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Bmo-miR-9a Down Regulates the Expression of *Bm-ase* Gene *in vitro*[1]

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Abstract—MicroRNAs (miRNAs) are a class of non-protein coding small RNAs of 18–24 nucleotides in length that regulate expression of genes at post-transcriptional levels and play multiple roles in biological processes. *Bm-ase* plays an important role in the course of nerve development of the silkworm, *Bombyx mori*. Bmo-miR-9a is a conservative miRNA. By using target prediction software RNA22 and RNAhybrid, we found a target site of Bmo-miR-9a in the 3'UTR of *Bm-ase* gene. To verify the regulation function of Bmo-miR-9a on the expression of *Bm-ase* gene, a Bmo-miR-9a over-expressing vector and *Bm-ase* 3'UTR fused firefly luciferase gene reporter plasmid were constructed, respectively. Then they were used to co-transfect the BmN cells. The result showed that luciferase activity in the co-transfected cells was suppressed compared with the control. A similar result was obtained when BmN cells were co-transfected with artificial synthetic Bmo-miR-9a mimics and *Bm-ase* 3'UTR fused luciferase reporter plasmid. These results suggest that Bmo-miR-9a can down regulate the expression of *Bm-ase* gene.

Keywords: Bombyx mori, microRNA, Bmo-miR-9a, Bm-ase, post-transcriptional regulation

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INTRODUCTION

MicroRNAs (miRNAs) are a class of non-protein coding small RNAs of 18–24 nucleotides in length that regulate the expression of target genes by binding on complementary sequences of target mRNAs [1–3]. The previous studies indicate that miRNAs are involved in lots of biological events, including development, hematopoietic lineage differentiation, organ formation, proliferation, apoptosis, host-viral interactions and tumorigenesis [4, 5]. Therefore, the research on miRNAs has become one of the hot fields of life sciences. So far, large numbers of miRNAs have been identified, but functions of few of them are well known, especially in the silkworm (*Bombyx mori*) miRNAs. It is crucial to identify target genes of miRNAs for understanding their biological functions and potential molecular mechanisms. Based on computational prediction approaches, the target gene of miRNA-13, *CG10222* was firstly verified with the luciferase reporter system in *Drosophila melanogaster* [6]. Now this luciferase reporter system is proven to be quite helpful to validate the target genes of miRNAs *in vitro* [7]. In our laboratory, expression vectors of miRNA-9a and miRNA-2b of the silkworm were constructed, and down regulation of the expression of *P25* gene by miRNA-2b was detected [8, 9].

The *achaete-scute* complex (AS-C) is a group of transcriptional factors involved in the formation of bristles *in Drosophila melanogaster*. In the silkworm, *Bombyx mori* there are four *achaete-scute* homologs (ASH), *Bm-ASH1, Bm-ASH2, Bm-ASH3* and *Bm-ase* [10]. These genes encode proteins containing bHLH domain, and genes of this family are involved in the regulation of the development of central nervous system and peripheral nervous system, myogenesis, blood corpuscles formation, sex determination, midgut development in metazoans [10]. *Bm-ase* is mainly expressed in the neural precursor cells, and it is a homolog of *asense*, which is essential for the development of neural precursor cells in *Drosophila melanogaster* [11]. According to the protein structure analysis, *Bm-ase* shares more than 70% identities with other insect Asense proteins within the bHLH region [10, 12]. During the embryonic development *Bm-ase* reveals two expression peaks, one on 3 d and the other on 6 d, and these two periods may be accompanied by two climaxes of nervous system development [10].

[1] The article is published in the original.

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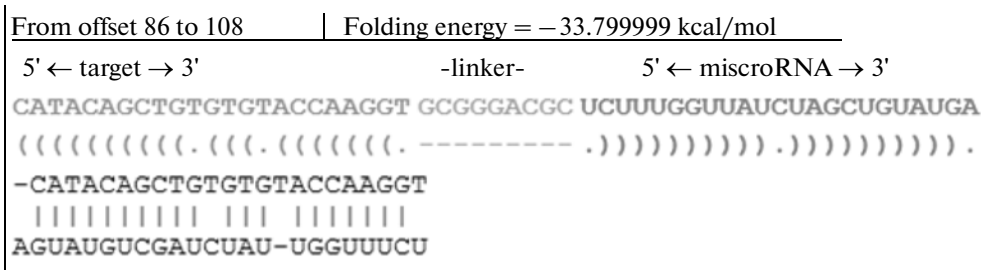
Result predicted by RNA22:



Result predicted by RNAhybrid:

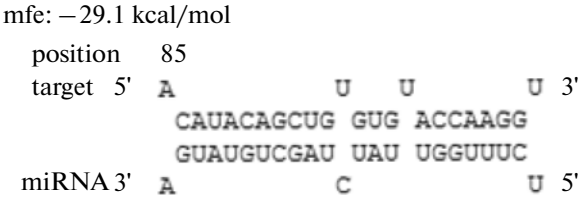


Fig. 1. Results predicted by miRNA target prediction software.

