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bmo-miR-0001 and bmo-miR-0015 down-regulate expression of *Bombyx mori* fibroin light chain gene in vitro*

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Abstract: Based on bioinformatic analysis, we selected two novel microRNAs (miRNAs), bmo-miR-0001 and bmo-miR-0015, from high-throughput sequencing of the *Bombyx mori* larval posterior silk gland (PSG). Firstly, we examined the expression of bmo-miR-0001 and bmo-miR-0015 in 12 different tissues of the 5th instar Day-3 larvae of the silkworm. The results showed that the expression levels of both bmo-miR-0001 and bmo-miR-0015 were obviously higher in the PSG than in other tissues, implying there is a spatio-temporal condition for bmo-miR-0001 and bmo-miR-0015 to regulate the expression of *BmFib-L*. To test this hypothesis, we constructed pri-bmo-miR-0001 expressing the plasmid pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0001-SV40] and pri-bmo-miR-0015 expressing the plasmid pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0015-SV40]. Finally, the BmN cells were harvested and luciferase activity was detected. The results showed that luciferase activity was reduced significantly ($P<0.05$) in BmN cells co-transfected by pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0001-SV40] or pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0015-SV40] with pGL3.0 [*A*3-*luc*-*Fib*-*L*-3'UTR-SV40], suggesting that both bmo-miR-0001 and bmo-miR-0015 can down-regulate the expression of *BmFib-L* in vitro.

Key words: *Bombyx mori*, MicroRNA, bmo-miR-0001, bmo-miR-0015, *BmFib-L*, Regulation of expression
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1 Introduction

MicroRNAs (miRNAs) are small non-coding RNA molecules about 19 to 22 nucleotides long, which mostly inhibit the expression or translation of target genes by complementary base pairing to the target mRNA 3' untranslated region (3'UTR) (Lee *et al.*, 2004). They are involved in a lot of biological processes, including development, hematopoietic lineage differentiation, organ formation, proliferation, apoptosis, host viral interactions, and tumorigenesis (Lim *et al.*, 2003; Poy *et al.*, 2004; Bushati and Cohen, 2007; Yuan *et al.*, 2014). Research on miRNAs has been conducted on a range of organisms including animals (Dai *et al.*, 2014; Lu *et al.*, 2014), plants (Mallory and Vaucheret, 2006), microbes (Liu *et al.*, 2016), and viruses (Jopling *et al.*, 2005). Studies on miRNAs will provide a theoretical basis for post-transcriptional regulation of genes and benefit our understanding of gene regulatory mechanisms.

The silkworm (*Bombyx mori*) is an insect that undergoes complete metamorphosis. It not only has the biological traits common to other insects, but also has its own characteristic properties making it an

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excellent model for the study of insect genetics and molecular biology (Xia *et al.*, 2004; 2009). With the completion of sequencing of the silkworm genome, the study of *B. mori* miRNA has entered a new development stage that has led to the discovery of novel miRNAs (Yu *et al.*, 2008; 2009; Cai *et al.*, 2010; Yang *et al.*, 2012), prediction of miRNA targets (He *et al.*, 2008; Zeng *et al.*, 2009; Huang *et al.*, 2012; Chen *et al.*, 2013), and identification and expression of miRNAs (Zhang *et al.*, 2009; Liu *et al.*, 2010; Li *et al.*, 2014). The mechanism of silk protein biosynthesis has been a popular focus of research among a large numbers of studies.

The silk glands, a special organ in the silkworm that synthesizes and secretes the silk protein, can be divided into the anterior silk gland (ASG), middle silk gland (MSG), and posterior silk gland (PSG) according to their morphological specificity and function (Inoue *et al.*, 2000). The ASG is a catheter of silk protein with no function in silk protein biosynthesis and the MSG mainly synthesizes and secretes sericin proteins, whereas the PSG synthesizes and secretes silk fibroin, which accounts for more than 70% of the total silk protein. Fibroin comprises the fibroin heavy chain protein (Fib-H), fibroin light chain protein (Fib-L), and P25/fibrohexamerin protein (P25) (Sprague, 1975; Yamaguchi *et al.*, 1989). Studies have shown that *B. mori* miRNAs are involved in regulation of fibroin gene expression. Four bmo-miRNAs have been predicted to have potential binding sites in the *BmFib-L* gene and might play important roles in the regulation of silk protein biosynthesis (Cao *et al.*, 2008). The miRNA bmo-miR-2b also regulates the expression of *BmP25* (Huang *et al.*, 2011). Both bmo-miR-965 and bmo-miR-1926 inhibit the expression of *BmFib-L* in vitro (Huang *et al.*, 2012). However, bmo-miR-2739 has been shown to up-regulate the expression of *BmFib-H* (Song *et al.*, 2014).

