Journal of Asia-Pacific Entomology 19 (2016) 439–445

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

Gene screening and differential expression analysis of microRNAs in the middle silk gland of wild-type and naked pupa mutant silkworms (*Bombyx mori*)

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ARTICLE INFO

Article history:
Received 26 November 2015
Revised 23 February 2016
Accepted 22 March 2016
Available online 2 April 2016

Keywords:
Bombyx mori
MicroRNA
Middle silk gland
Naked pupa mutant

ABSTRACT

MicroRNAs (miRNAs) are a class of endogenous, non-coding small RNAs that serve as important post-transcriptional gene expression regulators and play important roles in the silkworm (*Bombyx mori*) development, growth, and viral immunity. However, information on the diversity of these regulatory RNAs in the middle silk gland (MSG) of naked pupa (*Nd*) mutant silkworms is limited. In this study, by using Solexa high-throughput sequencing technology, we identified and compared small RNA libraries from the MSG of wild-type silkworm P50(MSG-P50) and the *Nd* mutant (MSG-Nd), respectively. A total of 272 conserved and 333 novel miRNAs were identified, in which 141 ones showed significantly different expression patterns between MSG-P50 and MSG-Nd, and 10 ones were randomly selected and validated by stem-loop quantitative reverse-transcription polymerase chain reaction (qRT-PCR). In addition, potential targets were predicted for differentially expressed miRNAs based on sequence complementation between miRNAs and their target genes. Gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation revealed miRNAs that actively participate in various life processes and three pathways associated with protein synthesis including endoplasmic reticulum pathway, ribosome pathway, and ribosome biogenesis in eukaryotes, were significantly disrupted in MSG-Nd. This is the first comprehensive description of miRNAs in the silkworm MSG. Overall, the results provide useful information for future studies on miRNAs and suggest that the fibroin synthetic deficiency in the posterior silk gland impairs the sericin secretion process in MSG.

Introduction

MicroRNAs (miRNAs) are endogenous, non-coding, approximately 18–24 nt RNAs that negatively regulate gene expression by specifically pairing to messenger RNAs (mRNAs) of protein-coding genes and promoting their degradation (Carrington and Ambros, 2003; Bartel, 2009). Since identification of the frist miRNA from *Caenorhabditis elegans*, numerous studies have been conducted in a variety of organisms, including plants, animals, and viruses (Lee et al., 1993; He and Hannon, 2004; Bushati and Cohen, 2007). To date, a total of 35,828 mature miRNAs has been identified from 223 species and submitted to miRBase (http://www.mirbase.org/). Due to their ubiquitous distribution, miRNAs participate in a wide range of biological processes, including cellular proliferation, growth, programmed cell death, and immune responses (White et al., 1999; Brennecke et al., 2003; Zhang et al., 2007; Wu et al., 2013a). Computational predictions have shown that over 30% of animal genes are regulated by miRNAs and that a single molecule can regulate several hundreds of target mRNAs at the post-transcriptional level (Lewis et al., 2005; Bushati and Cohen, 2007).

The domesticated silkworm, *Bombyx mori*, is one of the most important model organisms for investigating various biological processes, including development, gene regulation, and morphological innovation (Goldsmith et al., 2005). Additionally, the successful sequencing of the silkworm genome as well as the effective selection of mutants are ideal tools for genetic study (Xia et al., 2004). Previous studies have identified a considerable number of *B. mori* miRNAs (bmo-miRNAs) and verified their important role in lifespan control (Cao et al., 2008; He et al., 2008; Yu et al., 2008; Liu et al., 2009, 2010; Zhang et al., 2009; Huang et al., 2010; Jagadeeswaran et al., 2010).

Normal silkworm cocoons consist of sericin and fibroin, which are synthesized in the middle silk gland (MSG) and the posterior silk gland (PSG), respectively (Shimura, 1983). The naked pupa mutant (*Nd*) silkworm is deficient in fibroin-secretion, producing about 0.3% of normal strains, and spins a very poor cocoon (Inoue et al., 2005), due to a partial deletion of the fibroin heaven chain gene (*Bmfib-H*)

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http://dx.doi.org/10.1016/j.aspen.2016.03.009

that affects the disulfide linkage between heavy (H) and light (L) chains of fibroin (Tanaka et al., 1999). Previous studies on the *Nd* mutant mainly focused on PSG, which is directly responsible for the poor cocoon formation (Mori et al., 1995; Barbosa et al., 2008). Nevertheless, information on the location of sericin synthesis and fibroin accumulation in MSG is limited. In the present study, we used Solexa high throughput sequencing method to identify miRNAs in the MSG of wild-type P50 and the *Nd* mutant silkworm larvae and analyze differential expression genes to provide a better understanding of miRNA function and regulation mechanism of silk protein synthesis mechanism

Materials and methods

Silkworm strain

The silkworm strains used were P50 and the *Nd* mutant. silkworms raised on mulberry leaves at 25 °C and 80 ± 5% relative humidity with a 12 h light: 12 h dark photoperiod. MSGs of day-3 5th instar larvae were collected for RNA extraction or stored at − 80 °C for subsequent experiments.

