ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2012, Vol. 38, No. 4, pp. 417–421. © Pleiades Publishing, Ltd., 2012.

The Regulation of Silkworm Fibroin L Chain Production by miRNA-965 and miRNA-1926 in Insect Cells[1]

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Received September 18, 2011; in final form, November 20, 2011

Abstract—MicroRNAs (miRNAs) are an abundant class of approximately 22-nucleotide (nt)-short noncoding RNA molecules present in the genomes of all multicellular organisms that act through base pairing to partially complementary sequences of the 3'untranslated region (UTR) of targeted mRNAs. Using bioinformatic approach, we found that the 3' UTR of the Fibroin L chain (Fib-L) mRNA matches perfectly the nucleotides 2–8 at the 5' end of the miRNA-965 and miRNA-1926. These two miRNAs might act as regulators of Fib-L gene expression at the post-transcriptional level. To examine whether Fib-L is directly targeted by miRNA-965 and miRNA-1926 in vitro, miRNA expression vectors and target reporter vector with 3'UTR of Fib-L were constructed respectively. Two vectors were co~transfected into Sf21 cells. The luciferase assay showed that miRNA-965 and miRNA-1926 may down regulate the expression of Fib-L via complementary interaction with the target sites in 3'UTR.

Keywords: silkworm, miRNAs, targets, functional identification

DOI: 10.1134/S1068162012030168

INTRODUCTION

MicroRNAs (miRNAs) are 18–24 nucleotides non-coding small RNA molecules derived from a 70–90 nucleotides hairpin stem-loop pre-miRNA. The pre-miRNA is then recongnized by Dicer, and cleaved to produce a mature miRNA [1, 2]. The miRNAs are widely present in various eukaryotic organisms and play important roles in many biological processes as post-transcriptional regulators of gene expression [3–5]. Nowadays study of miRNAs is becoming one of the hotspots in biologcal research. Mature miRNAs recognize their target mRNAs by base-pairing interactions between nucleotides 2–8 (seed region) of the miRNA and the complementary nucleotides in the 3' untranslated region (3'UTR) of the mRNAs [6, 7]. MiRNAs inhibit gene expression by targeting mRNAs for translational repression or cleavage [8, 9]. Increasing attention has thus been paid to their identification of their target genes. Identification of the miRNAs is an important step in elucidating the function of miRNAs. Previous studies indicated that potential targets can be validated experimentally using report gene assays [10–12].

The silkworm (*Bombyx mori*), an insect that undergoes complete metamorphosis, not only has great agronomic value but is also an excellent model for the study of insect genetics and molecular biology [13, 14]. The silk gland of silkworm is a very important organ where fibroin is synthesized and secreted [15, 16]. Studies confirmed that many regulators are involved in regulation of silk protein gene expression [17, 18]. Until now, little is known about the mechanism of regulation of gene expression in silk gland.

In this paper, by bioinformatic search for miRNAs potentially interacting with the 3'UTR of the Fib-L, we found that the silkworm miRNA-965 and miRNA-1926 are perfectly complementary to the target in seed region. Thus, these two miRNAs may be involved in the post-transcriptional regulation of the expression of the Fib-L. In order to check whether these two miR-NAs may regulate the Fib-L gene expression the miRNAs expression vector and luciferase gene-based reporter vector were constructed respectively. The activities of luciferase were shown to decrease significantly implying that miRNA-965 and miRNA-1926 down regulate the expression of Fib-L gene via complementary interaction with the target site in 3'UTR. These data provide the experimental bases for further studies of transcriptional regulation of Fib-L gene by miRNAs targeting.

[1] The article is published in the original.

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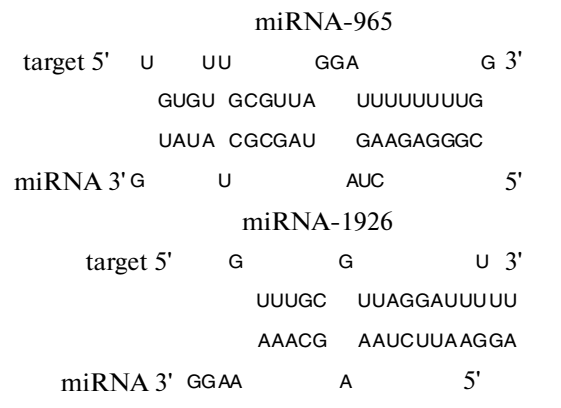
RNAhybrid prediction:



RNA22 prediction:

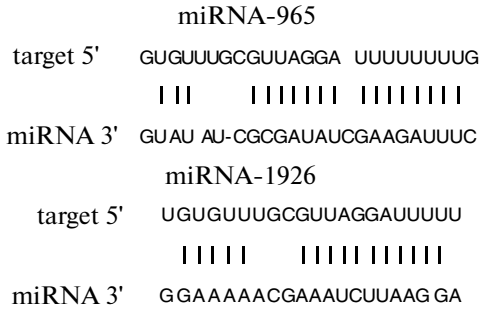


Fig. 1. Prediction of miRNA-965 and miRNA-1926 with a putative miRNA target site in the 3'UTR of Fib-L by RNAhybrid and RNA22. miRNA seed pairings with target site are highlighted by black blod characters and the pairing of adjacent nucleotides is marked.

RESULTS

Prediction of Silkworm miRNAs Targeting 3'UTR of Fib-L Gene

In order to explore whether any potential miRNAs associated with the Fib-L gene can be found, we utilized the RNA22 and RNAhybrid prediction software to predict the miRNAs which may target 3'UTR of Fib-L gene. As a result, the 2 miRNA candidates (miRNA-965 and miRNA-1926) were obtained. The two miRNAs recognize their target by base-pairing interactions between nucleotides 2–8 (seed region) and complementary nucleotides in 3'UTR of Fib-L (Fig. 1).

Expression of miRNA in Insect Cells

At forty-eight h post-transfection with the vectors (pcDNA3/A3-EGFP-miRNA-965 and miRNA-1926), a fluorescence signal was detected in the most of the cells. EGFP could be observed under a fluorescent microscope, showing the successful expression of EGFP (Fig. 2). Since genes of miRNA-precursors are inserted into the 3'-region of EGFP gene they should be expressed as well in Sf21 cells. Previous studies confirmed that EGFP marker gene placed at the 5'end of the miRNA expression cassette is expressed along with the mature miRNAs as was shown by northern blot assay [19–21].

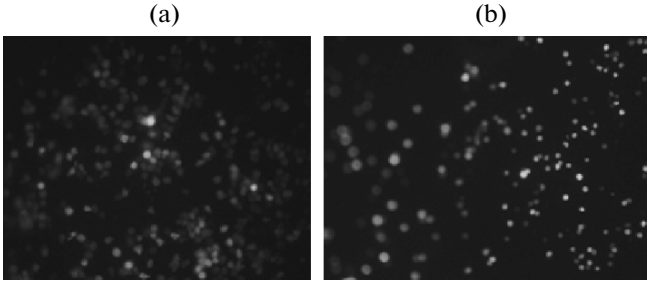


Fig. 2. Expression of green fluorescent protein in insect cells transfected with miRNA expression vectors. (a) Sf 21 cells transfected with pcDNA3/A3-EGFP-miRNA-965. (b) Sf 21 cells transfected with pcDNA/A3-EGFP-miRNA-1926.

Identification of miRNAs Targeting Fib-L Gene

The interaction of these miRNAs and potential Fib-L target in Sf21 cells were tested. As illustrated in Figure 3 ($p < 0.05$), in cells transfected with Fib-L 3'UTR reporter constructs and miRNA-965 or miRNA-1926 expression vector, the luciferase activity was repressed by 50% and 40% (miRNA-965 and miRNA-1926), respectively.

Luciferase activities of two positive controls were not significantly altered. Our results indicated that miRNA-965 and miRNA-1926 down regulate the expression of Fib-L gene via complementary interaction with the target site in 3'UTR of mRNA. Thus, we conclude that Fib-L is likely a direct target of miRNA-965 and miRNA-1926.