In the previous high-throughput sequencing of PSG small RNAs in our laboratory, 35 novel bmo-miRNAs were obtained, among which two, bmo-miR-0001 and bmo-miR-0015, are predicted to function in regulating the expression of *BmFib-L*. To validate this prediction, the expressions of bmo-miR-0001, bmo-miR-0015, and their potential target gene *BmFib-L* were examined by reverse transcription polymerase chain reaction (RT-PCR) at the mRNA level. The regulatory function of these two miRNAs was also tested using a dual luciferase reporter (DLR) system in BmN cells.

2 Materials and methods

2.1 Materials

The domesticated silkworm (*B. mori*) used in this experiment was of the strain P50, provided by the Sericultural Research Institute, Chinese Academy of Agricultural Sciences (CAAS; Zhenjiang, China). Plasmids pcDNA3.0 (Invitrogen, Shanghai, China), pRL-CMV (containing a Renilla luciferase gene), and pGL3.0 [*A3-luc-Fib-L*-3'UTR-SV40], and BmN cells were preserved or constructed by the Key Laboratory of Silkworm and Mulberry Genetic Improvement, Ministry of Agriculture, China. Restriction enzymes, T4 DNA ligase, PCR reagents, and a reverse transcription kit were purchased from TaKaRa (Dalian, China). Primers were synthesized by Sangon Biotech (Shanghai, China). Fetal bovine serum (FBS) and TC-100 cell culture medium were purchased from Invitrogen. Cell perfect transfection reagent was purchased from UcallM (Wuxi, China). A Dual-Luciferase® Reporter (DLR™) Assay System kit was purchased from Promega (Madison, USA).

2.2 Methods

2.2.1 Screening of *B. mori* miRNAs regulating *BmFib-L* expression

To screen *B. mori* miRNAs for those that may regulate the expression of *BmFib-L*, the *BmFib-L* sequence from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/gene) and sequences of *B. mori* mature miRNAs from Solexa sequencing were analyzed by RNAhybrid software (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid). The potential targets of miRNAs were identified as having perfect sequence complementarity between the seed region of the miRNA (a 7-nucleotide sequence from bases 2 to 8 at the 5' end of the miRNAs) and the 3'UTR of target (*BmFib-L*) mRNAs, and less than −20.0 kcal/mol (1 kcal/mol=4.182 kJ/mol) free energy in the secondary structure of the miRNA/mRNA duplex (Huang *et al.*, 2010).

2.2.2 Cloning of novel miRNAs and prediction of the secondary structures of their precursors

Total RNA was extracted from the PSG of Day-3 5th instar larvae using TRIzol (Invitrogen) according to the manufacturer's instructions. The complementary DNAs (cDNAs) were synthesized from total RNA using miRNA specific stem-loop primers according to the method of Kramer (2011). Subsequently, PCR was carried out with the following cycling conditions: initial denaturation at 94 °C for 5 min, 34 cycles of 94 °C for 30 s, 55 °C for 25 s, and 72 °C for 30 s, and final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis in 4% (0.04 g/ml) agarose gels. After purification, the gene fragments were cloned into a pMD18-T vector and sequenced. Using ClustalX 1.83, multiple sequence alignment software was applied for further confirmation of sequencing, and secondary structures were predicted using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The sequences of the primers are listed in Table 1.

