



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.

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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 *Hind*III and *Bam*HI, respectively, and ligated to pcDNA3.0 (IE-1-EGFP-SV40) plasmids which were previously digested with the same restriction enzymes.

The primers for cloning the precursors of the different miRNAs and *TOR2* 3' UTR are listed in Table S1.

2.7. MiRNA injection

The BmNPV-miR-415 mimic (miR-415m), BmNPV-miR-415 inhibitor (miR-415i), negative control (NC) mimic and the NC inhibitor were synthesised by RiboBio (Guangzhou, China). The miR-mimic is a dsRNA formed by the miRNA and its complementary sequence. The miRNA inhibitor consists of the antisense oligonucleotides of the miRNA with a 2'-O-methyl modification. The NC was designed based on a *Caenorhabditis elegans* miRNA sequence with no similarity to insect miRNAs.

Prior to the injection of the respective treatment, the BmNPV-infected fifth instar larvae were synchronised after they moulted. For each insect, 10 µg of the miRNA mimic or inhibitor was injected into the larvae. A total of 60 insects were injected for each miRNA. At 24 h after the injection, the animals were dissected. The total RNA was extracted from the obtained tissues using the TRIZOL reagent (Invitrogen), following the manufacturer's protocol. The experiment was repeated three times.

2.8. Transfection and dual luciferase reporter (DLR) assay

We co-transfected BmN cells with the following respective expression vectors: pcDNA3 and *TOR2* 3' UTR, bmo-miR-5738 and *TOR2* 3' UTR, BmNPV-miR-415 and *TOR2* 3' UTR and bmo-miR-5738 with BmNPV-miR-415 and *TOR2* 3' UTR. Each plasmid (5 ng) was transfected by mixing it with 0.8 µl Lipofectamine 2000 transfection reagent (Invitrogen) in 20 µl Opti-MEM I reduced serum medium (Gibco) in each well of a 96-well plate. The dual-luciferase reporter assay (Promega) was performed according to the manufacturer's protocol at 48 h after the transfection. The experiment was performed three times, each with three technical repeats.

2.9. Fluorescence quantification analysis

The quality of the total RNAs was determined at a 260/280 absorbance ratio and through electrophoresis. The RNA sample was stored at -80 °C until further use. After treatment

with DNase I, 1 µg of the total RNA was used to synthesise the first strand cDNA with the primerscript reverse transcriptase kit (TaKaRa) according to the manufacturer's protocol. The SYBR Green RT-PCR assay was conducted using an ABI PRISM 7300 sequence detection system (Applied Biosystems). The thermal profile for SYBR Green RT-PCR was 50 °C for 2 min and 94 °C for 30 s, followed by 40 cycles of 94 °C for 15 s and 58 °C for 40 s. The sequences of the primers are listed in Table S2.

3. Results

3.1. Differential expression protein analysis

miRNAs regulate gene expression at the post-transcriptional level. BmN cells were infected with BmNPV-miR-415 to study the target genes of BmNPV-miR-415. A 2-DE experiment was conducted to investigate the differential expression of proteins in the BmNPV-miR-415-transfected group and those in the control group. The results exhibited the presence of a differentially expressed protein in the BmNPV-miR-415 transfected group and the absence of a differentially expressed protein in the control group (see Fig. 1). This protein was identified through an ultraflex TOF/TOF mass spectrometer (Bruker) manufactured by the BGI-Beijing Company (<http://www.genomics.cn/index>). A mascot search showed that the protein is TOR2 (Figs. S1 and S2). The expression of TOR2 protein was also confirmed using Western blot (see Fig. 2). The RNA22 and RNA hybrid results revealed that BmNPV-miR-415 does not match the 3' UTR of *TOR2* mRNA. Therefore, we hypothesised that other miRNAs probably target *TOR2*.

