Genes Genom (2015) 37:703–712
DOI 10.1007/s13258-015-0300-7

RESEARCH ARTICLE

Characterization and profiling of MicroRNAs in posterior silk gland of the silkworm (*Bombyx mori*)

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Received: 26 December 2014 / Accepted: 25 May 2015 / Published online: 31 May 2015

Abstract MicroRNAs (miRNAs) regulate expression of genes at post-transcriptional level by binding on complementary sequences of target mRNAs and play multiple roles in biological processes. To investigate the differential expression of miRNAs in posterior silk gland (PSG) of silkworm (*Bombyx mori*) in different periods and regulation of miRNAs on the expression of fibroin genes, Solexa sequencing technology was used to detect miRNAs in PSGs of fourth-instar day-2 larvae and fifth-instar day-3 larvae, respectively. As a result, 466 previously reported miRNAs, and 35 novel miRNAs were detected, and 499 of these detected miRNAs are predicted to target 13,383 genes by target prediction softwares. Additionally, 29 miRNAs expressed differently between the PSG of fourth-instar day-2 larvae and fifth-instar day-3 larvae were found, and the differential expression of these miRNAs may play an important role in the expression of fibroin genes.

Keywords MicroRNA · *Bombyx mori* · Posterior silk gland · Differential expression · Solexa sequencing · Target gene

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Introduction

MicroRNAs (miRNAs) are a class of endogenous non-protein-coding small RNAs of ~22 nt that regulate the expression of target genes by binding on complementary sequences of target mRNAs (Bartel 2004). Following the initial discovery of miRNA, lin-4, in *Caenorhabditis elegans* in 1993 (Lee et al. 1993), an increasing number of miRNAs have been identified. Up to date, 567 miRNAs have been identified in the silkworm (*Bombyx mori*) (miRBase, http://www.mirbase.org/). The previous studies showed that miRNAs played crucial roles in various physiological processes, including development, hematopoietic lineage differentiation, organ formation, proliferation, apoptosis, host-viral interactions and tumorgenesis (Bushati and Cohen 2007; Guarnieri and DiLeone 2008).

Silkworm has been domesticated for over 5000 years and is well-known for its industrial importance in sericulture and economic value of silk production. The silk protein is composed of the silk fibroin (Fib) and the glue protein sericin (Ser), and the fibroin is composed of fibroin heavy chain (Fib-H), fibroin light chain (Fib-L) and fibrohexamerin (Fhx/P25) with a 6:6:1 molar ratio (Inoue et al. 2000). The silk gland is the site where silk proteins are synthesized and secreted, and can be divided into three distinct morphological and functional compartments: anterior silk gland (ASG), middle silk gland and posterior silk gland (PSG). The PSG exclusively synthesizes the fibroin proteins including Fib-H, Fib-L and Fhx or P25 (Bello and Couble 1990; Hui and Suzuki 1989; Suzuki et al. 1972; Suzuki and Suzuki 1974).

The expression of the fibroin genes is regulated spatially and temporally. They express in each period of larval stage, and the expression increases sharply in full appetite stage of the fifth-instar. This expression trait of fibroin genes is

co-regulated by some developmental stage-specific factors and tissue-specific factors such as POU-M1, Bm Fkh and FMBP-1 (Hui and Suzuki 1989; Takiya et al. 1997). The regulation on the expression of fibroin genes is mainly at the transcriptional level and 5′ flanking regions of fibroin genes are the major transcriptional regulatory regions, including the core promoters, the upstream enhancer elements, the PSG-specific enhancer elements, the intronic enhancer elements and the remote enhancer element REE. There are at least six cytokines involved in the regulation through binding to the upstream transcriptional modulator of *Fib-H* gene, such as SGF-1, SGF-2, SGF-3, SGF-4, FMBP-1, FBF-A1 (Hui and Suzuki 1989; Takiya et al. 1997).

The fibroin genes are expressed specifically in PSG of silkworm larvae. It is significant to analyze the expression profile of miRNA in the PSG and study their target genes. It was reported that bmo-miR-33 and bmo-miR-7 inhibited the expression of *Fib-L* gene by using GUS reporter gene experiment (Cao et al. 2008). In previously study, we found that silkworm miRNA bmo-miR-2b down-regulated *P25* expression (Huang et al. 2011), and bmo-miR-965 and bmo-miR-1926 inhibited the expression of *Fib-L* gene in vitro (Huang et al. 2012), suggesting that silkworm miRNAs involve in regulating expression of fibroin genes in PSG.