2.2.3 Detection of expression by RT-PCR

To investigate the possibility of spatio-temporal effects on the predicted function of bmo-miRNAs in regulating the expression of *BmFib-L*, the expression levels of potential bmo-miRNAs and *BmFib-L* in the silk gland of the silkworm larvae were analyzed. Firstly, total RNAs were extracted from different tissues including the head, epidermis, fat body, Malpighian tubule, ASG, MSG, PSG, trachea, testis, ovary, midgut, and haemolymph of the 5th instar Day-3 larva using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNAs were synthesized from total RNAs using a cDNA reverse transcription kit (TaKaRa). Subsequently, PCR was carried out and the products were detected as described above and photographed under UV light. Three parallel experiments were carried out and the *B. mori* U6 gene served as an internal control. Finally, the relative expression levels of the potential bmo-miRNAs and *BmFib-L* in different tissues were determined using Gel-Pro Analyzer software (Media Cybernetics, USA).

2.2.4 Construction of expression vectors

To validate the function of potential bmo-miRNAs in the regulation of *BmFib-L* expression in vitro, bmo-miRNA expression vectors were constructed as described previously (Huang *et al.*, 2011). The luciferase gene (*luc*) fused *BmFib-L* 3'UTR expression vectors pGL3.0 [*A3-luc-Fib-L*-3'UTR-SV40] constructed previously in our laboratory (Huang *et al.*, 2012) were used. The nucleotide sequences of precursors of the potential bmo-miRNAs and their up- and down-stream flanking regions were downloaded from SilkBase (http://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi). Then primers designed using Oligo 7 software (molecular Biology Insights, Inc., Cascade, CO, USA) were constructed for PCR of the potential bmo-miRNAs and the PCR products were cloned into the pET-18 vector for sequence determination. The pET-18-pri-miR-0001 and pET-18-pri-miR-0015 constructs were double digested with *Hin*dIII and *Bam*HI, separately. The nucleotide sequences of the mature bmo-miRNAs, bmo-miR-0001, and bmo-miR-0015, and their flanking regions (±100 bp) were cloned into the pCDNA3.0 plasmid downstream of the *egfp* gene controlled by the *B. mori* nucleopolyhedrovirus (BmNPV) *ie*1 promoter for construction of the bmo-miRNA expression vectors pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0001-SV40] and [*ie*1-*egfp*-pri-bmo-miR-0015-SV40]. The structure of these constructs was confirmed by sequencing (Sangon Biotech).

2.2.5 Cell culture, transfection, and transient expression assay

The method of Zhou *et al.* (2002) was used for routine BmN cell culture. Cells were seeded into 12-well plates (600 μl for each) at a density of about 5×10^5 cells/ml and cultured for 24 h (Song *et al.*, 2013). Before transfection, the medium was removed and the cells were washed twice with serum-free TC-100 medium. In the treatment group, cells in each well were co-transfected with a 1.6-μg mixture of DNAs including 0.7 μg pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0001-SV40] or pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0015-SV40], 0.7 μg pGL3.0 [*A3-luc-Fib-L*-3'UTR-SV40], and 0.2 μg internal plasmid pRL-CMV via PerFect Cellfectin reagent (UcallM), according to the manufacturer's manual (PerFect; UcallM). In the control group, cells in each well were co-transfected with 1.6 μg of a mixture DNAs including 0.7 μg pcDNA3.0 [*ie1-egfp*-SV40], 0.7 μg pGL3.0 [*A3-luc-Fib-L*-3'UTR-SV40], and 0.2 μg pRL-CMV. There were three replicates for each experiment.

Before harvesting, cells were observed under an inverted fluorescence microscope. At 72 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 luciferase activity assay using a Luc assay kit (Promega) in a GloMax® 20/20 Luminometer (Turner Biosystems Inc., Sunnyvale, CA, USA; 2 s delay and read at 10 s) as the relative luminescence unit (RLU) (Zhao *et al.*, 2007). The activity of firefly was normalized by the Renilla luciferase activity of pRL-CMV.