3.2. Regulation of bmo-miR-5738 on *TOR2*

Previous reports state that miRNAs usually bind to 3' UTR (Brennecke et al., 2003; Lin et al., 2003); thus, we focused on the 3' UTR of *TOR2*. Our data were filtered with the RNA22 and RNA hybrid software, and high-throughput sequencing revealed that the host bmo-miR-5738 miRNA perfectly matches the 3' UTR sequence of *TOR2* (Fig. 3), whose expression was induced by BmNPV infection. We then co-transfected BmN cells with the following group of expression vectors: Group A, pcDNA3 and *TOR2* 3' UTR; Group B, bmo-miR-5738 and *TOR2* 3' UTR; Group C,

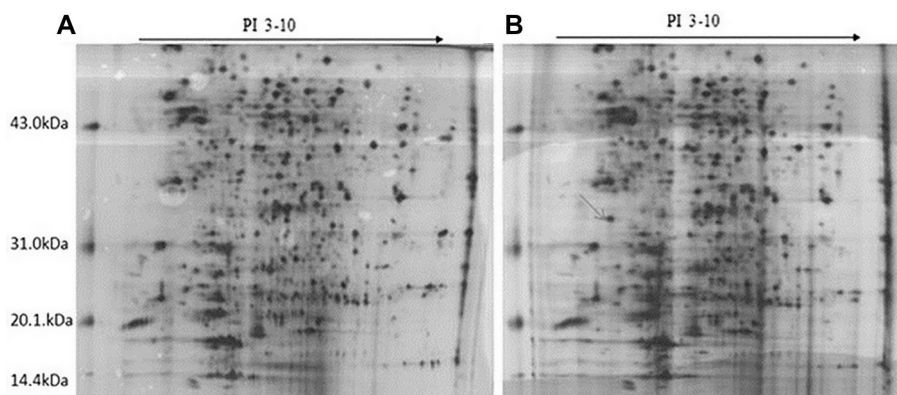


Figure 1 2-DE maps of proteins. (A) Control group without transfected miR-415. (B) Experimental group transfected with miR-415.

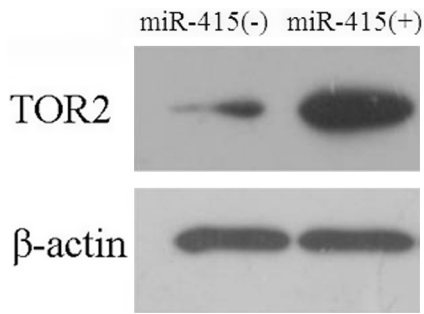


Figure 2 Western blot of TOR2 protein in miR-415-injected samples. Each blot represents a mixture of 3–9 samples, and β -actin was used as a loading control.

bmo-miR-5738 together with BmNPV-miR-415 and TOR2 3' UTR; and Group D, BmNPV-miR-415 and TOR2 3' UTR. Each group was co-transfected with a pRL-null internal control. The results of the luciferase activity assay showed that either bmo-miR-5738 or BmNPV-miR-415 significantly up-regulated the expression of TOR2. When BmN cells were co-transfected with the bmo-miR-5738 and BmNPV-miR-415 expression vectors, the up-regulation of TOR2 was amplified by more than threefold (Fig. 4), which implied that the expression of bmo-miR-5738 could be regulated by BmNPV-miR-415.

3.3. Expression of bmo-miR-5738, BmNPV-miR-415 and TOR2 in larval hemolymph after BmNPV infection

To confirm the regulatory functions of bmo-miR-5738, BmNPV-miR-415 and TOR2, their transcriptional levels in larval haemolymph were analysed by qPCR from 8 h to 96 h after BmNPV infection. The results showed that the transcription of TOR2 and bmo-miR-5738 increased from 8 h and 16 h and reached their respective maximum levels at 24 h, after which it decreased in a consistent trend. Meanwhile, the transcription of BmNPV-miR-415 constantly increased during the experiment (Fig. 5).

3.4. BmNPV-miR-415 up-regulates the expression of bmo-miR-5738 and TOR2 in vivo

Based on the abovementioned experimental results, we propose that BmNPV-miR-415 regulates the expression of

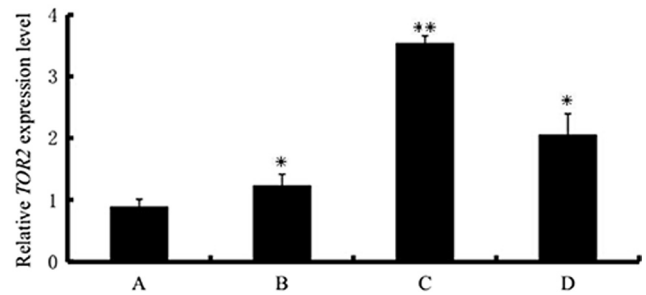


Figure 4 Relative TOR2 expression levels measured by dual luciferase activity. We co-transfected BmN cells with the following respective expression vectors: Group A, pcDNA3 and TOR2 3' UTR; Group B, bmo-miR-5738 and TOR2 3' UTR; Group C, bmo-miR-5738 together with BmNPV-miR-415 and TOR2 3' UTR; and Group D, BmNPV-miR-415 and TOR2 3' UTR. Each group was co-transfected using the pRL-null internal control. Data are represented as the mean value \pm SD of three independent experiments. Vertical bars represent the mean \pm SD ($n = 3$). The asterisks indicate the significant differences (* $P < 0.05$; ** $P < 0.01$).