Extraction of total RNA from MSG

MSGs of day-3 5th instar larvae were frozen in liquid nitrogen and ground for total RNA extraction using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). The quantity and integrity of total RNA were evaluated by Agilent 2100 (Agilent, Santa Clara, CA, USA).

Small RNA library construction and high-throughput sequencing

P50 and Nd mutant individuals were collected and homogenized in TRIzol RNAiso Plus (TaKaRa, Dalian, China). Total RNA was extracted, according to the manufacturer's instructions, and quantified by Agilent 2100 Bioanalyzer (Agilent). Small RNA fragments of 18–30 nt were isolated from the total RNA pool with polyethylene glycol precipitation and separated on 15% denaturing polyacrylamide gel electrophoresis (PAGE). Then, 59 and 39 adaptors were sequentially ligated to purified small RNAs, followed by reverse transcription to obtain sufficient products for Solexa high-throughput sequencing. The sequencing was performed by IlluminaHiSeq 2000 (Biomarker, Beijing, China).

Sequencing data analysis and identification of miRNAs

The sequencing of miRNAs generated millions of short reads. Raw sequencing data were filtered by eliminating adaptor contaminants to generate usable reads of 18–24 nt and exclude reads of low quality. Then, the clean reads were annotated and classified by Rfam (http://rfam.sanger.ac.uk), GenBank (http://blast.ncbi.nlm.nih.gov), miRBase, and RepBase (www.girinst.org/repbase). In some cases, one read was mapped to more than one category. To acquire the best annotation information of every unique small RNA, we established priority criteria as follows: ribosomal RNA (rRNA) > known miRNA > repeat > exon > intron. The reads corresponding to mRNA, rRNAs, transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), small conditional RNAs (scRNAs), repeat sequences, and small nucleolar RNAs (snoRNAs) were removed, and the residual sequences were further analyzed.

Detection of conserved and novel miRNA

The identification of conserved miRNAs was carried out by comparing the unique sequences with known miRNAs in miRBase 21. Sequences of reads identical (without or with one nucleotide substitution) to the mature miRNAs were identified as known miRNAs. Unmatched data were aligned to the silkworm genome to predict novel miRNA candidates using MIREAP (http://sourceforge.net/projects/mireap/) as described by Allen et al. (2005) and Friedländer et al. (2008). In detail, novel miRNAs were identified based on the miRNA sequence length (20–26 nt), the miRNA reference sequence length (20–24 nt), the minimal depth of Drosha/Dicer cutting site (3), the maximal copy number of miRNAs on reference (20), the maximal free energy allowed for a miRNA precursor (–18 kcal mol^{-1}), and the flank sequence length of miRNA precursor (10). Then, the secondary structure of individual miRNAs was analyzed using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) as described by Mathews et al. (1999). Potential miRNA-like hairpins were selected based on the number of base pairs in a stem (≥18 nt), the length of hairpin (53 nt), the percentage of miRNA in the stem (≥80%), the folding free energy of the hairpin (≤20 kcal mol^{-1}), and the minimal folding free energy index (MFEI; 0.85).

Verification of miRNAs by quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

To verify the presence and expression of identified miRNAs, 10 differentially expressed novel miRNAs were selected for qRT-PCR. The specific forward primers (Table S1) were designed based on miRNA sequences. Reverse transcription reactions were subsequently conducted using One Step PrimeScript RT reagent Kit (TaKaRa, Dalian, China), according to the manufacturer's instructions. The total RNA sample used in qRT-PCR was the same with that used in high-throughput sequencing. The qRT-PCR was performed using iCycleriQ real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with SYBR Premix ExTaq II (TaKaRa, Dalian, China). The U6 snRNA was selected as a reference gene for normalization, and the $2^{-\Delta\Delta Ct}$ method was used to evaluate the relative expression level of miRNA (Chen et al., 2005).

Target prediction of miRNAs

miRNAs negatively regulate gene expression by base pairing between the 5′ end of the miRNA (i.e., 2–8 nt, the“seed”region) and the 3′ untranslated regions (3′ UTR) of target mRNAs (Bartel, 2004; Wu et al., 2006; Easow et al., 2007). Mireap, miRanda (Bartel, 2009) and RNAhybrid(Kruger and Rehmsmeier, 2006) software were used to predict target genes of the miRNA by searching the Bombyx mori gene database (http://silkworm.genomics.org.cn/).

Analysis of miRNA expression levels in MSG-P50 and MSG-Nd libraries

We compared the expression pattern of miRNAs in MSG-P50 and MSG-Nd libraries to identify differences in expressed miRNAs between P50 and the *Nd* mutant. The expression levels of miRNAs in the two libraries were normalized as follows: Normalized expression = (Actual miRNA sequencing read count/Total mappable read count). The miRNA expression levels of zero in one library were modified in 0.01 to enable calculation, whereas the miRNA expression levels of less than 1 in both libraries were excluded from the analysis.