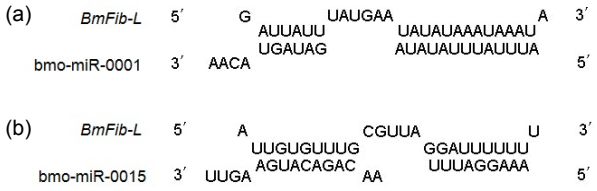

Fig. 1 Prediction of the binding sites of bmo-miR-0001 and bmo-miR-0015 in *BmFib-L* mRNA using RNAhybrid software

(a) Prediction of the binding site of bmo-miR-0001 in *BmFib-L* mRNA. The folding energy was −16.3 kcal/mol. The initiation binding site at the target gene was the 43rd base of the 3'UTR; (b) Prediction of the binding site of bmo-miR-0015 in *BmFib-L* mRNA. The folding energy was −15.2 kcal/mol. The initiation binding site at the target gene was the 5th base of the 3'UTR

3 Results

3.1 Prediction of novel *B. mori* miRNAs targeting *BmFib-L* 3'UTR

Target prediction software RNAhybrid was used to assess the scores and the complementarity between the miRNA seed regions and the target site. Among 35 novel *B. mori* miRNAs obtained from previous Solexa sequencing (Song *et al.*, 2015), bmo-miR-0001 and bmo-miR-00015 were predicted to have the potential to target *BmFib-L* 3'UTR. Moreover, pri-bmo-miR-0001 binds *BmFib-L* 3'UTR starting from the 43rd base, while pri-bmo-miR-0015 starts from the 5th base, suggesting that bmo-miR-0001 and bmo-miR-00015 function independently (Fig. 1).

3.2 Cloning of candidate miRNAs and prediction of secondary structures of their precursors

For each miRNA, RT-PCR was performed with the primers above using the total RNA of the 5th instar Day-3 larvae of the silkworm as a template, and a band of about 60–80 bp was separated by agarose gel electrophoresis, consistent with expectations (Fig. 2a). Sequencing results revealed that the nucleotide sequences of the two cloned miRNAs were the same as those from Solexa sequencing (Figs. 2b and 2c): bmo-miR-0001, AUU UAU UUA UAU AGA UAG UAC AA; bmo-miR-0015, AAA GGA UUU AAC AGA CAU GAA GUU. The secondary structures of their precursors were typical of miRNA precursors, with stem-loops (Fig. 3). The free energies of the bmo-miR-0001 and bmo-miR-0015 precursors were −18.10 and −25.80 kcal/mol, respectively.

3.3 Expression of bmo-miR-0001 and bmo-miR-0015 and their target gene *BmFib-L*

The RT-PCR results showed that in the 12 tissues tested bmo-miR-0001 expression was detected only in the PSG (Fig. 4a). In contrast, bmo-miR-0015 expression was detected not only throughout the silk

Table 1 Primers used in the experiments

Primer		Sequence (5'→3')
bmo-miR-0001	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTGTAC
	Forward	GCCCGATTTATTTATATAGATAGT
	Reverse	GTGCAGGGTCCGAGGT
bmo-miR-0015	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTTC
	Forward	GTGCAGGGTCCGAGGT
	Reverse	CCCAAGCTTAAGTCCATTCCAATAGC
U6	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACG
	Forward	CCTGCGCAAGGATGAC
	Reverse	GTGCAGGGTCCGAGGT
pri-bmo-miR-0001	Forward	CGGGATCCAGCTCACCTACATGTTA
	Reverse	CCCAAGCTTAAGTCCATTCCAATAGC
pri-bmo-miR-0015	Forward	CGGGATCCACAAGTACGCAAAAGCT
	Reverse	CCCAAGCTTAAGGCGCACCATCAATT