Through the target prediction software, *Bm-ase* was predicted to be one of the target genes of Bmo-miR-9a and experimental verification was conducted.

RESULTS

Prediction of Silkworm miRNAs Targeting 3'UTR of Bm-ase Gene

By using target prediction software RNAhybrid and RNA22, considering the scores and the perfect complementarities between the seed region of the miRNA (a 7-nucleotide sequence from base 2 to 8 in the 5'end of the miRNAs) and the target site, we found *Bm-ase* might be one of the target genes of Bmo-miR-9a (Fig. 1).

Construction of Expression Vectors

To confirm the expression vectors constructed, pGL3[FH-luc-ase3'UTR-SV40] and p[FH-EGFP-miR9a] were double digested with XbaI-FseI and BamH-HindIII respectively, and a *Bm-ase* 3'UTR fragment and a miR-9a fragment was identified, respectively (Fig. 2), implying that the expression vectors contain the interested gene fragments.

Regulation of Bm-ase Gene Expression by Bmo-miR-9a

In the transfection experiments p[FH-EGFP] (an EGFP gene was cloned into pCDNA3.0 plasmid and controlled by *BmFib-H* promoter) was served as a negative control for p[FH-EGFP-miR9a]. The pRL-CMV plasmid served as an internal control. Two independent experiments were carried out, and three replicates were set for each experiment. The results showed that at 48 h post infection the expression of pGL3[FH-luc-ase3'UTR-SV40] was down regulated to 87.4% by Bmo-miR-9a, compared to the negative controls (Fig. 3), suggesting that Bmo-miR-9a can suppress the expression of *Bm-ase* gene.

Regulation of Bm-ase Gene Expression by Artificial Bmo-miR-9a Mimics

The pGL3[FH-luc-SV40] and the "Bmo-miR-9a mimics negative control" served as negative controls for pGL3[FH-luc-ase3'UTR-SV40] and the artificial Bmo-miR-9a mimics, respectively. The pRL-CMV

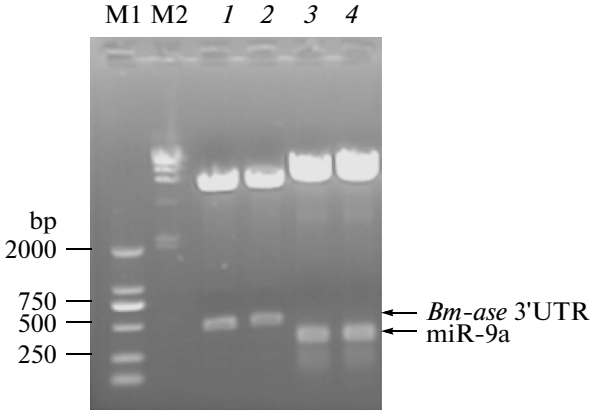


Fig. 2. Identification of the expression vectors pGL3[FH-luc-ase3'UTR-SV40] and p[FH-EGFP-miR9a]. M1—DL2000 DNA Marker; M2—λ-HindIII Marker; *1*, *2*—pGL3[FH-luc-ase3'UTR-SV40] digested by XbaI and FseI; 3, 4—p[FH-EGFP-miR9a] digested by BamHI and HindIII.

