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Research article

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Bmo-miR-2758 Targets *BmFMBP*-1 (Lepidoptera: Bombycidae) and Suppresses Its Expression in BmN Cells

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Abstract

MicroRNAs (miRNAs) are an abundant family of endogenous noncoding small RNA molecules. They play crucial roles on regulation of life processes both in plants and animals. Fibroin modulator binding protein-1 (FMBP-1) is a silk gland transcription factor of *Bombyx mori*, which is considered as a trans-activator of fibroin genes. And bioinformatics prediction showed that at the 3′ untranslated region (3′ UTR) of *BmFMBP*-1 there were binding sites for three bmo-miRNAs, bmo-miR-2b*, bmo-miR-305, and bmo-miR-2758, separately. In order to validate whether these bmo-miRNAs involved in the regulation of *BmFMBP*-1 expression, the expression levels of three bmo-miRNAs and *BmFMBP*-1 in the middle silk gland (MSG) and posterior silk gland (PSG) during the fourth- and fifth-larval stages of *B. mori* were measured by semi-quantitative reverse transcription polymerase chain reaction. The results revealed that the expression level of bmo-miR-2758 was the highest in the three, and it expressed higher in the PSG than in the MSG with a similar expression pattern as *BmFMBP*-1, implying that bmo-miR-2758 may involved in regulation of *BmFMBP*-1. To validate the regulation function of bmo-miR-2758 on *BmFMBP*-1, recombinant plasmids pcDNA3 [*ie*1-*egfp*-pri-bmo-miR-2758-SV40] and pGL3 [*A*3-*luc-FMBP*-1 3′ UTR-SV40] were constructed and co-transfected in BmN cells. The dual-luciferase reporter assay system was used for assay of transient expression. The results showed that the expression of the luciferase reporter was significantly decreased when pGL3 [*A*3-*luc-FMBP*-1 3′ UTR-SV40] co-transfected with pcDNA3 [*ie*1-*egfp*-pri-bmo-miR-2758-SV40] ($P < 0.01$). Furthermore, when the artificial antisense RNA of bmo-miR-2758 (inhibitor) was added to the above co-transfection, the expression of the luciferase reporter was recovered significantly ($P < 0.01$). These results suggest that bmo-miR-2758 represses the expression of *BmFMBP*-1 *in vitro.*

Key words: microRNA; *Bombyx mori*; Silk gland transcription factor; BmFMBP-1; Functional identification

MicroRNAs (miRNAs) are important gene posttranscriptional regulators involved in a variety of important physiological processes, including development, metabolism, and disease occurrence. Both miRNAs and transcription factors (TFs) function in regulation of target genes at the transcriptional level. And studies have shown that the interaction between miRNAs and TFs result in more accurate regulation of target genes (Shalgi et al., 2007; Pitto et al., 2008; Martinez et al., 2008; Re et al., 2009). The putative targets of most miRNAs are twice as many as associated TFs' general targets (Enright et al., 2003).

The silk gland of silkworm larvae, *Bombyx mori* is composed of three parts, anterior silk gland, middle silk gland (MSG), and posterior silk gland (PSG), which synthesize silk proteins efficiently. The expressions of fibroin protein genes, including fibroin light chain (*BmFib*-L), fibroin heavy chain (*BmFib*-H), and *BmP25* of *B. mori* are primarily regulated at the transcriptional level, and display a pattern of expression- expression inhibition circular from feeding period to molting period (Maekawa et al., 1980; Couble et al., 1983; Kimura et al., 1985). In the upstream of *BmFib*-H, e.g., there are binding sites for six transcriptional factors, *B. mori* fibroin modulator-binding protein-1 (BmFMBP-1), silk gland factors including BmSGF-1, BmSGF-2, BmSGF-3, and BmSGF-4, and fibroin-binding factor-A1 (BmFBF-A1) (Hui and Suzuki, 1989; Takiya et al., 1997). The *BmFMBP*-1 prefers AT-rich upstream elements which functions on the intragenic regions of the fibroin gene and enhance its transcription level (Hui and Suzuki, 1989; Takiya et al., 1990; Hui et al., 1992). BmFMBP-1 is therefore considered as a trans-activator of fibroin gene. The DNA-binding activity of BmFMBP-1 occurs in specific tissues and developmental stages associated with the expression of silk protein genes and it is probably regulated