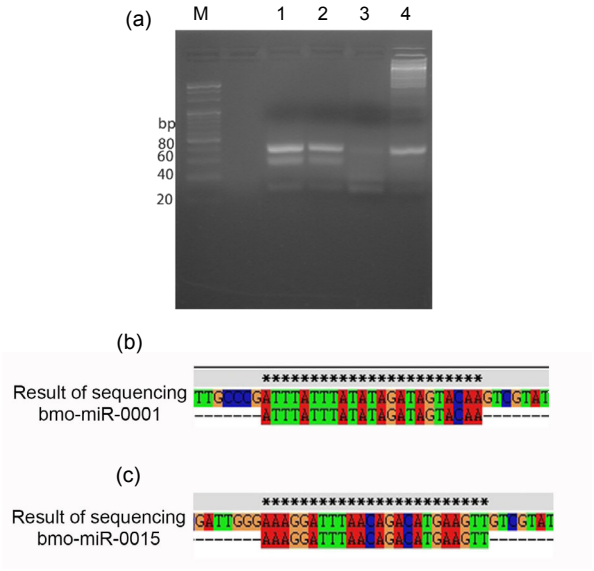

Fig. 2 Electrophoresis pattern of RT-PCR products and multiple sequence alignments of bmo-miR-0001 and bmo-miR-0015 by ClustalX 1.83

(a) Electrophoresis pattern of RT-PCR products of bmo-miR-0001 and bmo-miR-0015 (M: 20 bp marker; 1: bmo-miR-0001; 2: bmo-miR-0015; 3: bmo-miR-26* (control); 4: U6); (b) Sequence alignment result of bmo-miR-0001; (c) Sequence alignment result of bmo-miR-0015

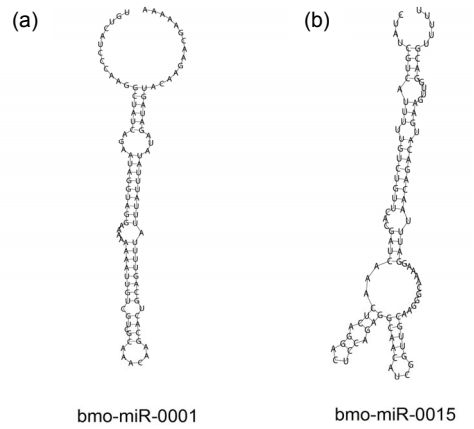

Fig. 3 Stem-loop structures of bmo-miR-0001 and bmo-miR-0015 predicted by RNAfold

(a) Secondary structure of the bmo-miR-0001 precursor; (b) Secondary structure of the bmo-miR-0015 precursor

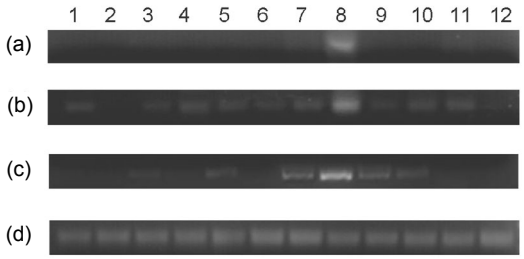

Fig. 4 Expression analyses of bmo-miR-0001, bmo-miR-0015, *BmFib-L*, and U6 in different tissues of 5th instar Day-3 larvae of *B. mori*

(a) Expression of bmo-miR-0001; (b) Expression of bmo-miR-0015; (c) Expression of *BmFib-L*; (d) Expression of internal gene U6. 1, head; 2, epidermis; 3, fat body; 4, Malpighian tubule; 5, testis; 6, anterior silk gland; 7, middle silk gland; 8, post silk gland; 9, trachea; 10, ovary; 11, midgut; 12, haemolymph

glands (ASG, MSG, and PSG), but also in the head, fat body, Malpighian tubule, testis, trachea, ovary, and midgut, but the expression level was highest in the PSG (Fig. 4b). *BmFib-L* expression was detected not only in the PSG, but also in the fat body, testis, MSG, trachea, and ovary (Fig. 4c). This is consistent with the results of Sun *et al.* (2009) and is known as "leaking expression" (Wang and Lu, 2006). The above experimental results show that spatio-temporal conditions affect the regulation of the expression of *BmFib-L* by bmo-miR-0001 and bmo-miR-0015. Together with the target prediction result, we deduced that bmo-miR-0001 and bmo-miR-0015 may be involved in the regulation of *BmFib-L* expression in *B. mori* larvae.

3.4 Validating the roles of bmo-miR-0001 and bmo-miR-0015 in regulating the expression of *BmFib-L*

3.4.1 Construction of expression vectors

The expression vectors pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0001-SV40] for miR-0001 and pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0015-SV40] for miR-0015 were successfully constructed to contain the BmNPV *ie*1 promoter and an *egfp* reporter gene. The inserted gene fragments were tested by electrophoresis in agarose gels after double digestion with *Hin*dIII and *Bam*HI (Fig. 5).

