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

Xin Wang, 1,2 Shunming Tang, 1,2 Fei Song, 1 Chen Chen, 1 Xijie Guo, 1,2 and Xingjia Shen 1,2,3

1 Jiangsu Key Laboratory of Sericultural Biology and Biotechnology, School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, China; 2 Key Laboratory of Silkworm and Mulberry Genetic Improvement, Ministry of Agriculture, Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, China and 3 Corresponding author, e-mail: shenxjsri@163.com

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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 [ie1-egfp-pri-bmo-miR-2758-SV40] and pGL3 [A3-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 [A3-luc-FMBP-1 3’ UTR-SV40] co-transfected with pcDNA3 [ie1-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; Pittro 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 BmFib2.5 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.
miRNAs, bmo-miR-2b*, bmo-miR-305, and bmo-miR-2758 were predicted to have binding sites in BmFMBP-1 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/mahybrid/) and RNA22 (http://cbcrsv.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.

![Table 1. List of bmo-miRNA primers](image)

| Bmo-miRNAs | Primer sequence (5'-3') |
|------------|------------------------|
| bmo-miR-2b* | RT Forward ggtgtCGACAGGTGGTTCAGTACGAGTGTT |
| bmo-miR-305 | RT Forward cccCGTGATCGGGGATGCTGGTTCAGTACGAGTGTT |
| bmo-miR-2758 | RT Forward cccCGTGATCGGGGATGCTGGTTCAGTACGAGTGTT |
| U6 rRNA | RT Forward cccCGTGATCGGGGATGCTGGTTCAGTACGAGTGTT |

Underlined parts: caagctt, ggatcc, tctaga, ggccggcc indicate restriction sites of HindIII, BamHI, KpnI, and EcoRI; 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 (Chen et al., 2005).

**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 [i5-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 sequences 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 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/mahybrid/) and RNA22 (http://cbcrsv.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.

Semi-quantitative 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 RNA 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 Bracta as an internal control. The primers for BmFMBP-1 were BmFMBP-1F (5'-GAAGGAAGAGGCTGAGGAC-3') and BmFMBP-1R (5'-GGGACATAGGCTTCGATGG-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 BamHI I and

![Table 2. Primer names lists of pri-bmo-miR-2758 and BmFMBP-1 3'UTR](image)

| Primer names | Primer sequences (5'-3') |
|--------------|------------------------|
| pri-bmo-miR-2758-F | cccgggACCGACCAAGGCTGAGGAC |
| pri-bmo-miR-2758-R | cccgggACCGACCAAGGCTGAGGAC |
| BmFMBP-1 3'UTR-F | cccgggACCGACCAAGGCTGAGGAC |
| BmFMBP-1 3'UTR-R | cccgggACCGACCAAGGCTGAGGAC |

Underlined parts: caagctt, ggatcc, tctaga, ggccggcc indicate restriction sites of HindIII, BamHI, KpnI, and EcoRI; the lower case letters uncrossed indicate the protective bases, the same as below.
cloned into pcDNA3 [ie1-egfp-SV40] vector. The BmFMBP-1 3’-UTR fragment was cut from the T-FMBP-1 3’-UTR using Fse I and Xho I restriction sites and cloned into pGL3 [A3-luc-SV40] vector.

Transfection and Dual Luciferase Assay
Recombinant plasmids pcDNA3 [ie1-egfp-SV40], pcGL3 [A3-luc-FMBP-1 3’-UTR-SV40], pcDNA3 [ie1-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 × 106 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 60–80bp 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 pattern 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 ever 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 BmFMBP-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 ie1 promoter and egfp
(enhanced green fluorescent protein) gene to construct the bmo-miR-2758 expression plasmid, pcDNA3 [ie1-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 [ie1-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
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 BmP25 (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

### 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 | RNAhybrid | RNA22 |
| bmo-miR-bantam   | −19.6     | −26.00 | 579       | 576...598 | target 5′′ A | A | C | C | C | C | C | C |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-2b*      | −20.2     | −26.60 | 639       | 640...661 | target 5′′ A | A | A | A | A | A | A | A |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-305      | −19.5     | −24.70 | 256       | 265...287 | target 5′′ A | A | C | C | C | C | C | C |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-1000     | −23.8     | −31.40 | 139       | 135...155 | target 5′′ A | A | A | A | A | A | A | A |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-2733d    | −21.7     | −24.90 | 261       | 262...283 | target 5′′ A | A | C | C | C | C | C | C |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-2733g    | −21.7     | −23.79 | 261       | 263...283 | target 5′′ A | A | C | C | C | C | C | C |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-2758     | −19.9     | −26.20 | 525       | 524...545 | target 5′′ A | A | A | A | A | A | A | A |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-2788     | −22.4     | −23.60 | 633       | 634...655 | target 5′′ U | A | A | A | A | A | A | A |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-2788*    | −19.3     | −27.40 | 688       | 682...703 | target 5′′ U | U | U | U | U | U | U | U |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-2804*    | −21.3     | −27.70 | 267       | 264...285 | target 5′′ U | C | A | A | A | A | A | A |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-3203*    | −21.3     | −27.70 | 143       | 142...164 | target 5′′ U | U | A | A | A | A | A | A |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-3245     | −22.0     | −28.70 | 143       | 142...164 | target 5′′ U | U | A | A | A | A | A | A |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-3265     | −20.6     | −23.79 | 242       | 239...260 | target 5′′ U | U | A | A | A | A | A | A |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
| bmo-miR-3397     | −22.7     | −28.60 | 517       | 517...539 | target 5′′ U | U | A | A | A | A | A | A |
|                  |           |       |           |       | GCC | GCC | GCC | GCC | GCC | GCC | GCC | GCC |
|                  |           |       |           |       | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ | 5′′ |
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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