Table 1. List of bmo-miRNA primers

Bmo-miRNAs		Primer sequence (5'-3')
bmo-miR-2b*	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC*CATGTC*
	Forward	gggtTCGACAAGGTGGTT
bmo-miR-305	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC*CAGAGC*
	Forward	cccATTGTACTTCATCAG
bmo-miR-2758	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC*CTTACT*
	Forward	ggggACTTGGTAGAACAC
U6 rRNA	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC*ACGATT*
	Forward	CCTGCGCAAGGATGAC
Reverse		GTGCAGGGTCCGAGGT

Table 2. Primer lists of pri-bmo-miR-2758 and *BmFMBP*-1 3′UTR

Primer names	Primer sequences (5′-3′)
pri-bmo-miR-2758-F	cccaagcttAAAATGTCTGCGAACCGATGAA
pri-bmo-miR-2758-R	cccggatccATGTAATGAACTTGTCCTGTCG
BmFMBP-1 3′UTR-F	ccctctagaTGTTCAGCAATCACCAATACTA
BmFMBP-1 3′UTR-R	gggggccggccTTACCCTGCCAAAGTTATCATT

Underlined parts: caagctt, ggatcc, tctaga, ggccggcc indicate restriction sites of *Hind*III, *Bam*H I, *Xba* I, and *Fse* I; the lower case letters uncrossed indicate the protective bases, the same as below.

posttranscription through interaction with RNA-binding proteins and other TFs (Takiya et al., 2005, 2009, Saito et al., 2007).

Studies of interaction between miRNAs and BmFMBP-1 may increase our understanding of the regulation mechanism of silk proteins biosynthesis. Based on bioinformatics analysis, three bmo-miRNAs, bmo-miR-2b*, bmo-miR-305, and bmo-miR-2758 were predicted to have binding sites in *BmFMBP*-1 3′-UTR. And the regulation function of bmo-miRNA-2758 on *BmFMBP*-1 was validated by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) method and transient expression assay with dual-luciferase reporter (DLR) assay system *in vitro*.

Materials and Methods

Silkworm Strains and Reagents.

Silkworm strain p50 was obtained from the Sericultural Research Institute, Chinese Academy of Agricultural Sciences. *Escherichia coli* DH10B, *B. mori*-derived cell line BmN, pGL3 [*A3-luc*-SV40], pcDNA3 [*ie*1-*egfp*-SV40], and pRL-CMV (*Renilla* luciferase plasmid, used as an internal control reporter) were constructed and preserved by the Key Laboratory of Silkworm and Mulberry Genetic Improvement, Ministry of Agriculture. Restriction enzymes, T4 DNA ligase, pMD18-T vector, and RT-PCR kit were purchased from TaKaRa (Shanghai, China). TC-100 medium was purchased from Applichem (Germany). Fetal Bovine Serum (FBS) (Qualified, Australia Origin) was purchased from Gibco (USA). Perfect transfection reagent was purchased from UCallM Biotech Co., Ltd. (Wuxi, China). The antisense RNAs of bmo-miR-2758, inhibitors were synthesized by the Biomics Biotechnologies Co., Ltd (Nantong, China). The DLR Assay System kit was purchased from Promega (USA).

Bioinformatic Analysis

All 563 mature bmo-miRNA sequences used were downloaded from the searchable database (miRBase 21, http://www.mirbase.org/). The sequence of *BmFMBP-1* (1481 bp, protein ID: NP_001036969.1) and the 3′-UTR (825 bp) were obtained from NCBI (http://www.ncbi.nlm.nih.gov/nuccore/NM_001043504.1) by a BLAST search. The online bioinformatics prediction software RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) and RNA22 (http://cbcsrv.watson.ibm.com/rna22.html) were employed for prediction of base pairing between *BmFMBP-1* 3′UTR and the seed regions (a conserved nucleotide sequence positions 2–8 in the 5′end of the miRNA) of the bmo-miRNAs.

Semiquantitative RT-PCR analysis

Total RNAs (1 μg) from the MSG and PSG during the fourth and fifth-instar larval stages, and the day-1 of cocooning, were extracted according to the manufacturer's instructions (TaKaRa), respectively. To investigate the expression pattern of candidate bmo-miRNAs, the RNA samples were converted into cDNA using mature miRNA RT primer, which were designed to add six reverse-complement nucleotides behind the general stem-loop sequence (Chen et al., 2005). The miRNA-specific forward and reverse universal primers were listed in Table 1. U6 rRNA was used to standardize among samples. The PCR verification was carried out under the following conditions: an initial denaturing step at 94°C for 5 min; 34 cycles of 94°C for 30 s, 65°C for 25 s, and 72°C for 30 s; and followed by a final extension step at 72°C for 10 min. Amplified DNA products (1 μg) were separated on 4% agarose gels and visualized via UV transillumination. The relative expressions of PCR products were analyzed by Gel-Pro Analyzer software (Media Cybernetics, USA).