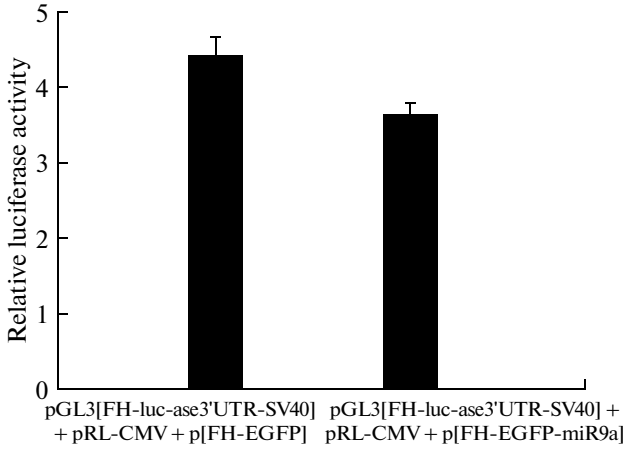


Fig. 3. Bmo-miR-9a expression vector inhibits the Bm-ase gene expression in BmN cells. Cells were co-transfected with p[FH-EGFP] or p[FH-EGFP-miR9a], firefly luciferase reporter containing *Bm-ase* 3'UTR (indicated as pGL3[FH-luc-ase3'UTR-SV40] on the X axis), and Renilla luciferase expression construct (as an internal control). Luciferase activity was assayed 48 h after transfection. Firefly luciferase values, normalized for Renilla luciferase, are presented. The data represent the mean values ± SD of 3 independent experiments done in duplicates.

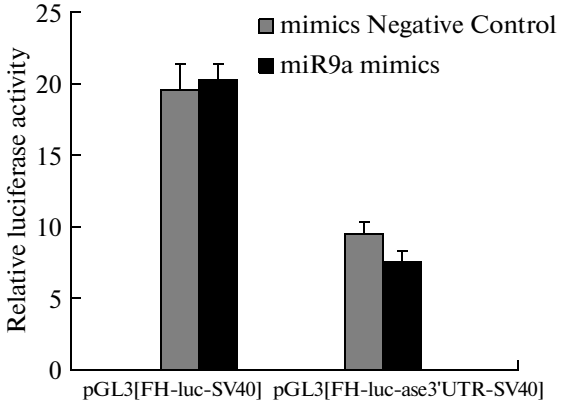


Fig. 4. Analysis of luciferase activity. Cells were co-transfected with mimics Negative Control or miR9a mimics, firefly luciferase reporter containing either *Bm-ase* 3'UTR or nothing (indicated as pGL3[FH-luc-ase3'UTR-SV40] or pGL3[FH-luc-SV40] on the X axis), and Renilla luciferase expression construct (as an internal control). Luciferase activity was assayed 48 h after transfection. Firefly luciferase values, normalized for Renilla luciferase, are presented. The data represent the mean values ± SD of 3 independent experiments done in duplicates.

served as an internal control. Firefly luciferase activity values, normalized by renilla luciferase activity, were presented as relative luciferase activity (firefly luciferase activity/renilla luciferase activity). The results revealed the relative luciferase activity of pGL3[FH-luc-ase3'UTR-SV40], in which *Bm-ase* 3'UTR was linked to the 3'end of *luciferase*, were suppressed compared to the relative luciferase activities of pGL3[FH-luc-SV40]. The effect of Bmo-miR-9a mimics on *Bm-ase* gene expression showed the same trend as the Bmo-miR-9a expression vector did (Fig. 4). The relative luciferase activities of cells co-transfected with pGL3[FH-luc-ase3'UTR-SV40] and Bmo-miR-9a mimics were suppressed down about 17.7% compared to those of cells co-transfected with pGL3[FH-luc-ase3'UTR-SV40] and mimics negative control ($P < 0.05$).

From the above results, a conclusion can be drawn that Bmo-miR-9a targets *Bm-ase* gene by interacting with its 3'UTR, i.e. *Bm-ase* is one of the target genes of Bmo-miR-9a, and Bmo-miR-9a can down regulate the expression of *Bm-ase* gene.

DISCUSSION

The miR-9a belongs to the mir-9 family. It is one of the miRNAs that is highly expressed in the mammalian brain and is 100% conservative at the nucleotide sequence from fly to human, suggesting that miR-9a plays an important role in brain development [13-15]. *Drosophila melanogaster* miR-9a regulates the development of sensory organ precursors via suppressing the expression of *sens* gene [16]. *Drosophila* mir-9a regulates wing development via fine-tuning of expression of *dLMO*, which is a transcription cofactor directly inhibiting the activity of *Apterous* [17]. All of these findings implied that a close relationship exists between miR-9a and nervous system development. In our study, Bmo-miR-9a down regulated the expression of *Bm-ase* which involved in neural development in the silkworm, *Bombyx mori* [9].

