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ORIGINAL ARTICLE

BmNPV-miR-415 up-regulates the expression of *TOR2* via Bmo-miR-5738

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Abstract MicroRNAs (miRNAs) have emerged as key players in host–pathogen interaction and many virus-encoded miRNAs have been identified (computationally and/or experimentally) in a variety of organisms. A novel *Bombyx mori* nucleopolyhedrosis virus (BmNPV)-encoded miRNA miR-415 was previously identified through high-throughput sequencing. In this study, a BmNPV-miR-415 expression vector was constructed and transfected into BmN cells. The differentially expressed protein target of rapamycin isoform 2 (TOR2) was observed through two-dimensional gel electrophoresis and mass spectrometry. Results showed that TOR2 is not directly a target gene of BmNPV-miR-415, but its expression is up-regulated by BmNPV-miR-415 via Bmo-miR-5738, which could be induced by BmNPV.

1. Introduction

Sericulture production is an important economic industry. *Bombyx mori* serves as a Lepidopteran model system for genetics and molecular studies. The *B. mori* nucleopolyhedrosis virus (BmNPV) is a natural pathogen of *B. mori*; it causes a very high mortality that in turn leads to a significant loss in silk cocoons and therefore a major economic damage to the silk industry. Varieties which have a strong resistance to the virus and which can eliminate the source of infection have been bred to control BmNPV damages; however the affected individuals cannot be cured once they are infected (Singh et al., 2010).

MicroRNAs (miRNAs) are non-coding RNA molecules of 20–23 nt in length. These molecules regulate the expression of genes by guiding the RNA-induced silencing complex to a target sequence, which is usually located at the 3′ UTR of mRNAs (Bartel, 2004; Pedersen et al., 2007). Increasing

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evidence suggests that miRNAs have indispensable roles in host–virus interactions (McClure and Sullivan, 2008; Zhumur et al., 2009). Several experiments have been performed to study anti-viral miRNAs. For example, the miR-199a recombinant plasmid has been transfected into cells to significantly reduce hepatitis C virus replication (Murakami et al., 2009). During viral infections, a virus can use the endogenous miRNA biosynthetic machinery of the host cell to encode viral miRNAs, attack the host cell defence system to protect the virus itself, or adjust the viral or host gene expression, thereby creating an increasingly conducive environment for the survival of the virus. The miRNAs encoded by Kaposi's sarcoma herpes virus down-regulate intracellular glycoprotein thrombospondin-1 with anti-angiogenic and anti-proliferative activities. The main purpose of miRNAs is to evade the immune system of cells (McClure and Sullivan, 2008).

Studies on the function of viral miRNAs demonstrate that some miRNAs have important roles in regulating the viral life cycle and the interaction between viruses and their hosts (Zhumur et al., 2009; Cai et al., 2006). The first in vivo antagomir provides the first step towards using miRNA therapy; the design of molecular medicines based on the modulation of miRNAs ultimately exhibits good potential (Pfeffer et al., 2004). Viruses are obligate intracellular parasites whose replication depends on their hosts. This interplay has important consequences to both the virus and the host (Cai et al., 2006).

In our previous studies, we obtained a novel BmNPV-encoded miRNA, the miR-415, through high-throughput sequencing (data not shown). miRNAs are small regulatory molecules that function at the post-transcriptional level and may cause translation inhibition. We therefore speculated that, when extra miRNA is present in cells, the protein level of the target gene may differ. As such, we transfected BmNPV-miR-415 into BmN cells and observed the differential expression of the TOR2 protein through two-dimensional gel electrophoresis (2-DE) and an ultraflex TOF/TOF mass spectrometer. *TOR2* is not the target gene of BmNPV-miR-415 but is the target of the Bmo-miR-5738, a *B. mori* miRNA obtained via high-throughput sequencing. We validated in this study that Bmo-miR-5738 up-regulates the expression of *TOR2*, whereas BmNPV-miR-415 amplifies this regulatory effect.

2. Materials and methods

2.1. Silkworm strains and BmNPV infection

The domesticated silkworm strain hybrid S16·S17 × A1·A16 was used in this study. The larvae were reared on fresh mulberry leaves at 25 °C with 80 ± 5% relative humidity and at intervals of 12 h light:12 h dark. Freshly enclosed fifth instar larvae were orally fed with the purified BmNPV suspension of occlusion bodies (OBs; 20,000 OBs/larva).

2.2. RNA extraction

Tissue samples were extracted from the BmNPV-infected fifth instar larvae. The extracted tissue was homogenised in TRIzol reagent (Invitrogen). The total RNA was extracted using standard protocol.

2.3. 2-DE analysis

2-DE was used to analyse the differential expression of BmN cells in both the transfected BmNPV-miR-415 group and in the control group (no miR-415 treatment). The proteins were extracted from BmN cells at 48 h post-transfection. The protein concentration was quantified using a Bradford kit (Sangon). 2-DE was performed according to the manufacturer's recommendations (GE Healthcare). The 2-DE gels were visualised by silver staining. The obtained images were then scanned with an HP Scanjet G2410 scanner (Hewlett Packard) and analysed through the ImageMaster™ 2D Platinum 6.0 software (GE Healthcare).

2.4. Western blot analysis

For the Western blot analysis, the proteins were separated by 10% SDS–PAGE and transferred to a nitrocellulose membrane (GE Healthcare). The TOR2 protein on the membrane was detected with a rabbit anti-TOR2 antibody (1:1000 dilution) as the primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution; ABmart) as the secondary antibody. The signal detection was performed using the ECL Plus Western Blotting Detection Kit (GE Healthcare). The detection of β-actin as a control was performed as described above using rabbit anti-β-actin as the primary antibody.

2.5. Filter target miRNAs

We searched all the known silkworm miRNA sequences from the miRNA database (http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=bmo) to obtain the miRNA that matched the 3′ UTR sequence of TOR2. The novel miRNAs were obtained through high-throughput sequencing. The RNA22 (http://cbcsrv.watson.ibm.com/rna22.html) and RNA hybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) software programs were jointly used to confirm the target miRNAs. The 3′ UTR of TOR2 was downloaded from SilkDB (http://silkworm.genomics.org.cn).

2.6. Cell culture and construction of recombinant vectors

The *B. mori*-derived cell line (BmN) was originally conserved in our laboratory. BmN cells were cultured in TC100 medium (Gibco, Invitrogen Corporation, USA) at 27 °C, as described by standard methods (Zhao et al., 2007). The pcDNA3.0(IE-1-*EGFP*-SV40), PGL3(A3-*luc*-SV40) and pRL-null vectors were constructed and preserved in our laboratory (Chen et al., 2013). To construct PGL3 (A3-*luc*-TOR2-3′ UTR-SV40) plasmids, the 3′ UTR fragment of *TOR2* was cleaved by *Xba*I and *Fse*I and ligated to pGL3 (A3-*luc*-SV40) plasmids which were previously digested by the same restriction enzymes. To construct the pcDNA3.0 (IE-1-*EGFP*-miR-415-SV40) and pcDNA3.0 (IE-1-*EGFP*-miR-5738-SV40) plasmids, the precursors of BmNPV-miR-415 and bmo-miR-5738 were cleaved by *Hin*dIII and *Bam*HI, respectively, and ligated to pcDNA3.0 (IE-1-*EGFP*-SV40) plasmids which were previously digested with the same restriction enzymes.