3.4.2 Regulation of *BmFib-L* expression by bmo-miR-0001 and bmo-miR-0015 in BmN cells

In the treatment group, BmN cells were co-transfected with a mixture of pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0001-SV40] or pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0015-SV40], pGL3.0 [*A3-luc-Fib-L*-3'UTR-SV40], and pRL-CMV. In the control group, BmN cells were co-transfected with a mixture of pcDNA3.0 [*ie*1-*egfp*-SV40], pGL3.0 [*A3-luc-Fib-L*-3'UTR-SV40], and pRL-CMV.

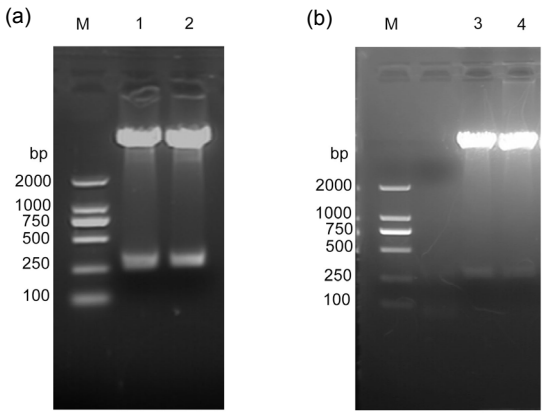

Fig. 5 Double enzyme digestion of recombinant plasmid pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0001-SV40] and pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0015-SV40] with *Hin*dIII and *Bam*HI

(a) Identification of *ie*1-*egfp*-pri-bmo-miR-0001-SV40 structure (M: DL 2000 DNA marker; 1, 2: pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0001-SV40] after double digestion with *Hin*dIII and *Bam*HI); (b) Identification of *ie*1-*egfp*-pri-bmo-miR-0015-SV40 structure (M: DL 2000 DNA marker; 3, 4: pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0015-SV40] after double digestion with *Hin*dIII and *Bam*HI)

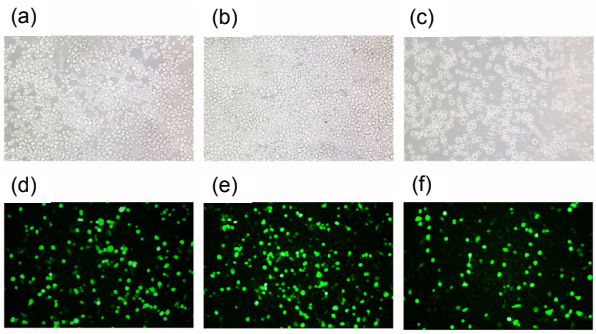

Fig. 6 Expression of enhanced green fluorescent protein in BmN cells transfected with recombinant plasmids

(a) pcDNA3.0 [*ie*1-*egfp*-SV40]+pGL3.0 [*A*3-*luc*-*Fib*-*L*-3'UTR-SV40]+pRL-CMV, bright light; (b) pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0001-SV40]+pGL3.0 [*A*3-*luc*-*Fib*-*L*-3'UTR-SV40]+pRL-CMV, bright light; (c) pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0015-SV40]+pGL3.0 [*A*3-*luc*-*Fib*-*L*-3'UTR-SV40]+pRL-CMV, bright light; (d) pcDNA3.0 [*ie*1-*egfp*-SV40]+pGL3.0 [*A*3-*luc*-*Fib*-*L*-3'UTR-SV40]+pRL-CMV, fluorescence; (e) pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0001-SV40]+pGL3.0 [*A*3-*luc*-*Fib*-*L*-3'UTR-SV40]+pRL-CMV, fluorescence; (f) pcDNA3.0 [*ie*1-*egfp*-pri-bmo-miR-0015-SV40]+pGL3.0 [*A*3-*luc*-*Fib*-*L*-3'UTR-SV40]+pRL-CMV, fluorescence