In our experiments, the basic expression level of pGL3[FH-luc-ase3'UTR-SV40] and pGL3[FH-luc-SV40] showed some difference when they co-transfected with artificial mimics negative control, respectively. This is probably due to the difference of structure and size of the fusion reporter gene in the vector caused by insertion of 3'UTR of *Bm-ase* gene.

The luciferase activities from BmN cells co-transfected with different kinds of exogenous miRNAs were different, i.e. the artificial synthetic miRNAs exerted much stronger effect on the target reporter than that of over-expression of the recombinant plasmids. This was possibly caused by different concentration of miRNA in BmN cell, since the transfection efficiencies of artificial Bmo-miR-9a mimics and Bmo-miR-9a expression vector p[FH-EGFP-miR9a] are different. On the other hand, after transfection of BmN cells with p[FH-EGFP-miR9a] the amount of Bmo-miR-9a increased gradually and its concentration in BmN cells was not as high as in the cells transfected with artificial Bmo-miR-9a mimics at 48 hpt.

The experimental results revealed that Bmo-miR-9a could repress the firefly luciferase activity of the target reporter, pGL3[FH-luc-ase3'UTR-SV40] *in vitro*, indicating that Bmo-miR-9a could down regulate the

List of primers

Primers	Sequences, 5'–3'
Fib-H-F	AGATCTGCAGTATGTCGTGCTAA
Fib-H-R	CCATGGTGAGAGTTGGAACCGAAC
EGFP-F	CCATGGGCGTGAGCAAGGGCGAGGAGCT
EGFP-R	AAGCTTTTACTTGTACAGCTCGTCCA
Bm-ase 3'UTR-F	TCTAGATAATATTTATGAACAATAATTAC
Bm-ase 3'UTR-R	GGCCGGCCAAATTTACAAAATTTATTACA
miR-9a-F	AAGCTTAATTGAATAGAAAAAAAATATAAC
miR-9a-R	GGATCCGACAGATATAGGGAAAGAG

Note: the underlined parts indicate the restriction sites of HindIII, BamHI, NcoI, BglII, XbaI and FseI.

expression of *Bm-ase* gene. But Bmo-miR-9a is not the only factor which regulates the expression of *Bm-ase* gene, to understand *Bm-ase* gene regulation further study need to be done.

EXPERIMENTAL

Materials

Domesticated silkworm (*Bombyx mori*) strain P50, provided by the Sericultural Research Institute, Chinese Academy of Agricultural Sciences. Plasmid pcDNA3.0 (Invitrogen), pRL-CMV (contains a renilla luciferase gene), pGL3-Basic (Promega), EGFP-N3 (Clontech) (contains *egfp* gene) and BmN cells were preserved in the Key Laboratory of Silkworm and Mulberry Genetic Improvement, Ministry of Agriculture, Sericultural Research Institute, Chinese Academy of Agricultural Sciences.

The Bmo-miR-9a mimics and negative control were synthesized by Guangzhou RiboBio Co., Ltd. The miRNA mimics negative control was a kind of miRNA of *Caenorhabditis elegans*, Bioinformatic analysis showed that the negative control had the least homology with all of the miRNAs in miRBase database (http://www.mirbase.org/). Its sequence was as follows: UUUGUACUACACAAAAGUACUG.

Prediction of miRNAs Targets

The sequences of silkworm mature miRNAs and their pre-miRNAs were downloaded from the miRBase Sequence Database (http://www.mirbase.org/; released on 3 November, 2011). The *Bm-ase* mRNA sequence and *BmFib-H* promoter sequence were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/gene/). RNA22 (http://cbcsrv.watson.ibm.com/rna22.html) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid) softwares were used to predict the potential targets of miRNAs, and the parameters utilized were described as defaults. The potential targets of miRNAs were identified with perfect complementarities between the seed region of the miRNA (a 7-nucleotide sequence at base 2 to 8 in the 5' end of the miRNAs) and the 3'-UTR of the target mRNAs, and with less than –25.0 kcal/mol free energy of the secondary structure of the miRNA/mRNA duplex.

Design of PCR Primers

According to the sequences information, the PCR primers were designed and synthesized for *BmFib-H* promoter, EGFP ORF, *Bm-ase* 3'UTR, and the 302-bp DNA fragment containing miR-9a precursor, respectively as listed in the table.

DNA Extraction

The silkworm genomic DNA was isolated from 0.5 g posterior silk gland according to the method described previously [18].