To investigate the expression pattern of *BmFMBP*-1, the total RNAs were converted into cDNA using an oligo (dT) primer with *Bmactin* 3 as an internal control. The primers for *BmFMBP*-1 were *BmFMBP*-1F (5′-GAAGGAAAGGCTCAGGAC-3′) and *BmFMBP*-1R (5′-CGGACATACGCTTCAGT-3′) in the forward and reverse directions, respectively. PCR was performed as the following conditions: an initial denaturing step at 94°C for 5 min; 30 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 10 min. Amplified DNA products (1 μg) were separated on 1% agarose gels and analyzed as described earlier.

Construction of Recombinant Vectors

The primary sequence of bmo-miR-2758 (pri-bmo-miR-2758) that 100-bp extended at upstream and downstream of the precursor sequence was obtained from the miRBase database and Silkworm Genome Database (SilkDB, http://www.silkdb.org/silkdb/). The *BmFMBP*-1 3′-UTR sequence was obtained from NCBI. Primers for pri-bmo-miR-2758 and the *BmFMBP*-1 3′-UTR with restriction sites at the 5′ and 3′end, respectively, were designed using Oligo 6 (Table 2). After PCR process, two gene fragments of PCR products were cloned into a pMD18-T vector, respectively for sequencing. Then the pri-bmo-miR-2758 fragment was cutout from pMD-T-pri-bmo-miR-2758 at restriction sites using *Hind* III and *Bam*H I and

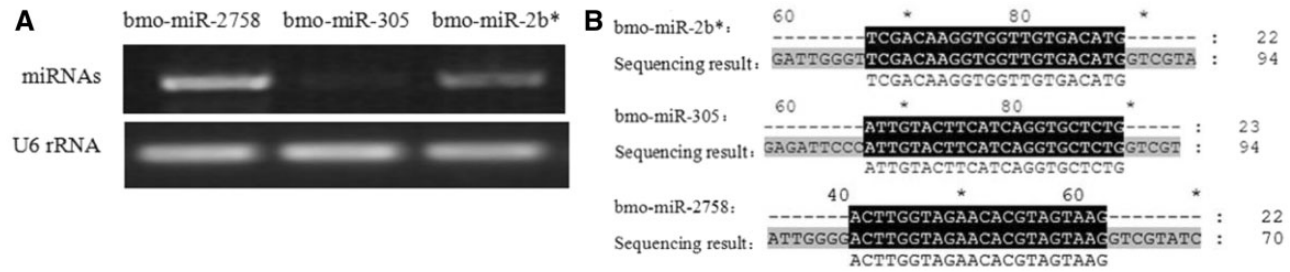


Fig. 1. RT-PCR results and sequence alignments by Genedoc of bmo-miR-2b*, bmo-miR-305, and bmo-miR-2758. (A) RT-PCR results of bmo-miR-2b*, bmo-miR-305, and bmo-miR-2758; (B) Sequence alignment results of bmo-miR-2b*, bmo-miR-305, and bmo-miR-2758.

Table 3. The plasmids of three experimental groups for transfection

Plasmid names	Group 1	Group 2	Group 3
pGL3 [*A3-luc-FMBP*-1 3′-UTR-SV40]	950 ng	950 ng	950 ng
pcDNA3 [*ie*1-*egfp*-SV40]	950 ng	–	–
pcDNA3 [*ie*1-*egfp*-pri-bmo-miR-2758-SV40]	–	950 ng	950 ng
bmo-miR-2758 inhibitor	–	–	100 nM
pRL-CMV	100 ng	100 ng	100 ng

cloned into pcDNA3 [*ie*1-*egfp*-SV40] vector. The *BmFMBP*-1 3′-UTR fragment was cut from the T-*FMBP*-1 3′-UTR using *Fse* I and *Xba* I restriction sites and cloned into pGL3 [*A3-luc*-SV40] vector.