Gene ontology (GO) enrichment and KEGG pathway analysis of target genes

To identify biological processes regulated by differentially expressed miRNAs, we conducted GO enrichment and KEGG pathway analyses by locally running BLAST against UniProt (http://www.uniprot.org). The number of all target gene candidates in GO terms was calculated, and then hypergeometric testing was used to identify significantly altered GO terms as follows:

$$P = 1\text{-}\sum_{i=0}^{m-1}\frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{M}{n}}$$

In the formula above, N is the number of all genes with GO annotation; n is the number of target gene candidates in N, M is the number

Table 1
Read distribution of small RNAs in the middle silk gland (MSG) of wild-type (P50) and naked pupa (*Nd*) mutant silkworms.

	MSG-P50		MSG-Nd	
	Total reads	Percent	Total reads	Percent
Total Tags number	17,842,102	100.00%	18,040,932	100.00%
Filter having 'N' Tags	10,491	0.06%	10,588	0.06%
Filter low-quality Tags	1,060,284	5.94%	1,049,283	5.82%
Adaptor3 insert null	1	0.00%	0	0.00%
5′ adaptor contaminants	4	0.00%	5	0.00%
Length <18	1,744,451	9.78%	2,223,137	12.32%
Length >24	2,669,309	14.96%	2,132,837	11.82%
PloyA	243,548	1.37%	126,163	0.70%
Clean number	10,807,806	60.57%	11,735,160	65.05%
Uniq number	1,306,208	7.32%	763,759	4.23%

of all genes that are annotated to a certain GO term, and m is the number of target gene candidates in M. We used the Bonferroni correction for the p-value to obtain a corrected p-value. GO terms with corrected p-values of ≤ 0.05 are defined as significantly enriched in target candidate genes. This analysis is able to recognise the main biological functions for target gene candidates.

KEGG pathway analysis was carried out as described for GO. To determine significant enrichment of the gene sets, we selected a corrected *p*-value of <0.05 as the threshold in GO (Xue et al., 2010) and a false discover rate (FDR) of <0.05 in KEGG analyses.

Results

High-throughput sequencing of silkworm small RNAs

To systematically identify miRNAs involved in silkworm development, MSG-P50 and MSG-Nd were sequenced side by side by Illumina HiSeq 2000 and information or read distribution is summarized in Table 1. A total of 17,842,102 and 18,040,932 raw reads was produced from MSG-P50 and MSG-Nd, respectively. After filtering reads with low quality, length lower than 18 nt, length higher than 24 nt, poly A contamination, adapter sequences, and junk sequences, a total of 10,807,806 and 11,735,160 clean reads was generated from MSG-P50 and MSG-Nd, respectively. The reads were subjected to analysis of length distribution (Fig. 1A), which showed a unimodal distribution pattern in both libraries with a peak around 19–21 nt. By classifying reads with non-redundancy, 1,306,208 and 763,759 unique reads, accounting for 7.32% and 4.23% of the total reads, were generated from MSG-P50 and MSG-Nd, respectively. The length distribution of unique reads showed that the major size in both libraries was 20 nt (Fig. 1B). Comparison of the total reads with unique reads in the two libraries (Fig. 2) revealed common sequences in 39.98% of the total reads, whereas MSG-P50- and MSG-Nd-specific reads accounted for 9.67% and 50.35%, respectively. Common sequences had only 11.19% of unique reads, and the majority of unique reads were library-specific, indicating the differential small RNA expression patterns in MSG-P50 and MSG-Nd. (See Fig. 3.)

Identification of conserved and novel miRNAs in silkworm

To identify conserved miRNAs in silkworm, 1,178,761 filtered unique sequences from MSG-P50 and MSG-Nd were aligned against 567 mature miRNAs and 489 precursors in miRBase. Information on the conserved miRNA is summarized in Table S2. A total of 272 miRNAs was identified, of which 186 were common in both libraries. The position of mature miRNA in each stem-loop precursor is presented in Table S3. In 272 conserved miRNAs, the expression number varied from 100,000 to less than 10 sequence reads (Table S2). In detail, six of the most highly expressed miRNAs (miR-9c, bantam, miR-2766, miR-276, miR-100, and miR-279b) exceeded 100,000 reads. In contrast, 104 miRNAs were infrequently sequenced with reads of less than 10 in the two libraries. We also identified 86 miRNAs specifically expressed in MSG-P50 or MSG-Nd, but all were at low frequency, except for miR-2836 (Table S4).