To investigate the expression profile of miRNAs in PSG and the regulation of miRNAs on the fibroin genes, we sequenced small RNAs in PSGs of fourth-instar day-2 larvae and fifth-instar day-3 larvae respectively by Solexa, identified miRNAs and compared the expression of miRNAs between two samples and predicted target genes as well. It will provide new experimental data for clarifying the function of miRNAs and benefit to elaborate the regulatory mechanism of fibroin synthesis.

Materials and methods

Materials

Domesticated silkworm (*B. mori*) strain P50 is obtained from the Sericultural Research Institute, Chinese Academy of Agricultural Sciences. The silkworms were reared on mulberry leaves at 25 °C, 80 ± 5 % relative humidity, with a 12 h light: 12 h dark photoperiod.

Extraction of total RNAs from PSG

PSGs of fourth-instar day-2 larvae and fifth-instar day-3 larvae were collected respectively, for RNA extraction or stored in −80 °C for subsequent experiments. Then they were separately frozen in liquid nitrogen and grinded for total RNA extraction using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). Total RNA samples were immediately stored at −80 °C for deep sequencing.

Solexa sequencing

Sequencing was conducted in Illumina HiSeqTM 2000 by Biomarker Co. Ltd (Beiing China) according to the method previously described (Ma et al. 2011). Small RNAs in the desired size range (15–30 nt) were purified from denaturing 15 % polyacrylamide gel and sequentially ligated with the 3′ and 5′ adapters as follows: (5′rApp/TGGAATTCTCGGGTGCCAAGG/3′ddC) and (5′GUUCAGAGUUCUACAGUCCGACGAUC). Then reverse transcription reaction and PCR amplification was performed. The PCR products in the desired size ranging from 140 to 160 bp were purified from polyacrylamide gel. Finally these PCR products were shipped to Illumina HiSeqTM 2000 for sequencing.

Bioinformatic analysis of sequencing data from PSG

Firstly, the raw data were processed to obtain clean reads through the elimination of the following aspects: (a) low quality reads; (b) 3′ and 5′ adaptor sequences; (c) empty vector sequences; (d) reads shorter than 16 nt; (e) reads longer than 30 nt; (f) reads with poly(A); (g) contaminants. Then, the clean reads were mapped to the silkworm genome to analyze their distribution on the genome. Sequences with perfect matches were retained for further analysis. Afterwards, the clean reads were compared against the Rfam databases (http://rfam.sanger.ac.uk/) to annotate the known non-coding RNAs (rRNAs, tRNAs, snRNAs, snoRNAs, etc.). The sequences were aligned against the reported miRNA precursors and mature miRNAs deposited in the miRBase v20. (http://www.mirbase.org/) to identify known miRNAs, and aligned to RepBase (http://www.girinst.org/repbase/index.html) to annotate the repetitive sequences. Degraded fragments of mRNAs were identified by aligning the clean reads with exons and introns of mRNAs annotated on the silkworm genome. Finally all the reads annotation information were integrated, and the following priority rules were used to ensure that every unique small RNA was mapped to only one annotation: rRNA etc. > known miRNA > repeat > exon > intron.

Identification of novel miRNAs from PSG

The unannotated sequences were used to predict potential novel miRNA candidates by the Mireap software (http://sourceforge.net/projects/mireap/). RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) was used to further analyze the secondary structures of miRNA candidates.

Prediction of miRNA target genes

The miRanda (Bino et al. 2004) (http://www.microrna.org/microrna/home.do) was used to detect potential target sites of the known miRNAs and the predicted novel miRNAs. The parameters employed were as follows: match score $S \geq 100$ and target duplex free energy $\Delta G \leq -20$ kcal/mol. To functionally categorize the target genes, gene ontology (GO) and chip on glass (COG) analysis were conducted. The functions of target genes were annotated with the GO and COG pathway annotations of homologous genes, and in accordance with that miRNAs were categorized.