Transfection and Dual Luciferase Assay

Recombinant plasmids pcDNA3 [*ie*1-*egfp*-SV40], pGL3 [*A3-luc-FMBP*-1 3′-UTR-SV40], pcDNA3 [*ie*1-*egfp*-pri-bmo-miR-2758-SV40], bmo-miR-2758 inhibitor, and pRL-CMV were arranged into three groups for co-transfection (Table 3). Transfection was performed as previously described (Huang et al., 2010, Chen et al., 2013). Each experimental group was combined with 4 µl perfect transfection reagent, separately diluted in 50 µl unsupplemented TC-100, mixed gently, and incubated for 20 min at room temperature (Zhao et al., 2007). The Cellfectin-DNA complexes were added dropwise to BmN cells (1.0–1.5 × 10^6 cells per 35-mm cell culture dish) and incubated at 27°C for 5 h. Then the FBS free medium was substituted by TC-100 medium containing 10% FBS and 1% antibiotics, and incubated at 27°C. At 48 h posttransfection the fluorescence of cells were examined by a fluorescence microscope Olympus IX7 (Olympus, Japan). Then, cells were harvested and treated with 250 µl of 1 × Passive Lysis Buffer for 30 min until fully lysed.

The expression activity of *luc* reporter gene of each group was detected via DLR according to manufacturer instructions (Promega, USA) by a 20/20 n Luminometer (Turner BioSystems, USA). The activity of firefly luciferase was normalized by renilla luciferase of pRL-CMV. Three independent experiments were carried out and all transfections were performed in triplicate in each experiment. Data are presented as mean ± SD. Significant differences between the groups were assessed using the Student's *t*-test with a cutoff of $P < 0.05$.

Results

Prediction of Candidate bmo-miRNAs

By using the RNAhybrid combined with RNA22 software, 13 candidate bmo-miRNAs were predicted to be perfectly complementary with the target site of *BmFMBP-1* 3′-UTR, based on the level of minimum free energy (MFE) and 2-8 complementary base pairing interactions in the seed regions. The positions of each bmo-miRNA that matched the *BmFMBP*-1 3′-UTR sequence were shown in Table 4.

Identification of Candidate bmo-miRNAs by Stem-Loop RT-PCR

The results of stem-loop RT-PCR showed that nine candidates' products of 60–80 bp were detected via gel electrophoresis. However, sequencing results revealed that only three candidates, bmo-miR-2b*, bmo-miR-305, and bmo-miR-2758 were in accordance with those in the database records (Fig. 1A and B), illustrating that these three bmo-miRNAs were expressed in the silk gland of day-3 of fifth-instar larva. Moreover, the expression level of bmo-miR-2758 was much higher than that of either bmo-miR-2b* or bmo-miR-305 under the same template concentration (Fig. 1A), suggesting that bmo-miR-2758 may involve in the regulation of *BmFMBP*-1 in the *B. mori* larva.

Expression Analysis of bmo-miR-2758 and BmFMBP-1 in the PSG and MSG

The relative expression levels of both bmo-miR-2758 and BmFMBP-1 in MSG and PSG at different developmental stages were analyzed via semi-quantitative RT-PCR, respectively. The bmo-miR-2758 expressed in both MSG and PSG, while its relative expression in PSG was 2.16–15.72 folds than that in MSG (Fig. 2A). The expression of bmo-miR-2758 in the PSG increased gradually along with the development of larva in the fifth instar, reaching the highest levels at day-5 of fifth instar. Thereafter it sharply declined to the levels even lower than that in the day-2 of the fourth instar until pupation. The expression of bmo-miR-2758 in MSG was generally in a low level but revealed the same trend as in PSG.

BmFMBP-1 expressed both in MSG and PSG (Fig. 2B). In PSG its highest expression level was on day-1 of fifth instar and decreased gradually until day-5 of fifth instar. Thereafter its expression levels decreased rapidly to the lowest on the day-1 of wandering stage with about 5% of the total. The expression pattern of *BmFMBP*-1 in MSG was similar to that in PSG with the peak on day-5 of fifth instar but decreased sharply in the wandering stage. However, on day-2 of fourth instar, expression of *BmFMBP*-1 revealed the greatest difference between PSG and MSG, in PSG its expression level was 12.44-fold higher than that in MSG. Bmo-miR-2758 displayed prominent and coincident expression patterns with its predicted target gene *BmFMBP*-1, implying that bmo-miR-2758 has time-space expression situation to regulate Bm*FMBP*-1, which means that bmo-miR-2758 may involved in posttranscriptional regulation of *BmFMBP*-1.