By systematic analysis of dicer digestion sites and hairpin structures of pre-miRNAs, we identified 333 novel silkworm miRNAs in MSG-P50 and MSG-Nd libraries (Table S5). However, MSG-P50 and MSG-Nd shared less than 50% of novel miRNAs; 192 of them were MSG-P50 specific, and 16 were MSG-Nd specific. Compared with conserved miRNAs, almost all novel miRNAs exhibited relatively low read counts (the average read counts of conserved miRNAs were 3514 and of novel miRNAs were only 328). Only four novel miRNAs exceeded 10,000 reads, whereas the other 179 novel miRNAs had less than 10 reads each. The secondary structure of the three most abundant novel miRNAs, novel-m0129, novel-m0233, and novel-m0094, was tested, and the results showed that all three sequences formed typical stem-loop hairpins. The folding free energy of novel-m0129, novel-m0233, and novel-m0094 was −42.55, −28.38, and −32.84 kcal/mol, respectively, and their MFEI was 3.86, 3.14, and 2.09 kcal mol^{-1}, respectively.

Differentially expressed miRNAs in MSG-P50 and MSG-Nd

The expression levels of miRNAs in the two libraries were measured by Illumina sequence frequencies and further normalized in the form of Fragments Per Kilobase of transcript per Million mapped reads (FPKM) (Table S6). Using high-throughput sequencing data, we first searched for miRNAs that were present only in one library and detected 11 miRNAs specifically expressed in MSG-P50 or MSG-Nd (sequence read

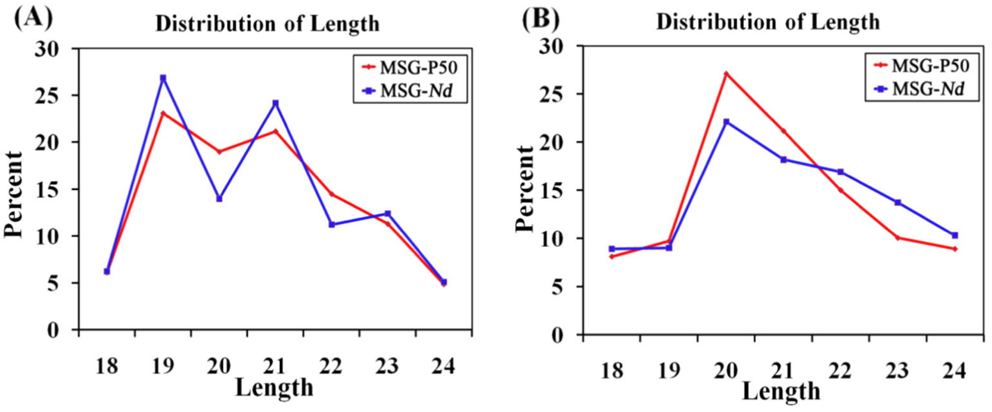

Fig. 1. Sequence length distribution of microRNAs in the middle silk gland (MSG) of wild-type (P50) and naked pupa (*Nd*) mutant silkworms. A: Total read length distribution. B: Unique read length distribution.

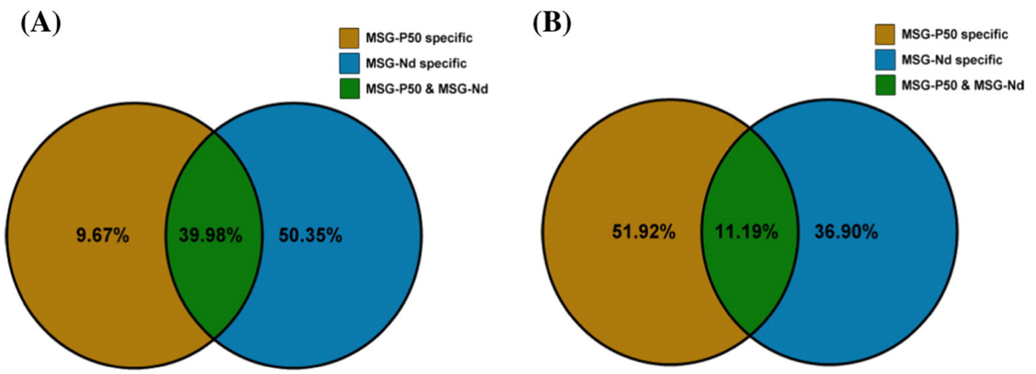

Fig. 2. Venndiagram of common and specific sequences in the middle silk gland (MSG) of wild-type (P50) and naked pupa (*Nd*) mutant silkworms (MSG-P50 and MSG-Nd, respectively). A: total reads; B: unique reads.