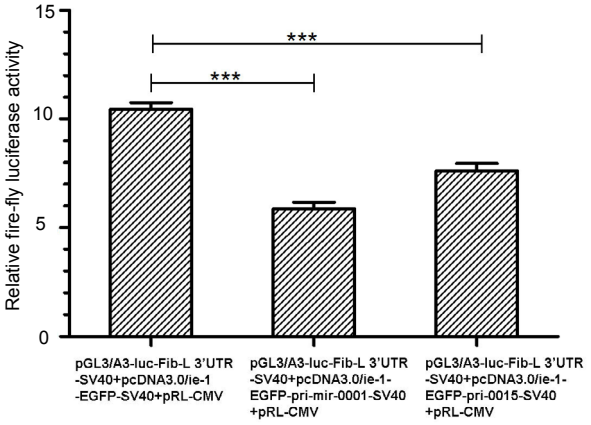

Fig. 7 Effect of bmo-miR-0001 and bmo-miR-0015 expression on luciferase activity in transfected BmN cells

Data represented as the mean±standard deviation (SD) from three independent experiments. *** represents $P<0.001$ in unpaired *t*-tests

Observations were carried out under an inverted fluorescence microscope. Results showed that recombinant plasmids had been transfected into cells and the majority of cells emitted green fluorescence (Fig. 6). Cells were then harvested and luciferase activity was assayed using a Luc assay kit (Promega) in a Luminometer 20/20, as described above. The results showed that luciferase activity in the two experimental groups decreased to 60% and 69%, respectively, of that of the control group. The differences between the treatment groups and the control groups were significant ($P<0.05$; Fig. 7), indicating that bmo-miR-0001 and bmo-miR-0015 can down-regulate the expression of *BmFib-L* in vitro.

From the above results, we conclude that *BmFib-L* is one of the target genes of bmo-miR-0001 and bmo-miR-0015, and that both bmo-miR-0001 and bmo-miR-0015 can down-regulate the expression of the *BmFib-L* gene by interacting with its 3'UTR of mRNA in vitro.

4 Discussion

miRNAs participate in a variety of biological activities, including cell proliferation, embryonic development, individual growth, occurrence and development of disease, and apoptosis (Johnston and Hobert, 2003; Lim *et al.*, 2003; Poy *et al.*, 2004). There are 563 silkworm mature miRNA sequences registered in the MiRBase (http://www.mirbase.org). Studies have shown that *B. mori* miRNAs play essential roles in silkworm development and growth. At least four bmo-miRNAs, miRNA-33, miRNA-190,

miRNA-276, and miRNA-7, participate in the regulation of *BmFib-L* expression (Cao *et al.*, 2008). miR-965 and miR-1926 have been shown to down-regulate the expression of *BmFib-L* in vitro (Huang *et al.*, 2012).

Based on Solexa sequencing results, we obtained two novel candidate bmo-miRNAs, bmo-miR-0001 and bmo-miR-0015, complementary to the 3'UTR of *BmFib-L* mRNA using RNAhybrid target prediction software. The DLR system was applied to validate the role of these two miRNAs in regulating the expression of *BmFib-L* in cells. For the first time, we reported that bmo-miR-0001 and bmo-miR-0015 down-regulate the expression of *BmFib-L* in vitro. This result is similar with that described by Huang *et al.* (2012) and is consistent with the finding that the majority of miRNAs inhibit expression of their target gene by binding to its 3'UTR (Fabian *et al.*, 2010). These results will not only enrich the database of silkworm miRNAs, but also provide new experimental data for further study of miRNA function and the regulatory mechanism of silk protein biosynthesis.

B. mori silk fibroin comprises BmFib-H, BmFib-L, and BmP25 proteins (Sprague, 1975; Couble *et al.*, 1983; Yamaguchi *et al.*, 1989), which are produced abundantly in the silk glands of 5th instar larvae (Wang *et al.*, 2011). However, in this experiment bmo-miR-0001 and bmo-miR-0015 down-regulated the expression of the *BmFib-L* gene. Recent studies have identified a competing endogenous RNA (ceRNA) gene regulatory mechanism, in which ceRNAs serve as miRNA absorbing sponges to vie with miRNAs by sharing miRNA response elements (MREs) in mRNAs (Khan *et al.*, 2009; Ebert and Sharp, 2010). These ceRNAs act as modulators of miRNAs by lowering the available miRNA levels for the target mRNA, resulting in increased translation (Sen *et al.*, 2014).

