



Construction and detection of expression vectors of microRNA-9a in BmN cells*

Yong HUANG^{1,3,4}, Quan ZOU², Sheng-peng WANG^{1,3}, Shun-ming TANG^{1,3},
 Guo-zheng ZHANG^{1,3}, Xing-jia SHEN^{†‡1,3}

(¹Key Laboratory of Silkworm and Mulberry Genetic Improvement, Ministry of Agriculture,
 Jiangsu University of Science and Technology, Zhenjiang 212018, China)

(²School of Information Science and Technology, Xiamen University, Xiamen 361005, China)

(³Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, China)

(⁴Animal Science and Technology College, Henan University of Science and Technology, Luoyang 471003, China)

[†]E-mail: shenxj63@yahoo.com.cn

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Abstract: MicroRNAs (miRNAs) are small endogenous RNAs molecules, approximately 21–23 nucleotides in length, which regulate gene expression by base-pairing with 3' untranslated regions (UTRs) of target mRNAs. However, the functions of only a few miRNAs in organisms are known. Recently, the expression vector of artificial miRNA has become a promising tool for gene function studies. Here, a method for easy and rapid construction of eukaryotic miRNA expression vector was described. The cytoplasmic actin 3 (A3) promoter and flanked sequences of miRNA-9a (miR-9a) precursor were amplified from genomic DNA of the silkworm (*Bombyx mori*) and was inserted into pCDNA3.0 vector to construct a recombinant plasmid. The enhanced green fluorescent protein (EGFP) gene was used as reporter gene. The *Bombyx mori* N (BmN) cells were transfected with recombinant miR-9a expression plasmid and were harvested 48 h post transfection. Total RNAs of BmN cells transfected with recombinant vectors were extracted and the expression of miR-9a was evaluated by reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blot. Tests showed that the recombinant miR-9a vector was successfully constructed and the expression of miR-9a with EGFP was detected.

Key words: miRNA-9a (miR-9a), EGFP gene, *Bombyx mori* N (BmN) Cells, Expression vector

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1 Introduction

MicroRNAs (miRNAs) are endogenous small RNA molecules (~22 nt) that post-transcriptionally regulate expressions of target genes (Bartel, 2004). miRNA genes are transcribed by RNA polymerase II in the nucleus to give primary miRNA transcripts,

which are capped at 5' end and polyadenylated at 3' end (Bushati and Cohen, 2007). These primary miRNA transcripts are processed by the RNase III enzyme Drosha into ~70-nt precursor miRNAs, which form an imperfect stem-loop structure (Filipowicz *et al.*, 2008). Precursor miRNAs are then transported into the cytoplasm by the transporter exportin-5, where they are cleaved by the cytoplasmic RNase III enzyme Dicer to yield ~22-nt miRNA duplexes. This duplex is then loaded into the multiprotein RNA-induced silencing complex (miRISC) which regulates the stability and translation of mRNAs that are partially or fully complementary to specific miRNA (Pillai *et al.*, 2007; Tang *et al.*, 2008).

* Corresponding author

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miRNAs play a crucial role in regulating development, cell differentiation, proliferation, species evolution, and diseases pathogenesis (Liu, 2008; Bartel, 2009). Some studies confirmed that artificial miRNA can be processed in a similar biogenesis pathway of natural miRNA and generate functional mature miRNA, as long as the basis of precursor remains unchanged or very few alterations within an miRNA sequence occur (Vaucheret *et al.*, 2004; Trujillo *et al.*, 2010). This result makes it possible to construct artificial miRNA expression vector targeting any gene of interest for further functional study (Parizotto *et al.*, 2004; Niu *et al.*, 2006; Park *et al.*, 2009).