>100), of which 10 were novel, suggesting their pivotal role in the development of MSG. Then, the up- or down-regulated miRNAs were screened based on an absolute value of log2 ratio fold-change of >1.0 for $P < 0.01$. We searched for miRNAs that were present in both libraries and detected 130 differentially co-expressed miRNAs, of which 79 were conserved, and 51 were novel. Most miRNAs were down-regulated in MSG-Nd, whereas only seven (miR-2780b, miR-2849, miR-2780a-3p, miR-2998, novel-m0225-3p, novel-m0225-5p, and novel-m0179-3p) were up-regulated. Therefore, the miRNA concentration and composition were significantly altered when silkworms naturally mutated to naked pupae. To verify the Illumina sequencing data, 10 differentially expressed novel miRNAs were selected for qRT-PCR. The results demonstrated that all of them were amplified (data not shown), and that the miRNA expression patterns were similar to those obtained by high-throughput sequencing. Both novel-m0225-5p and novel-m0179-3p were up-regulated, whereas the others were down-regulated in the *Nd* mutant (Fig. 4).

Target prediction of miRNAs and gene enrichment analysis

In the MSG-P50 and MSG-Nd, 2522 target genes were predicted for 272 conserved miRNAs, whereas 1376 target genes were predicted for 333 novel miRNAs.

To validate target prediction, qRT-PCR was performed on the predicted target gene of representative miRNAs. We predicted that *cactus* and *REL1* were target genes of miR-375, a miRNA with a 5-fold higher expression level in MSG-P50 compared with that in MSG-Nd. Therefore, we quantified the expression level of *cactus* and *REL1* in both MSG-P50 and MSG-Nd and found that both were significantly down-regulated in MSG-P50 compared with MSG-Nd. We also predicted that the juvenile hormone methyltransferase gene (*JHAMT*) was a target of miR-2998, a miRNA that was significantly up-regulated in MSG-Nd. Therefore, we quantified the expression level of *JHAMT* in both MSG-P50 and MSG-Nd and found that it was significantly up-regulated in MSG-P50 compared with MSG-Nd. (Fig. 5)

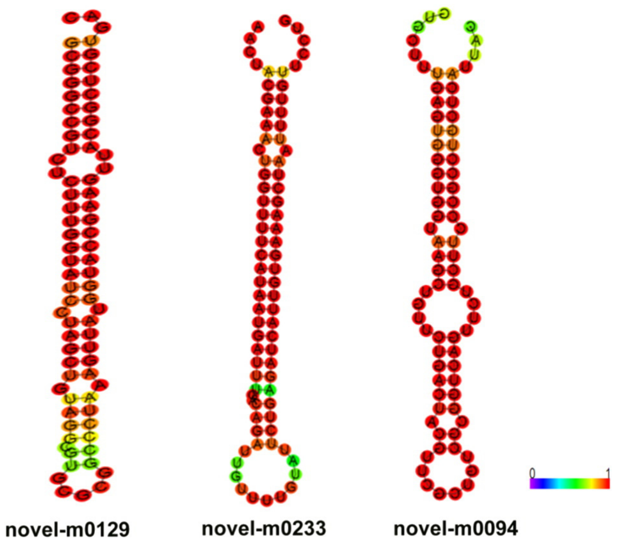

Fig. 3. Predicted secondary structures of three novel microRNAs in silkworm.

GO enrichment and KEGG pathway analyses

To gain a better insight into the functional roles of miRNAs, all target genes regulated by the differentially expressed miRNAs were subjected to GO enrichment and KEGG pathway analyses. The three main categories (biological processes, cellular components, and molecular functions) of GO are summarized in Fig. 6. The analysis of target genes based on biological processes showed that the most significantly enriched GO terms were cellular process, metabolic process, biological regulation, respond to stimulus, and localization, whereas based on molecular functions, the four most enriched GO terms were binding, catalytic activity, transporter activity, and structural molecular activity. KEGG results showed that several pathways were altered, and the 20 most disrupted pathways are listed in Table S7. Some of them were identical to biological processes revealed by GO analysis. We identified 133 target genes involved in protein processing in the endoplasmic reticulum, 128 target genes involved in RNA transportation, and 119 target genes involved in the spliceosome.

Discussion

Silkworm miRNAs have been studied more extensively than many other insect species, and as a result, 489 precursors and 567 mature miRNAs has been submitted to miRBase. Previous studies mainly focused on the expression patterns of miRNAs in different developmental stages, viral immunity, or specific tissues (Zhang et al., 2009; Cai et al., 2010; Huang et al., 2010; Jagadeeswaran et al., 2010; Liu et al., 2010; Wu et al., 2013a). However, few studies have focused on miRNAs in specific tissues of silkworm mutants such as the PSG of the fibroin-secretion-deficient *Nd* mutant.