BmN cells used in this experiment were obtained from *B. mori* ovarian tissue instead of silk gland cells and might lack corresponding interference endogenous miRNAs. This may have provided an opportunity for the exogenous miRNAs (bmo-miR-0001 and bmo-miR-0015) to bind the mRNA of the target gene (*BmFib-L*), resulting in down-regulation of target gene expression. However, it is also highly likely that there are real differences between expression in the silkworm larvae and in the in vitro gene model. Therefore, further study is needed on the regulation of *BmFib-L* expression by bmo-miR-0001 and bmo-miR-0015 in vivo via over expression of silkworm miRNAs following infection by a recombinant *B. mori* baculovirus expression vector, or injection of synthetic miRNA analogues or antisense RNA of miRNAs into the 5th instar larvae.

The expression and regulation of genes producing silk protein are very complex. However, Shalgi *et al.* (2007) have proposed the existence of hundreds of "target hub" genes, each potentially subject to massive regulation by dozens of miRNAs. Therefore, the study of individual miRNAs is insufficient to uncover the complicated and precise regulatory mechanisms of silk protein biosynthesis. Studies of other transcription factors and more information about the regulation of silk protein biosynthesis by miRNAs are needed. Besides binding at the 3'UTR of target mRNAs, miRNAs can bind at the 5'UTR of target mRNAs and usually promote expression or translation of the target gene (da Sacco and Masotti, 2012). Identification of *B. mori* miRNAs that up-regulate *BmFib-L* expression will be important in the study of the mechanisms of silk protein biosynthesis.

Compliance with ethics guidelines

Chen CHEN, Yang-yang FAN, Xin WANG, Fei SONG, Tao JIANG, Ping QIAN, Shun-ming TANG, and Xing-jia SHEN declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：家蚕 miR-0001 和 miR-0015 体外下调 *BmFib-L* 基因的表达

目　的：探究新发现的两个 miRNA 对家蚕丝素轻链基因 *BmFib-L* 的调控作用。

创新点：在家蚕后部丝腺中发现两个新的 miRNA，并首次证明它们对家蚕丝素蛋白轻链基因 *BmFib-L* 有负调控作用。

方　法：本实验通过生物信息学分析，从家蚕后部丝腺 miRNA 高通量测序获得的新 miRNA 中，筛选出两个可能对家蚕丝素蛋白轻链基因 *BmFib-L* 有调控作用的 miRNA，即 bmo-miR-0001 和 bmo-miR-0015。设计茎环引物，采用反转录聚合酶链反应（RT-PCR）方法对家蚕 5 龄 3 d 头部、表皮、脂肪体、马氏管、精巢/卵巢、丝腺（前、中、后）、气管、中肠和血淋巴细胞等 12 个不同组织的 bmo-miR-0001 和 bmo-miR-00015 进行半定量表达分析。并采用双荧光报告基因检测系统进一步在细胞水平上验证 bmo-miR-0001 和 bmo-miR-0015 对 *BmFib-L* 表达的调控作用。

结　论：本实验中 RT-PCR 结果显示，这两个新 miRNA 在家蚕后部丝腺中表达量最高（图 4）。双荧光报告基因检测结果显示，报告基因荧光素酶活性明显低于阳性对照组（图 7），转染 bmo-miR-0001 和 bmo-miR-0015 表达载体的细胞，报告基因和荧光素酶活性分别只及对照组 60%和 69%。*t* 检验分析结果显示两个实验组与对照组之间差异都达到显著水平（$P<0.05$）。由此可见，bmo-miR-0001 和 bmo-miR-0015 在体外对 *BmFib-L* 的表达具有显著的抑制作用。

关键词：家蚕；miRNA；bmo-miR-0001；bmo-miR-0015；*BmFib-L*；功能验证