, cells were cultured in medium supplemented with 10% FBS. This time point was marked as 0 h p.i. At 72 h p.i., BmNPV infection rates (EGFP-positive, EGFP+) in amiRNA-expressing cells (DsRed-positive, DsRed+) and in amiRNA-non-expressing cells (DsRed-negative, DsRed−) were viewed and counted under a fluorescence microscope by selecting five different fluorescent fields. The assay was repeated three times and the standard deviation calculated using Microsoft Excel.

4.7. Quantification of viral DNA load

At the indicated time points, BmNPV-infected BmN-SWU1 cells were harvested, washed with PBS, and suspended in sterile water. Total DNA from each sample was prepared with a Wizard® Genomic DNA Purification Kit (Promega) according to the protocol in the manual. Quantitative PCR was performed with a SYBR Premix Ex Taq™ II Kit (TaKaRa) using primers targeting a 120-bp region of the BmNPV *GP41* gene (GP41-F: 5′-AGCGGTTTGAATCGGAGGAGAC-3′; GP41-R: 5′-TTGAACGGTAACGGAATGGGAA-3′). Reaction mixtures (15 μL) containing 20 ng total DNA, 0.5 mM each primer, and 7.5 μL 2× SYBR Select Master Mix (Life Technology) underwent PCR amplification on a StepOnePlus™ Real-Time system (Applied Biosystems). All DNA samples were normalized to cell equivalents using the silkworm glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (BmGAPDH F: 5′-CATTCC GCGTCCCTGTTGCTAAT-3′; BmGAPDH-R 5′-GCTGCCTCCTTGACCT TTTGC-3′) as a genome standard for BmN-SWU1 cells. RT-qPCR data were analyzed using system software. Each assay was repeated three times.

4.8. Quantification of mature amiRNAs

Total RNA was extracted using TRIzol (Invitrogen) and treated with RQ1 RNase-Free DNase (Promega) to remove DNA contamination. Then, 1 μg total RNA was reverse transcribed using a One Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa) according to the manufacturer's protocol. For real-time PCR, 1 μL cDNA was mixed with 12.5 μL SYBR Premix Ex Taq II (2×), 1 μL Universal_RU6B_ Primer (10 μM), 1 μL amiR279-forward primer (5′-CGCUAU ACGCUCAAACGACACG-3′) and 10.5 μL diethylpyrocarbonate-treated H_2O according to the TaKaRa protocol. PCR was performed using a common program: initial incubation of 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. Standard curves were obtained by 10-fold serial dilution of pooled cDNA. Quantitative mRNA measurement was performed in triplicate and normalized to the *B. mori* U6 small nuclear RNA (5′-CGCAAGGATGACACGCAA-3′) internal control.

4.9. BmNPV BV production assay

Cells were seeded in 24-well culture plates and infected with v39K^{prm}-EGFP for 1 h at 4 °C at 20 MOI. Infected cells were washed three times with medium. Then, cells were cultured with medium supplemented with 10% FBS. This time point was marked as 0 h p.i. At the indicated hours, supernatant containing BVs were harvested for $TCID_{50}$ titration (Wu et al., 2010). Virus infection was evaluated by observing EGFP expression in BmN-SWU1 cells under fluorescence microscopy. The assay was performed in triplicate.

Author contributions

Conceived and designed the experiments: J Zhang, C-D Zhang, C Lu, and M-H Pan. Performed the experiments: J Zhang, Q He, X-M Chen, Z-Q Dong and X-Y Chen. Analyzed the data: J Zhang, N Li, X-X Kuang, M-Y Cao and X-L Dong. Wrote the paper: J Zhang, Q He, C-D Zhang, C Lu, and M-H Pan.

Competing interests

The authors have declared that no competing interests exist.

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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.2014.01.017.

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