DISCUSSION

In plants, the miRNAs often show perfect or near-perfect complementarity with its target, resulting in target degradation, whereas animal miRNAs form imprecise base-pairing and cause translational repression. Identification target genes that miRNAs regulated by miRNAs is important for understanding their specific biological functions. Experimental validation of targets is currently done through in vitro reporter assays [19, 22–24]. For example, luciferase or enhanced green fluorescent protein (EGFP)-based reporter assay in cell, in which the entire 3'UTR of the putative target gene is cloned downstream of reporter gene, after the introduction of a miRNA to the cell, has been used for rapid detection of miRNA-target interaction in vitro. Previous studies showed that constructing miRNA expression vector is highly flexible and reliable with the insertion sequence of miRNA precursors and will facilitate the application of miRNA in functional targets research. Using eukaryotic vector to expressing

miRNAs is considered to suppress target genes in vitro because insertion of precursor miRNA allows it to be processed in a similar biogenesis pathway similar as to that of the natural miRNA in vivo which results in functional mature miRNAs.

Increasing attention has thus been paid to their regulation mechanisms and the identification of target genes [25, 26]. Recently, Cao et al. using the heterologous (gus) reporter gene expression system by Agrobacterium-mediated method have also confirmed that at least 4 miRNAs (miRNA-33, miRNA-190, miRNA-276, and miRNA-7) might play an important role in the regulation of Fib-L expression [27]. It was also reported for the first time that miRNAs can regulate target Fib-L in the silkworm. Between miRNA and regulated target were both one-to-many and many-to-one relationships. We speculate that there are still other miRNAs which may regulate Fib-L gene. In this paper, by bioinformatic analysis using RNA22 and RNAhybird target prediction software, two candidate miRNA (miRNA-965 and miRNA-1926) targeting Fib-L was identified. To validate that 3'UTR of Fib-L is a functional target of these two miRNAs, we constructed miRNA expression vectors and reporter vector containing the 3'UTR of Fib-L at the 3'position of the luciferase gene. The recombinant vectors were co-transfected into Sf21 cells and relative luciferase activity were measured. It was shown that activity of luciferase was significantly decreased and was not affected by other endogenous miRNAs. Overall, the current study suggests that miRNA-965 and miRNA-1926 are likely to be involved in the regulation of Fib-L at the post-transcriptional level.

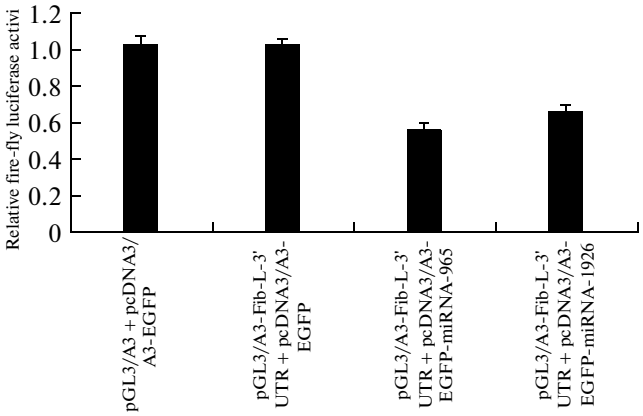


Fig. 3. Effect of miRNA expression on luciferase activity in transfected Sf21 cells. Data are represented as the mean value ± SD from three independent experiments.

EXPERIMENTAL

Materials

The silkworm strain P50 was provided by the Sericultural Research Institute of the Chinese Academy of Agricultural Sciences, Zhenjiang, China. The posterior silk glands of the fifth instar larvae 3 day were dissected, frozen immediately in liquid nitrogen, and stored at –80°C. A modified pcDNA3/A3-EGFP (Invitrogen) vector was previously constructed in our laboratory. The pGL3-basic vector, pRL-TK vector and Dual-luciferase assay kit were from Promega. Restriction enzymes, T4 DNA ligase, PCR reagents were obtained from TaKaRa Company (Dalian), Sf21 cells and cell culture Grace's medium, fetal bovine serum (FBS) and Lipofectamine were from Invitrogen.

Computational Predictions of miRNAs Targeting 3'UTR of Fib-L

The 3'UTR of silkworm Fib-L gene was obtained from the NCBI Database. The silkworm miRNA sequence database (miRBase) was obtained from the University of Manchester. Based on the regulation mechanism of miRNAs, two targets prediction software (RNA22 and RNAhybrid) were used to find potential miRNAs targeting with sequence of 3'UTR of Fib-L. The websites for these programs are as follows: RNAhybrid http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/ and RNA22 http://cbc-srv.watson.ibm.com/rna22/ [28, 29].