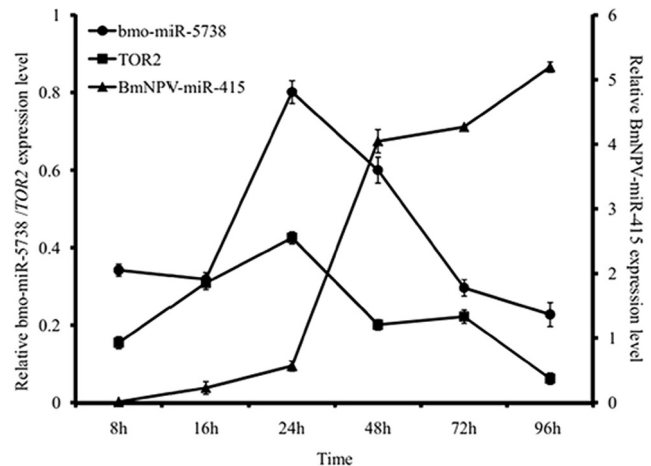


Figure 5 Expression of TOR2, bmo-miR-5738 and BmNPV-miR-415 in silk worm haemolymph.

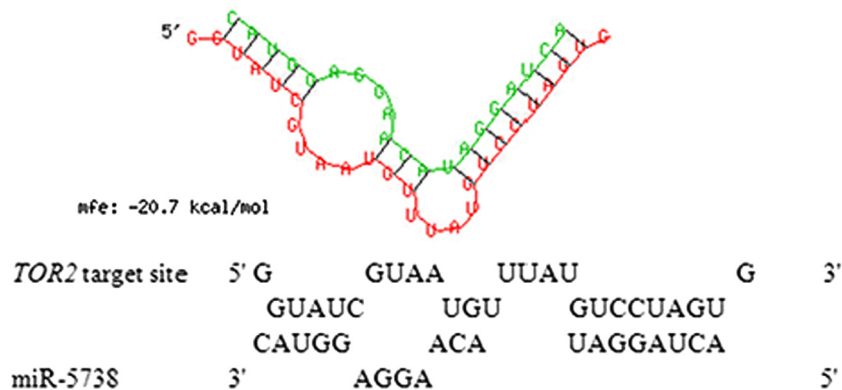


Figure 3 Diagram of miR-5738 targeting the 3' UTR sequence of TOR2. The miR-5738 sequence is indicated by green.