Amplification of Gene Fragments

The gene fragments were amplified from the genomic DNA of the silkworm or plasmid by PCR.

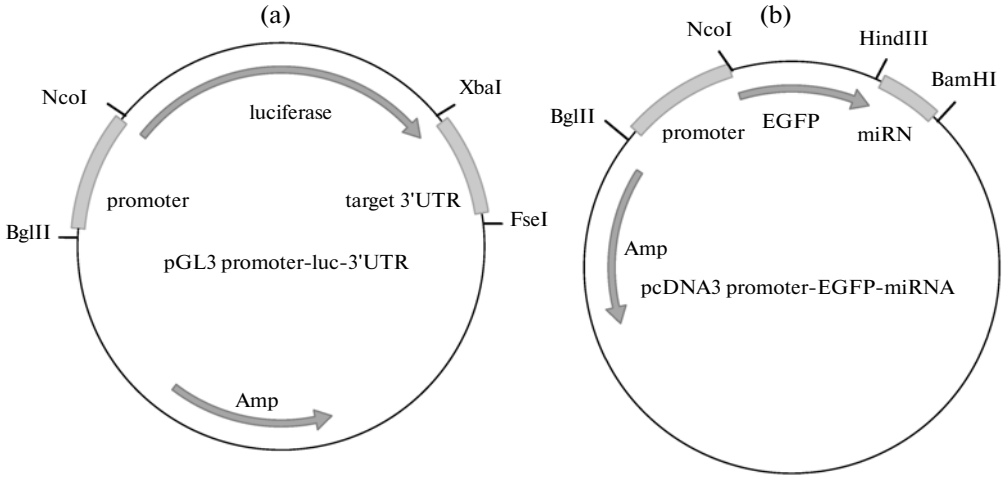


Fig. 5. (a) The structure of pGL3[FH-luc-3'UTR-SV40] vector. (b) The structure of miR-9a expression vector p[FH-EGFP-miRNA].

The PCR cycling conditions were as follows: initial denaturation at 94°C for 4 min, 25 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, and final extension at 72°C for 5 min. The PCR products were separated by electrophoresis in 1% agarose gel. After purification, the gene fragments containing *BmFib-H* promoter, EGFP ORF, *Bm-ase* 3'UTR and Bmo-miR-9a precursor were cloned into pMD18-T vector and sequenced, respectively.

Construction of Expression Vectors

The pGL3-Basic plasmid was used for construction of expression vectors. The 3'UTR fragment of *Bm-ase* gene was inserted into the pGL3-Basic plasmid downstream the stop codon of firefly *luciferase (luc)* gene, which was driven by *BmFib-H* promoter, for constructing of pGL3[FH-luc-ase3'UTR-SV40] vector (Fig. 5a).

The DNA sequence including mature miR-9a and its flanking regions (±100 bp) were cloned into pCDNA3.0 plasmid downstream the *EGFP* gene, which was controlled by *BmFib-H* promoter, for constructing of Bmo-miR-9a expression vector p[FH-EGFP-miR9a] (Fig. 5b).

Cell Culture and Transfection

The method for routine BmN cell culture was as described previously [19, 20]. Cells were seeded into 24 wells plate (600 μL for each) at a density of about 5×10^5 cells/mL and cultured for 24 h. Before transfection, the medium was removed and the cells were washed twice with serum-free TC-100 medium. Each well of cells was transfected with 0.1 μg reporter plasmid pGL3[FH-1uc-ase-3'UTR-SV40], 10 ng internal control plasmid pRL-CMV, 0.4 μg miRNA expressing plasmid (or 0.05 nmol miRNA mimics), respectively via lipofectamine 2000 (Invitrogen) as described in the manufacturer's manual (lipofectamine 2000; Invitrogen). Three replicates were done for each experiment.

Transient Expression Assay

At 48 h post transfection (hpt), cells were harvested by centrifugation at 9000 g for 5 min at 4°C, washed once in phosphate-buffered saline and lysed in 150 μL of passive lysis buffer (Promega). The supernatants were used for the luciferase assay. Assays for luciferase were performed with a Luminometer 20/20 (Turner Biosystems Inc., Sunnyvale, CA; 2 s delay and read at 10th s) as the relative luminescence unit (RLU) per 4 μL lysates [21] by using a Dual-Luciferase Reporter Assay System kit (Promega).

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