Construction of Expression Vectors

The primary sequence pri-bmo-miR-2758 was cloned into the expression vector pcDNA3 containing the *ie*1 promoter and *egfp*

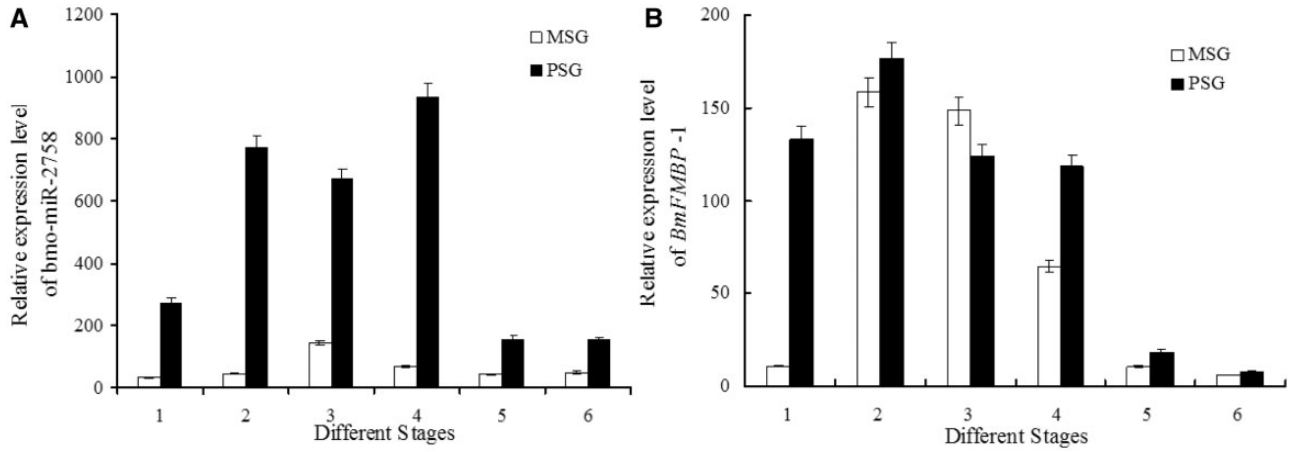


Fig. 2. The relative expression of Bmo-miR-2758 and *BmFMBP*-1 in MSG and PSG at different development stages of silkworm, respectively. (A) The relative expression of Bmo-miR-2758. (B) The relative expression of *BmFMBP*-1. 1. day-2 of fourth instar; 2. day-1 of fifth instar; 3. day-3 of fifth instar; 4. day-5 of fifth instar; 5. day-7 of fifth instar; 6. day-1 of cocooning.

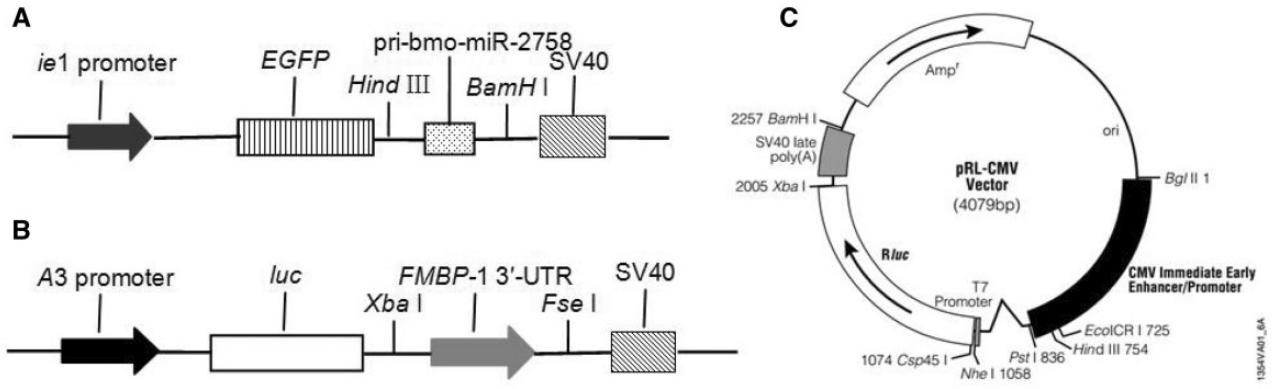


Fig. 3. Diagrams of the recombinant plasmids and internal control plasmid pRL-CMV. (A) Recombinant plasmid pGL3 [*A3-luc-FMBP*-1 3′-UTR-SV40]; (B) Recombinant plasmid pcDNA3 [*ie*1-*egfp*-pri-bmo-miR-2758-SV40]; (C) The internal control plasmid pRL-CMV.