The silkworm, *Bombyx mori*, is used as a model organism to represent lepidopteran insects that have been used for study of insect physiology, genetics and molecular biology. At present, by using computational and experimental methods, a total of 487 silkworm miRNAs have been identified on the miRBase database (<http://www.mirbase.org/>) (Tong *et al.*, 2006; Cao *et al.*, 2008; He *et al.*, 2008; Yu *et al.*, 2008; 2009; Huang *et al.*, 2009; Zhang *et al.*, 2009; Cai *et al.*, 2010; Jagadeeswaran *et al.*, 2010; Liu *et al.*, 2010). However, their functions in the silkworm are largely unknown. The miRNA-9a (miR-9a) is highly conservative and spreads in animals from insects to human. Ambros (2003) showed that the high conservation of miRNA is involved in physiological functions. Previous studies indicated that the miR-9a is involved in the developmental regulation of the wings and nervous system differentiation in *Drosophila* (Li *et al.*, 2006; Bejarano *et al.*, 2009; Biryukova *et al.*, 2009). Liu *et al.* (2009) found that miR-9a was highly expressed in the silkworm embryos, implying that miR-9a may play an important functional role during embryogenesis. Up until recently, few functional studies were performed in the silkworm miRNAs, leaving function of miR-9a unknown. We were interested in constructing a eukaryotic expression vector system containing the silkworm miR-9a gene. The miR-9a expression vector contains the silkworm actin 3 (A3) promoter and an enhanced green fluorescent protein (EGFP) reporter gene. In this study, an miR-9a expression vector was successfully constructed based on the flanked sequence of miR-9a precursor, expressed in *Bombyx mori* N (BmN) cells and confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blot. These

results will make this expression vector to facilitate further functional study of miR-9a in the silkworm.

2 Materials and methods

2.1 Materials

The silkworm strain used in this study was p50 and was reared in the Sericulture Research Institute, Chinese Academy of Agriculture Sciences. The plasmids of pCDNA3.0 (Invitrogen) and pEGFP-N3 (Clontech) were preserved in our laboratory. The silkworm ovary cells line BmN was gifted by the Institute of Life Sciences, Jiangsu University. Restriction enzymes, T4 DNA ligase and PCR reagents were obtained from TaKaRa. Primer was purchased from Sangon. Fetal bovine serum (FBS), the cell culture medium TC-100 and transfection reagent were from Invitrogen.

2.2 PCR amplifications of A3 promoter, EGFP and pre-miR-9a fragments

A3 promoter fragment was amplified from silkworm genomic DNA with the primer (up) 5'-AGATCTCCGCTACGATATCATTATCATA-3' and (down) 5'-AAGCTTCTTGAATTAGTATAGTATTATTAATAAGT-3', which contained *Bg*III and *Hind*III sites, respectively. The EGFP open reading frame (ORF) from pEGFP-N3 plasmids was amplified with the primer (up) 5'-AAGCTTGCGTGAGCAAGGGCGAGGA-3' and (down) 5'-GGATCCTTACTTGACAGCTCGTCCATGC-3', which contained *Hind*III and *Bam*HI sites, respectively. The 290-bp DNA fragment containing miR-9a precursor was amplified with the primer (up) 5'-GGATCCAA TTGAATAGAAAAAATATAACTTATC-3' and (down) 5'-GAATTCGACAGATATAGGGAAAGAGAA-3', which contained *Bam*HI and *Eco*RI sites, respectively. The PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 40 s, and final extension at 72 °C for 5 min.

2.3 Generation of plasmids

The cytomegalovirus (CMV) promoter was replaced by *Bombyx mori* A3 promoter in pCDNA3.0 vector because CMV did not have the biological activity in insect cells. The A3 promoter fragment was

treated with *Bgl*II and *Hind*III and then ligated into the pCDNA3.0 vector, which was treated using same restriction enzyme to form pBmA3 vector. The EGFP ORF was obtained from the pEGFP-N3 plasmid by PCR, and it was subcloned into the downstream of the A3 promoter at *Hind*III and *Bam*HI sites of pBmA3 vector to generate pBmA3-EGFP vector. Then, the expression vector pBmA3-EGFP-miR-9a was generated by inserting the miR-9a gene fragment into the *Bam*HI/*Eco*RI sites of pBmA3-EGFP. The EGFP was used as a reporter gene in this vector. The construction process is shown in Fig. 1. These constructs were identified by sequencing performed by a commercial service provider (Sangon Biotech).