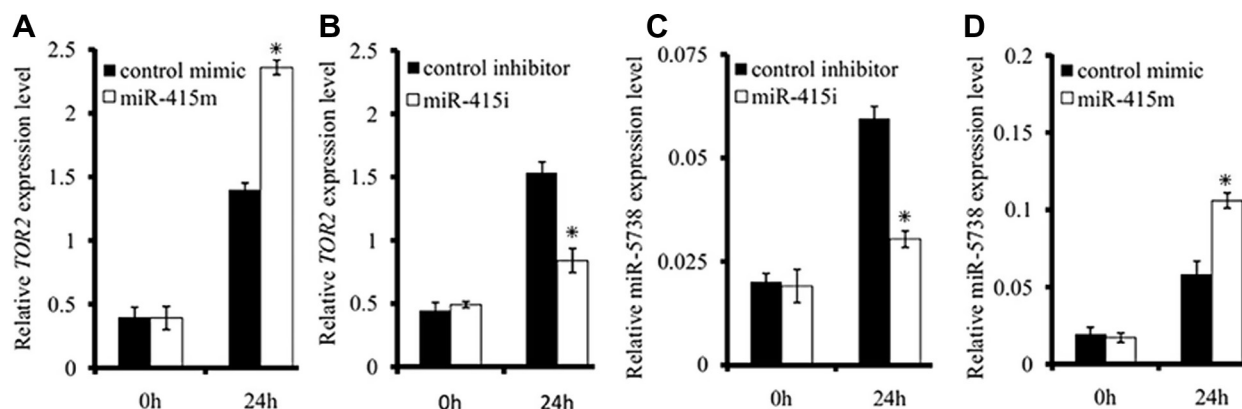


Figure 6 BmNPV miR-415 regulates *TOR2* and bmo-miR-5738 expression in silkworm after miR-415m or miR-415i injection. The asterisks (*) indicate the significant differences ($P < 0.05$) relative to the relevant control based on a two-tailed *t*-test. Each histogram bar represents the mean relative expression of the indicated transcripts (at least three replicates for each bar); the error bars represent the SD. MiR-5738 expression was normalised to U6, whereas the genes encoding *TOR2* were normalised to A3.

TOR2 via bmo-miR-5738. We injected a miR-415m or miR-415i on day 1 of the fifth instar larvae infected with BmNPV and determined the outcome in the haemolymph at 24 h post-treatment. A higher expression of miR-5738 and *TOR2* was detected in the miR-415m treatment group than in the control mimic-injected insects (Fig. 6A and C). The expression of both also decreased in the miR-415i treatment group (Fig. 6B and D).

4. Discussion

The computationally identified targets in the present study suggest that virus-encoded miRNAs probably have important roles in regulating a range of cellular activities for the easy establishment of the virus in the host (Adam et al., 2006; Stern et al., 2007).

Most of the previously studied miRNAs down-regulate the expression of target genes, but some miRNAs can up-regulate the expression of target genes. For example, the hepatitis C virus-induced up-regulation of miR-155 promotes hepatocarcinogenesis by activating *wnt* signalling (Zhang et al., 2012) and the down-regulation of miR-181a up-regulates sirtuin-1 (SIRT1) and improves hepatic insulin sensitivity (Zhou et al., 2012). BmNPV-miR-415 is located in *ORF37* of the BmNPV genome. *ORF37* encodes the occlusion-derived virus (ODV)-E66, which is a structural protein of the ODV. The secondary structure of the BmNPV-miR-415 precursor is a typical hairpin (Fig. S3). *TOR2* is a member of the phosphatidylinositol kinase-related kinase family and a key regulator which senses nutritional status. *TOR2* has a critical role in the regulation of growth, metabolism, development and survival at the cellular and organism levels (Hietakangas and Cohen, 2009; Wang and Proud, 2009). A recent study has shown that *TOR2* up-regulates either starvation or the moulting hormone 20-hydroxyecdysone (20E) (Zhou et al., 2010).

When infecting silkworms, BmNPV produces BmNPV-miR-415 to better infect the host cells. Meanwhile, bmo-miR-5738 is produced by the host cells upon the induction of BmNPV-miR-415. bmo-miR-5738 up-regulates the expression of *TOR2* by targeting its 3' UTR to ensure

the stability of the host environment. With the increased number of virus particles, the host immune system gradually collapses. Consequently, the expression of bmo-miR-5738 and *TOR2* begins to decline, whereas that of BmNPV-miR-415, along with the number of viral copies, continues to increase. Bmo-miR-5738 up-regulates the expression of *TOR2* through its 3' UTR. This regulatory effect is increased by BmNPV-miR-415. To verify this phenomenon, we treated silkworms with BmNPV and then injected miR415m or miR415i into the insects. The results showed that miR415m promotes the expression of *TOR2* and bmo-miR-5738, whereas miR415i significantly inhibits the expression of *TOR2* and bmo-miR-5738. Thus, we speculate that BmNPV-miR-415 promotes the expression of *TOR2* via bmo-miR-5738.

Viral miRNAs do not down-regulate the expression of host immune-related genes to avoid stimulating the immune system of the host during early viral infection. By contrast, these miRNAs promote *TOR2* expression to avoid any response from the immune system. When BmNPV invades the silkworm body, the miRNAs control the reproduction of the virus particle itself to avoid stimulating the host immune system as soon as possible, thereby achieving the goal of evading the immune system. We believe that this study serves as a useful reference for understanding the molecular mechanisms involved in host-pathogen interactions and enables future studies to identify methods of applying miRNAs against BmNPV infection in *B. mori*.

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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.sjbs.2015.09.020>.

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