Vector Constructions

DNA fragments of 300 bp containing miRNA-965 or miRNA-1926 precursors were amplified from silkworm genomic DNA. The PCR primers were as follows: up: (5'–3') GAATTCCGATCTTAACAGGCCAGAAATGC, down: AGATCTCCACCAGACAGAAGGACCAGAGT, containing the EcoRI and BglII sites. The amplified fragments were cloned into the pcDNA3/A3-EGFP vector between the EGFP gene and the BGH poly(A) signal to generate the pcDNA3/A3-EGFP-miRNA-965 and miRNA-1926 constructs. The scheme of miRNAs expression vectors is shown in Fig. 4. To construct target vector with Fib-L 3'UTR sequence, pGL3-basic vector ontaining the luciferase reporter gene was used (Fig. 5). Silkworm cytoplasmic actin A3 promoter from silkworm DNA was inserted into the BglII and NcoI sites of pGL3-basic to create pGL3/A3. The resulting vector was subsequently digested with XbaI and FseI and then the 340 bp of 3'UTR sequence form Fib-L gene was inserted downstream of the luciferase gene at the XbaI and FseI sites to create pGL3/A3-Fib-L-3'UTR. The PCR primers were as follows (5'–3'): A3-up AGATCTCCGCTACGATATCATTATCATA; and A3-down, CCATGGCTTGAATTAGTATAGTATTATTAAATAAGT.

The PCR cycles were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 58°C for 45 s, and final extension at 72°C for 1 min. Fib-L 3'UTR-up, 5'-TCTAGAATAAGAACTGTAAATAATGTA; and Fib-L

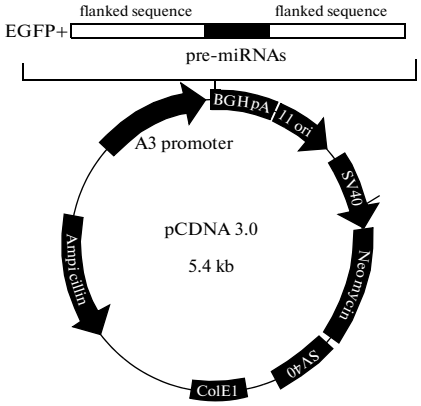


Fig. 4. Schematic map of miRNAs vector construction.

3'UTR-down, 5'-GGCCGGCCATCTGGAAAACTG-GATACA. The PCR cycles were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 64°C for 45 s, 72°C for 1 min, and final extension at 72°C for 8 min. The sequence of the PCR products and resulting vectors were confirmed by sequencing performed by a commercial service provider.

Cell Culture, Transfection and Dual Luciferase Assay

The insect Sf21 cells were grown in Grace's medium supplemented with 10% (v/v) FBS, 200 μg/mL penicillin, and 100 μg/mL streptomycin. Before transfection, the medium was removed and the cell was washed three times by serum-free Grace's medium. For miRNA expressed vectors and targets vectors co-transfection, p cDNA3/A3-EGFP-miRNAs (miRNA-965-1926; 500 ng each) and 50 ng pGL3/A3-Fib-L-3'UTR were mixed with 20 μL Lipofectamine2000. Two positive controls were used: (1) pGL3/A3 and pcDNA3/A3-EGFP vectors and (2) pGL3/A3-Fib-L-3'UTR and pcDNA3/A3-EGFP vectors, respectively. The pRL-TK vector of (5 ng) harboring the Renilla luciferase gene was co-transfected as an internal control for the transfection efficiency. Forty-eight hours after transfection, expression of EGFP was observed in cells, and then cells were washed with phosphate-buffered saline (PBS, pH 7.4) and treated with lysis buffer for luciferase assays. Luciferase activities were measured according to the manufacturer's instructions (Dual-luciferase assay system; Promega). All transfections were performed in three independent experiments with in triplicate each. Data are presented as mean ± SD. Significant differences between the samples were assessed using the Student *t* test. *P* values < 0.05 were considered statistically significant.

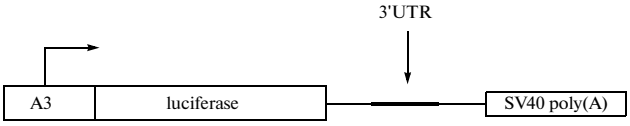


Fig. 5. Schematic map of luciferase assay strategy.

ACKNOWLEDGMENTS

This research was supported by the Natural Science Foundation of China (31172266) and Doctoral Science Foundation of He Nan University of Science (09001578).

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