(enhanced green fluorescent protein) gene to construct the bmo-miR-2758 expression plasmid, pcDNA3 [*ie*1-*egfp*-pri-bmo-miR-2758-SV40] (Fig. 3A). The 3′-UTR sequence of *BmFMBP*-1 was cloned into the pGL3 vector containing the A3 promoter and *luc* (luciferase) gene to construct the *BmFMBP*-1 3′-UTR expression plasmid, pGL3 [*A3-luc-FMBP*-1 3′-UTR-SV40] (Fig. 3B). The recombinant plasmids and internal control plasmid pRL-CMV were co-transfected into BmN cells as described earlier (Section 'Transfection and Dual Luciferase Assay'), respectively (Fig. 3C).

At 48 h posttransfection, fluorescence of cells was detected, indicating that the recombinant plasmids pcDNA3 [*ie*1-*egfp*-pri-bmo-miR-2758-SV40] were successfully transfected into BmN cells and it can express bmo-miR-2758 effectively in BmN cells (Fig. 4).

Regulatory Effect of bmo-miR-2758 on the Expression of BmFMBP-1

To normalize the data of luciferase activity obtained, the ratio of firefly luciferase activity/renilla luciferase activity (Luc/Rlu) was presented as relative luciferase activity. Luciferase activity of Group 2 was obviously decreased compared with that of Group 1, and the differences were extremely significant ($P < 0.01$) (Fig. 5), indicating that expression of *luc*, which fused with *BmFMBP*-1 3'-UTR sequence, could be repressed by bmo-miR-2758. Meanwhile, expression of *luc* was recovered and showed a significant increase in Group 3 ($P < 0.01$) (Fig. 5), which validated distinctly that the bmo-miR-2758 inhibitor (anti-sense RNA) could restrain the posttranscriptional regulation function of bmo-miR-2758. The above results demonstrated that bmo-miR-2758 significantly down regulate the expression of *BmFMBP*-1 at posttranslational level by binding on the 3'-UTR complementary site of *BmFMBP*-1.

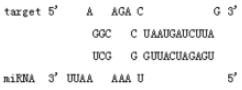
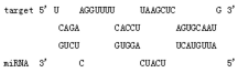
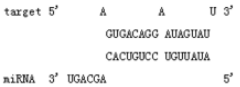
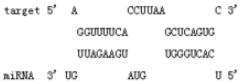
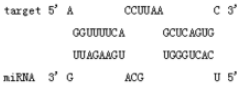
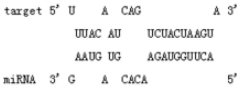
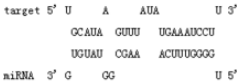
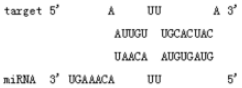
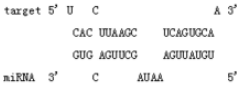
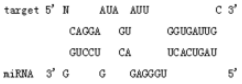
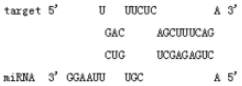
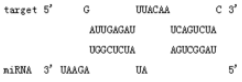
Discussions

Previous reports confirmed that the expression level of FMBP-1 in the larval was higher than in the embryonic and larval diapause period (Suzuki et al., 1986).

Electrophoretic mobility shift assay observed that BmFMBP-1 binds to the upstream regulatory region of fibroin and it expressed faintly in larval fatty body and MSG on day-2 of the fifth instar (Shigeharu et al., 1997). Although the expression level of *BmFMBP*-1 was slightly higher in the ovarian tissue, and highest in the PSG (Shigeharu et al., 1997). These conclusions are supported by our results, which confirmed the expression specificity of the *BmFMBP*-1 gene.