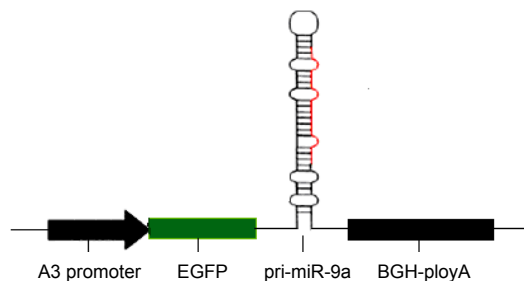


Fig. 1 Schematic map of pBmA3-EGFP-miR-9a vector construction

2.4 Cell transfection and detection of the EGFP fluorescence

The BmN cells were grown in a TC-100 medium supplemented with 10% (v/v) FBS, 200 µg/ml penicillin, and 100 µg/ml streptomycin at 27 °C with 5% CO₂. Transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions. Cell images with fluorescence were taken using Nikon E600 microscope equipped with Olympus camera and were processed by OpenLAB software.

2.5 RT-PCR assay

The BmN cell small RNA samples were isolated using mirVana™ miRNA isolation kit (Ambion) according to the manufacturer's instructions. The cDNAs were synthesized from small RNAs by using miRNA specific stem-loop primers according to the criteria as described previously (Chen *et al.*, 2005; Feng *et al.*, 2009). The stem-loop RT-primer was 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATAC-3'; the primer was 5'-GCGGCTCTTTGGTTATCTAG-3' (up); and the

primer was 5'-GTGCAGGGTCCGAGGT-3' (down). The cDNAs synthesis was performed using 1 µl of diluted first-strand cDNA (1/10, v/v). PCRs were performed in a 20-µl mixture containing 1 µl cDNA, 0.5 µmol/L up and down primers, 10× PCR buffer, 0.25 µmol/L each of deoxyribonucleoside triphosphate (dNTPs) and 2 U Taq polymerase, under the following conditions: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s.

2.6 Northern blotting

The expression of miR-9a was detected by Northern blotting analysis at 48 h after transfection. Total RNA was extracted from BmN cells using a TRIzol reagent and quantified by GeneQuant spectrophotometer (Bio-Rad) before loading sample. For each sample, 20 µg of total RNAs were run on 12% (v/v) polyacrylamide gel (SequaGel, National Diagnostics) and transferred to a Nytran SuPerCharge Signal membrane (Schleicher & Schuell). The miR-9a was detected with a purified 3' P-end-labelled oligo probe, 5'-TCATACAGCTAGATAACCAAAGA-3', which is complementary to the mature miR-9a sequence.

3 Results

3.1 Identification of recombinant plasmid

The miR-9a expression vector was successfully constructed based on the flanked sequence of the miR-9a precursor, and this vector contained the silkworm A3 promoter and an EGFP reporter gene (Fig. 1). The miR-9a precursor was amplified by the use of recombinant plasmid template. The PCR products were detected in a 0.01 g/ml agarose gel by electrophoresis containing ethidium bromide, and photographed under ultraviolet (UV) light. A band at approximately 290 bp was separated, which was consistent with the expected molecular mass (Fig. 2a). The miR-9a precursor fragment could also be isolated from the pBmA3-EGFP-miR-9a vector after the recombinant plasmid was digested with *Bam*HI and *Eco*RI (Fig. 2b). These results implied that the recombinant plasmid pBmA3-EGFP-miR-9a was successfully constructed.

3.2 EGFP reporter gene expression

EGFP was observed under a fluorescent

microscope. At 48 h post transfection with the recombinant miR-9a expression plasmid, a fluorescence signal was detected in the most of the cells (Fig. 3).