In this study, the majority of miRNAs was detected in both libraries and shared similar expression patterns. These results were consistent with recent reports in silkworm, locust, and brown planthopper (Jagadeeswaran et al., 2010; Chen et al., 2012). The sequenced miRNAs encompassed almost 50% of known silkworm miRNAs, whereas another 295 miRNAs were not detected, probably because they were expressed at extremely low levels or only during specific developmental stages (Li et al., 2012; Liu et al., 2013). Among the 20 most abundant miRNAs, miR-9c, bantam, miR-2766, miR-276, miR-100, and miR-279b were

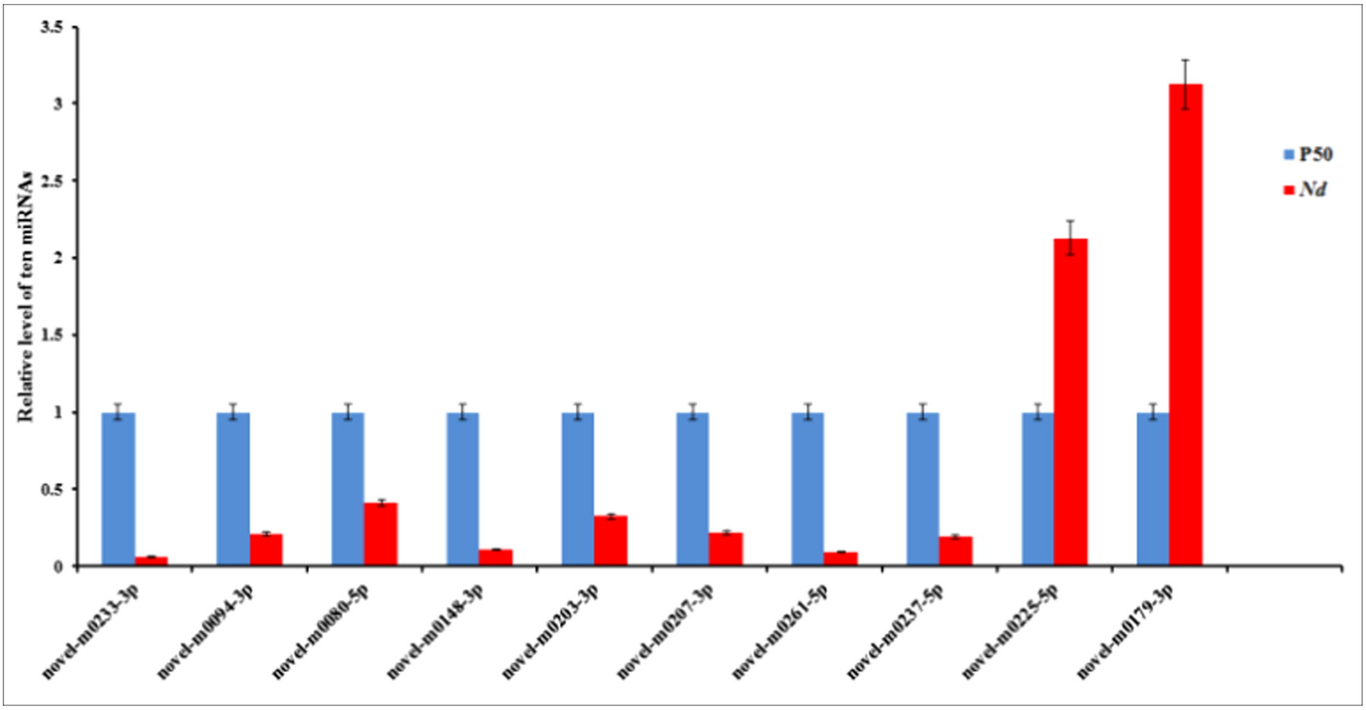

Fig. 4. Expression analysis of 10 differentially expressed novel microRNAs (miRNAs) in wild-type (P50) and naked pupa (*Nd*) mutant silkworms. Error bars indicate the 95% confidence interval of the mean.

very frequently sequenced with more than 100,000 reads. These results were in agreement with those reported previously in silkworm and other insect species (Khajuria et al., 2013; Wu et al., 2013a, 2013b). The six most abundant miRNAs, namely, miR-9c, miR-276, miR-2766, miR-100, miR-281, and mir-14, are metamorphosis-related miRNAs. Insect metamorphosis is a complex, highly conserved, and strictly regulated developmental process (Jagadeeswaran et al., 2010). Numerous predicted targets of miRNAs are essential for insect development, molting, and metamorphosis, especially those related to ecdysone and juvenile hormone (JH) (Riddiford, 1996). In silkworm, miR-9c and miR-276 are preferentially expressed in spinning larvae, and the expression level is altered during pupal metamorphosis (Liu et al., 2009; Zhang et al., 2009). The juvenile hormone esterase (JHE) is a target of mir-2766, which is involved in JH biosynthesis and degradation (Jagadeeswaran et al., 2010). The ecdysone receptor (EcR) is a target of miR-281 and mir-14, which play a crucial role in metamorphosis (Varghese and Cohen, 2007; Jiang et al., 2013). Moreover, miRNAs related to sense organ development, energy homeostasis, insulin signaling,