In this study, we identified three potential bom-miRNAs targeting on *BmFMBP*-1 3′-UTR by using bioinformatics methods. And we showed that the expression of *BmFMBP*-1 in silk glands is highest at the initial stage of the fifth instar, but gradually reduces to a

Table 4. Candidate bmo-miRNAs targeting the 3′-UTR sequence of the *BmFMBP*-1, and the binding sites

Bmo-miRNAs	MFEs (Kcal/mol)		Binding sites		Bmo-miRNAs matching with the 3′-UTR
	RNAhybrid	RNA22	RNAhybrid	RNA22	
bmo-miR-bantam	−19.6	−26.00	579	576 . . . 598	
bmo-miR-2b*	−20.2	−26.60	639	640 . . . 661	
bmo-miR-305	−19.5	−24.70	256	265 . . . 287	
bmo-miR-1000	−23.8	−31.40	139	135 . . . 155	
bmo-miR-2733d	−21.7	−24.90	261	262 . . . 283	
bmo-miR-2733g	−21.7	−23.79	261	263 . . . 283	
bmo-miR-2758	−19.9	−26.20	525	524 . . . 545	
bmo-miR-2788	−22.4	−23.60	633	634 . . . 655	
bmo-miR-2804*	−21.3	−27.40	688	682 . . . 703	
bmo-miR-3203*	−21.3	−27.70	267	264 . . . 285	
bmo-miR-3245	−22.0	−28.70	143	142 . . . 164	
bmo-miR-3265	−20.6	−23.79	242	239 . . . 260	
bmo-miR-3397	−22.7	−28.60	517	517 . . . 539	

minimum value during cocooning, whose pattern is consistent with biosynthesis of fibroin, supporting that BmFMBP-1 acts as a positive regulator of fibroin expression. To our knowledge, this is the first time to discuss on the bmo-miR-2758 expression in the fifth-instar larvae of the silkworm. Moreover, the expression of bmo-miR-2758 in the PSG was higher than that in the MSG whose expression pattern was similar to *BmFMBP*-1, implying that bmo-miR-2758 probably regulates the expression of *BmFMBP-1* mainly in PSG.

Silk gland TFs are required for silk protein biosynthesis, but the scientific questions were hitherto unexplored, such as, their potential regulation by bmo-miRNAs; the interrelationships among TFs, silk gland proteins, and bmo-miRNAs. Previous studies revealed that bmo-miR-2b can inhibit the expression of *BmP*25 (Huang et al., 2011), miR-33/-190/-276/-7 can target *BmFib*-L transcripts (Cao et al., 2008), miRNA-965 and miRNA-1926 can down-regulate the expression of the *BmFib*-L (Huang et al., 2012), two novel miRNAs Bmo-miR-0001 and Bmo-miR-0015 can down-regulate expression of *BmFib*-L in vitro (Chen et al., 2016), while bmo-miR-2739 can up-regulate the expression of *BmFib*-H (Song et al., 2014), suggesting that miRNAs might play an important role in the regulation of silk protein production. We report here that bmo-miR-2758 significantly inhibits the expression of *BmFMBP*-1