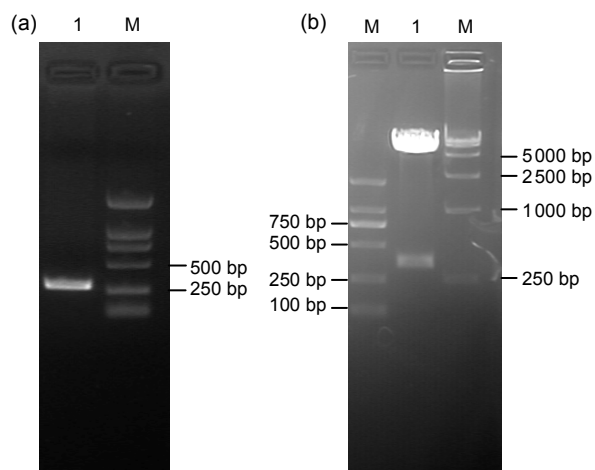


Fig. 2 Amplification of miR-9a precursor and double enzymatic digestion

(a) PCR amplification of the miR-9a precursor DNA (M: DL2000 maker; Lane 1: PCR products); (b) Characterization of the recombinant plasmids after digestion with restriction enzymes (M: DNA maker; Lane 1: pBmA3-EGFP-miR-9a digested by *Bam*HI and *Eco*RI)

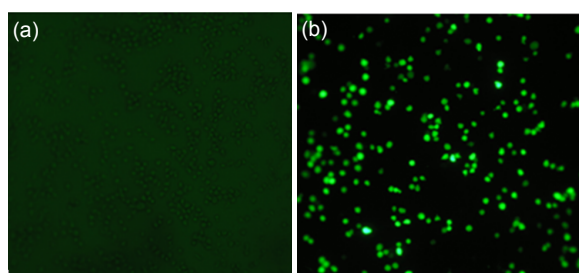


Fig. 3 BmN cells transfected with pBmA3-EGFP-miR-9a plasmid-bright light (a) and pBmA3-EGFP-miR-9a plasmid-fluorescence (b)

3.3 Detection of expression by RT-PCR

The BmN cells transfected with the recombinant miR-9a expression plasmid were harvested 48 h post transfection. The RT-PCR results showed that a ~60-bp fragment was amplified, suggesting that the recombinant plasmid of miR-9a was successfully transfected into BmN cells and miR-9a precursor can be cut into mature miR-9a (Fig. 4). The results implied that the maturity of approximately 20 bp miR-9a was successfully expressed in BmN cells.

3.4 Northern blot assay

To accurately assess whether the recombinant plasmid was appropriately processed into the mature miRNA, the pBmA3-EGFP-miR-9a vector was transfected into BmN cells and the total RNA was isolated. Northern blot analysis showed that miR-9a was expressed most obviously in the transfected BmN cells (Fig. 5), but miR-9a was not detected in untransfected BmN cells or BmN cells transfected with pBmA3-EGFP plasmid, suggesting that endogenous miR-9a was not expressed in this cell line. This suggests that miR-9a expression vector is recognized by Drosha and Dicer as a miRNA precursor and processed into mature miRNA-9a in BmN cells. These results show that the plasmid can express miR-9a gene effectively in BmN cells.

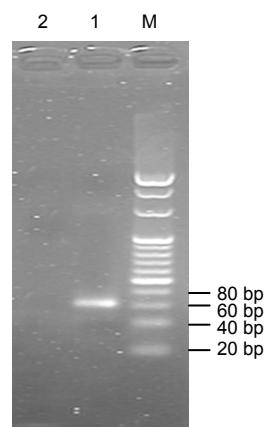


Fig. 4 RT-PCR amplification of mature miR-9a

M: 20-bp ladder maker; Lane 1: PCR products amplified with recombinant plasmid in transfected BmN cells; Lane 2: no PCR products amplified in untransfected BmN cells