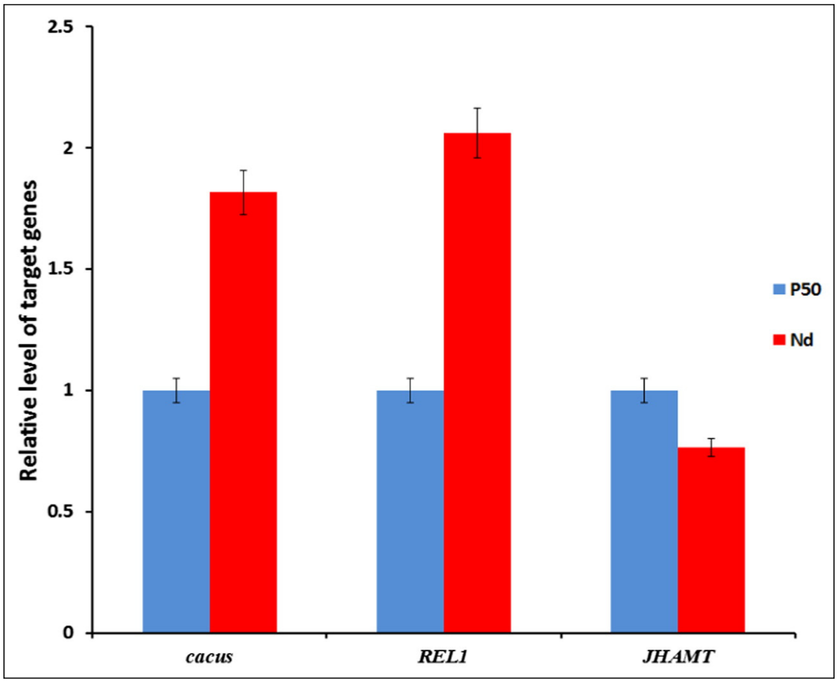

Fig. 5. Expression analysis of three micro RNA target genes in wild-type (P50) and naked pupa (*Nd*) mutant silkworms. Error bars indicate the 95% confidence interval of the mean.

