Antiviral Research 104 (2014) 143–152

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

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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ARTICLE INFO

Article history:
Received 25 November 2013
Revised 17 January 2014
Accepted 22 January 2014
Available online 31 January 2014

Keywords:
BmNPV
RNA interference
Artificial microRNA
Baculovirus-induced RNA interference system

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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http://dx.doi.org/10.1016/j.antiviral.2014.01.017
0166-3542/

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,

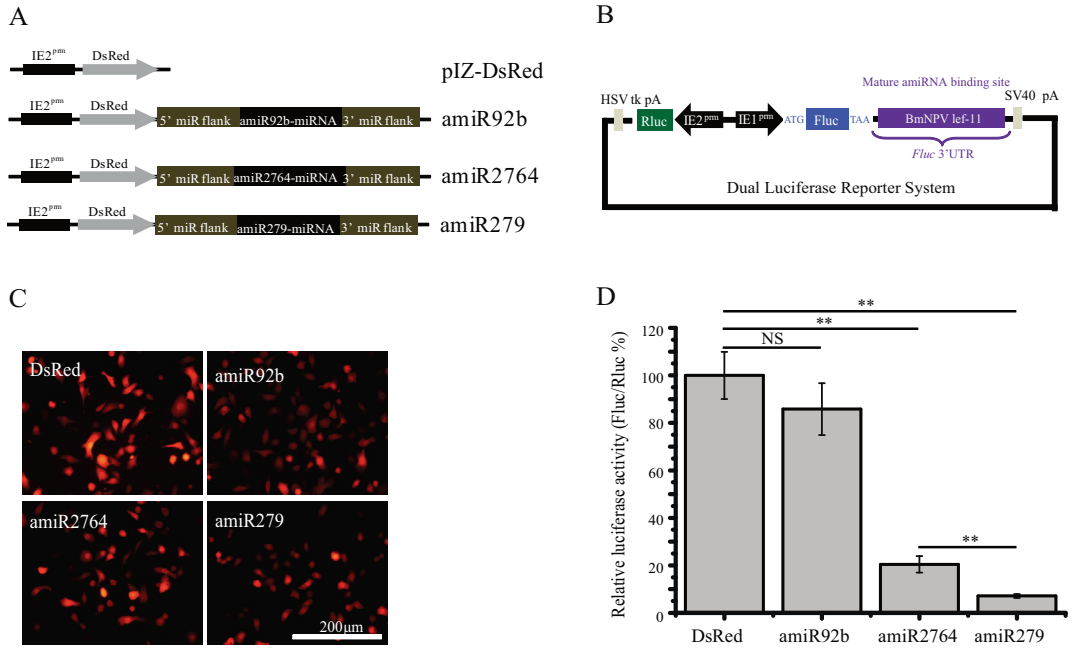

Fig. 1. Construction of amiRNA cassettes. (A) Structure of three amiRNA expression cassettes with 5′ and 3′ miRNA flanking sequences based on the silkworm endogenous miRNAs bmo-mir-92b, bmo-mir-264, and bmo-mir-279. These backbones were inserted into pIZ/V5-His, which contained the OpMNPV IE2 promoter 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.)