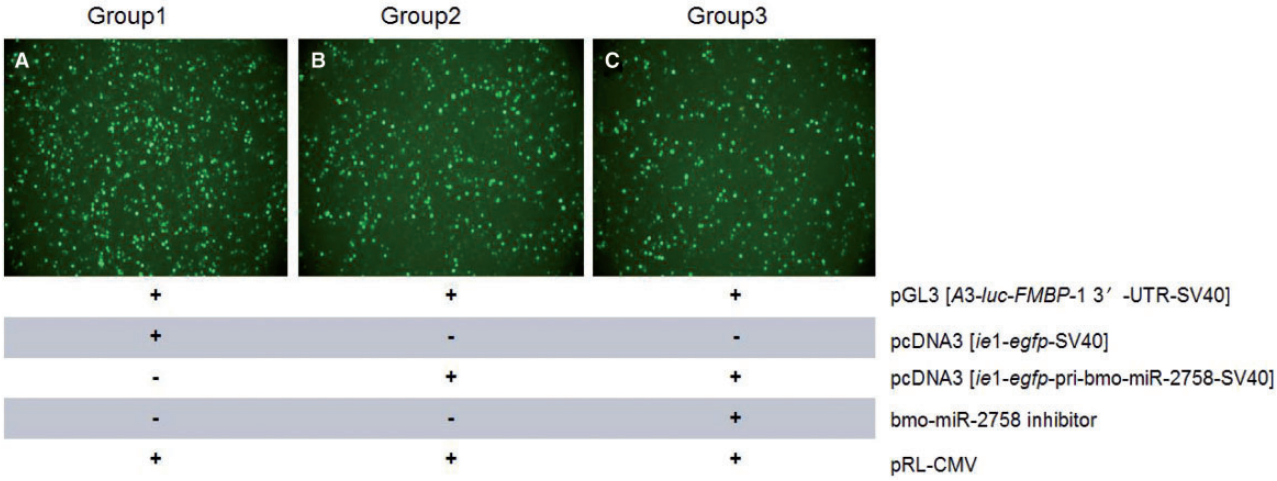


Fig. 4. Photographs of BmN cells transfected with different recombinant plasmids. (A) BmN cells transfected with pcDNA3 [*ie*1-*egfp*-SV40], pGL3 [*A3-luc-FMBP*-1 3′-UTR-SV40] and pRL-CMV. (B) BmN cells transfected with pcDNA3 [*ie*-1-*egfp*-pri-bmo-miR-2758-SV40], pGL3 [*A3-luc-FMBP*-1 3′-UTR-SV40] and pRL-CMV. (C) BmN cells transfected with pcDNA3 [*ie*-1-*egfp*-pri-bmo-miR-2758-SV40], pGL3 [*A3-luc-FMBP*-1 3′-UTR-SV40], bmo-miR-2758 inhibitor and pRL-CMV.

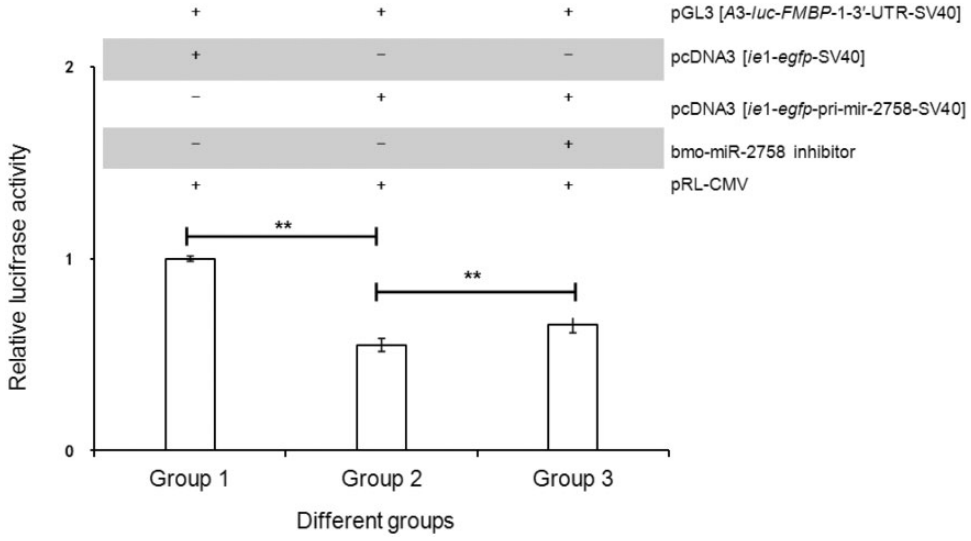


Fig. 5. Effect of bmo-miR-2758 expression on luciferase activity in transfected BmN cells. (A) The effect of bmo-miR-2758 on BmFMBP-1. (B) The effect of bmo-miR-2758 inhibitor on bmo-miR-2758. Data are represented as the mean value±S.D. **, $P < 0.01$ from three independent experiments. Group 1: BmN cells transfected with pGL3 [*A3-luc-FMBP*-1 3′-UTR-SV40], pcDNA3 [*ie*1-*egfp*-SV40], and pRL-CMV; Group 2: BmN cells transfected with pGL3 [*A3-luc-FMBP*-1 3′-UTR-SV40], pcDNA3 [*ie*1-*egfp*-pri-bmo-miR-2758-SV40], and pRL-CMV; Group 3: BmN cells transfected with pGL3 [*A3-luc-FMBP*-1 3′-UTR-SV40], pcDNA3 [*ie*1-*egfp*-pri-bmo-miR-2758-SV40], pRL-CMV and bmo-miR-2758 inhibitor.

in vitro ($P < .01$). These results provide new evidence toward better understanding of the regulatory mechanism of silk protein biosynthesis and the function of bmo-miRNAs, to improve silkworm breeds for cocoon production. Next *in vivo* study of the regulation of bmo-miR-2758 on the expression of *BmFMBP*-1 and other silk gland TFs need be carried out, which will provide a more complete explanation of the mechanisms regulating eukaryotic gene expression.

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