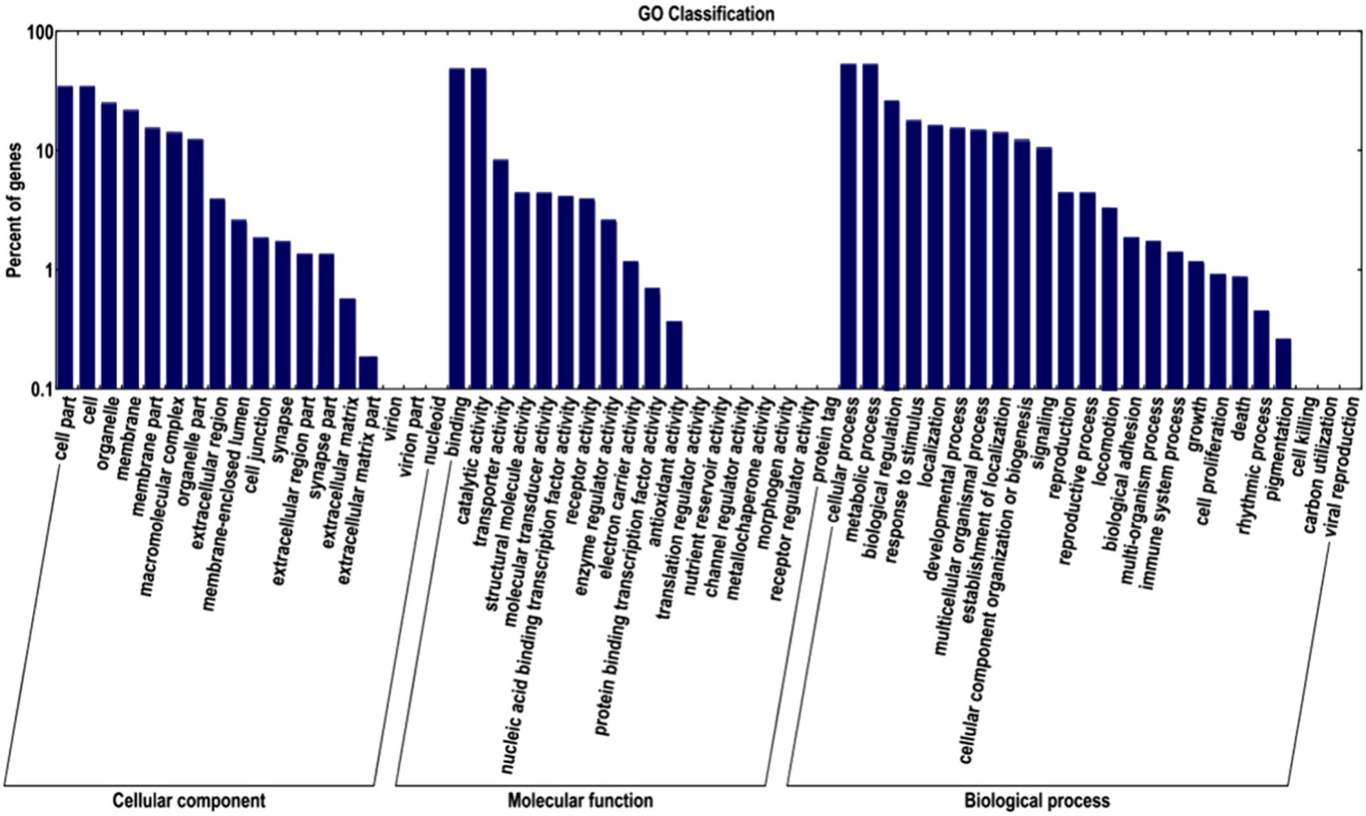

Fig. 6. Gene ontology (GO) categories and sub-categories of target genes of differentially expressed micro RNAs in the middle silk gland (MSG) of wild-type (P50) and naked pupa (*Nd*) mutant silkworms.

and immune response are also abundantly expressed. These findings suggest that miRNAs in MSG actively participate in diverse life processes, and several targets of miRNAs may interact with each other to complete a single physiological process.

We also identified 123 down-regulated and seven up-regulated miRNAs in MSG-Nd. These aberrantly expressed miRNAs might play roles in morphological changes between P50 and the *Nd* mutant. The most significantly up-regulated miRNA was miR-2998, which has been reported to interact with *JHAMT*, a gene that participates in the conversion of JH acids or farnesoic acid (FA) to JH in various insect species (Kinjoh et al., 2007; Kenny et al., 2013). The novel miRNA m0225 was also up-regulated in the *Nd* mutant, and target analysis revealed that it was related to multiple targets such as ecdysteroid kinase, fatty acid CoA ligase, sulfate transporter, and RNA polymerase II. These targets correspond to insect molting, energy metabolism, and the nutrient transport process (Wojtczak and Schönfeld, 1993; Arif et al., 2003; Vincourt et al., 2003).

Gene silencing occurs when miRNA binds to the target mRNA and prevents mRNA from being translated. The up-regulation of miRNAs in the *Nd* mutant resulted in the lower expression of target mRNAs. Additionally, 123 miRNAs were down-regulated in MSG-Nd, and the range of their predicted functions included viral immunity, osteoclast differentiation, glycosphingolipid-biosynthesis-associated apoptosis, metamorphosis, and circadian rhythm. For example, the transcription level of mir-375 was 5-fold higher in MSG-P50 than in MSG-Nd. In a previous study, the complementary target sites of mir-375 were identified and confirmed in the 5′ untranslated region of the Toll immune pathway genes, *cactus* and *REL1*. Elevating expression of miR-375 results in the reduction of *cactus* and the increase of *REL1* transcripts in *Aedes aegypti* (Lucas et al., 2013). The different miRNA repertoires in MSG-P50 and MSG-Nd indicate that the *Nd* mutant has an altered miRNA expression pattern that affects its biology.

The KEGG analysis of up-regulated and down-regulated targets revealed that protein processing in the endoplasmic reticulum pathway was significantly different between P50 and the *Nd* mutant. The endoplasmic reticulum is a factory where secretory proteins are manufactured and stringent quality-control systems ensure that only correctly folded proteins are sent to their final destinations (Sitia and Braakman, 2003). In silkworm, fibroin proteins are synthesized in PSG and transported to the lumen of MSG, where fibroin is coated with sericin secreted by cells of MSG (Shimura, 1983). The participation of fibroin is required in the synthesis and folding process of three forms of sericin layers (types I, II, and III) (Mondal et al., 2007). The poorly developed PSG of the *Nd* mutant barely secrets fibroin, which results in decreased fibroin accumulation in the lumen of MSG. As a result, the sericin secretion process in the endoplasmic reticulum pathway is disrupted because of the absence of fibroin in MSG.

Two other pathways associated with ribosomes (ribosome pathway and ribosome biogenesis in eukaryotes) were also significantly different between P50 and the *Nd* mutant. Ribosomes are the integration point of the regulatory control of translation in eukaryotes, and a large number of functional ribosomes is required for protein production (Blanchard, 2009). Previous studies in other low-fibroin-production silkworm strain showed that numerous ribosomal proteins and transcripts were down-regulated (Wang et al., 2014). Fibroin and sericin are the main proteins secreted by silkworms, and they interact with each other closely to form the functional cocoon. In the *Nd* mutant, sparse secretion of fibroin in PSG inevitably disrupts the regulation network of miRNAs in MSG, especially of those that participate in protein synthesis. The altered post-transcriptional regulatory molecules of the endoplasmic reticulum and ribosomes may decrease their productivity and quantity, which inevitably impacts the efficiency of protein synthesis and secretion. Therefore, we conclude that the *Nd* mutant is not only fibroin-secretion deficient, but also unable to produce high-quality sericin.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aspen.2016.03.009.

Acknowledgments

This work was supported by the National Nature Science Foundation of China (no.31402143) and the National Nature Science Foundation of China (no.31172266).

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