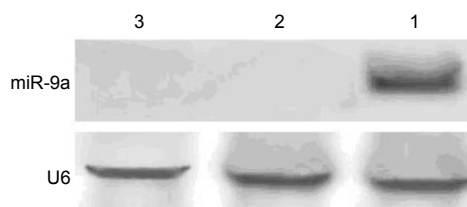


Fig. 5 Northern blot detection of miR-9a expression in BmN cells

Lane 1: BmN cells transfected with pBmA3-EGFP-miR-9a plasmid; Lane 2: BmN cells transfected with pBmA3-EGFP plasmid; Lane 3: normal BmN cells. U6 served as an internal control

4 Discussion

Research evidence indicates that miRNAs participate in a variety of biological activities, including cell differentiation, biological development, genesis of disease, tumorigenesis, and host-viral interactions (Bartel, 2004; Stefani and Slack, 2008). In the past few years, the important role played by miRNAs in gene regulation has been recognized (Filipowicz *et al.*, 2008). It is estimated that miRNAs comprise about 1%–3% of genes in animal and may regulate 10%–30% protein-coding genes (Friedman *et al.*, 2009; Hong *et al.*, 2009). Although the role played by miRNAs in target regulation has been recognized, very little is known about their functions.

Currently, there are primarily three approaches for the functional research of miRNAs: construction of eukaryotic expression vectors, in vitro transcription (Hu *et al.*, 2010; Schmollinger *et al.*, 2010), and direct chemical synthesis (Shibata *et al.*, 2007; Hu *et al.*, 2009; Shan *et al.*, 2009; 2010). Compared with the chemical synthesis or in vitro transcription, construction of the eukaryotic expression vector should be more economical, and applied in long-term research of its biological functions. Endogenous primary miRNA transcripts share some similar structural characteristics that comprise a stem, a terminal loop, long flanking sequences, and some internal loops or bulges, which likely contribute to efficient processing in miRNA pathways (Ritchie *et al.*, 2007; Scherr and Eder, 2007; Qiu *et al.*, 2008). Previous experiments confirmed that the flanking sequences at 5' and 3' ends of miRNA precursors are necessary for miRNA expression (Chen *et al.*, 2004; Zhou *et al.*, 2005; Chang *et al.*, 2006; Fukuda *et al.*, 2006). Zeng *et al.* (2002) first successfully utilized miR-30 precursor to express functional mature miRNA. The sequence flanking miRNA precursor has also proven critical for primary miRNA transcripts to be processed into the miRNA precursor by Drosha complex (Zeng and Cullen, 2005; Rumi *et al.*, 2006). Sun *et al.* (2006) also used an extended miR-30 hairpin of 118 nt as a backbone for artificial miRNA expression. Based on these previous results, various studies have been carried out successfully with regards to the construction of miRNA expression vectors (Han *et al.*, 2006; Molnar *et al.*, 2009; Xu *et al.*, 2009; Sun *et al.*, 2010). So, we reasoned that the flanked sequence of the

extended miR-9a hairpin of 100 nt on each side of miR-9a is essential and sufficient for the expression of miR-9a. The eukaryotic expression method is considered fairly inexpensive and can generate functional miRNAs to suppress target genes in cells (Silva *et al.*, 2005; Gou *et al.*, 2007). In addition, there are also commercial kits for miRNA expression vector construction, but these expression vectors can express miRNAs only in mammalian cells and are not suitable for miRNA expression in insect cells.

Exogenous genes can be faithfully and efficiently transcribed in BmN cells derived from the ovarian tissue of the silkworm (Sakurai *et al.*, 1990). Previous studies have confirmed that EGFP ORF was placed at the 5' end of miRNA gene, and EGFP and mature miRNAs were expressed (Cai *et al.*, 2004; Du *et al.*, 2006). In our study, based on knowledge on the primary miRNAs driven by polymerase II and natural miRNA biogenesis, a method for the generation of miRNA expression vectors was developed, which can efficiently expressed miRNAs in silkworm BmN cells. This study demonstrated that the construction of miRNA expression vectors is a better approach for further function study of miRNAs in vitro or in the silkworm.

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