Expression profile of miRNAs in PSG

The expression profiles of miRNAs in PSG of fourth-instar day-2 larvae and fifth-instar day-3 larvae were compared by IDEG6 software (Chiara et al. 2003) (http://telethon.bio.unipd.it/bioinfo/IDEG6/). The general Chi-square method were used to detect the differential expression of miRNAs, and miRNAs with a $P \leq 0.01$ and ratio of TPM values (Tags Per Million reads: TPM is a normalized tag count calculated by dividing the number of reads mapped to the miRNAs by the total clean reads, then multiplying by a million) more than 2 times were deemed to be significantly different between the two samples.

Results

Distribution of small RNAs from PSG

The result of Solexa sequencing showed that totally 22,042,778 and 21,442,934 raw reads were obtained in PSGs of fourth-instar day-2 larvae and fifth-instar day-3 larvae, respectively. After removing the low quality reads, adaptors and etc., 13,891,284 and 12,561,241 clean reads of the two samples were ultimately obtained, and totally 1,323,648 and 1,140,082 unique sequences were obtained by clustering (Table 1). Then, the unique sequences were mapped to the silkworm genome to analyze their distribution on the genome, leading to 1,210,069 (91.42 %) and 1,046,397 (91.78 %) genome-matched reads (Table 2). Sequences with perfect matches were retained to compare against the Rfam databases to annotate the known non-coding RNAs, and classified as rRNA, tRNA, snRNA and snoRNA, as shown in Table 3.

Length distribution of unique sequences from PSG

The result of length distribution of unique sequences in PSGs showed that the lengths of the majority small RNAs were 16–30 nt in PSGs of both fourth and fifth instar larvae with an even distribution, and the most abundant small RNA sequences was 20 nt (Fig. 1). The unique sRNA sequences in the PSG of fifth instar showed no significant difference with those of fourth instar.

Identification of known miRNAs from PSG

The unannotated reads compared against the Rfam database were aligned against the reported miRNAs precursors and mature miRNAs deposited in the miRBase to identify known miRNAs. As shown in Table 4, 796,397 and 949,619 sequences (representing 10,863 and 11,633 unique reads, respectively) in PSGs of fourth and fifth instar larvae matched known miRNAs of the silkworm, belonging to 71 and 72 families respectively. The members in different families vary widely, and large differences of expression abundance existed among these families with the reads number ranging from 1 to 150,592.

The largest reads number identified in the PSG of fourth-instar day-2 larvae is the bantam family with 116,806 reads, followed by mir-8 family with 89,507 reads. In the PSG of fifth-instar day-3 larvae, the largest reads number is mir-8 family with 150,592 reads, followed by bantam family with 109,487 reads. Thus, after throwing off overlapping reads, totally 466 known miRNAs were identified from PSGs of fourth and fifth-instar larvae of *B. mori*.

Prediction of novel miRNAs from PSG

The unannotated small RNAs were used to predict potential novel miRNAs candidates by mireap as previously reported (Yue et al. 2012), and 17 and 23 potential novel miRNAs candidates were obtained from the PSG library of fourth-instar day-2 and the fifth-instar day-3 larvae, respectively (Table 5). In which 5 miRNAs were detected in both libraries, thus a total of 35 novel miRNAs were predicted. Of which Bmo-m0001 expresses in rather high level both in PSGs of fourth-instar day-2 and fifth-instar day-3 larvae, reaching 145 and 224 reads respectively. However, most of these 35 novel miRNAs showed very low expression abundance. Then these novel miRNAs were aligned against known miRNAs of other species in miRBase, and it was found that Bmo-m0006 showed high homology with known miRNAs of many other species, even showed 100 % homology with hme-miR-193-3p of *Heliconius melpomene* and mse-miR-193 of *Manduca sexta*. We explored the hairpin structures and minimal free energies of these novel miRNAs using RNAfold software. The free energy of 35 candidates obtained ranged from −18.1 to −47.1 kcal/mol with the average of −26.36 kcal/mol (Table 6). In addition, the lengths of these novel miRNAs ranged from 20 to 26 nt, and the most abundant class was 23 nt, accounting for 31.42 % (Table 6).

Table 1 Summary of Solexa sequencing data

Type of fragments	Fourth-instar day-2 PSG		Fifth-instar day-3 PSG	
	Reads number	Percentage	Reads number	Percentage
Total tags number	22,042,778	100.00	21,442,934	100.00
Filter low quality reads	1,192,805	5.41	1,171,017	5.46
Adaptor3 insert null	727	0.00	3054	0.01
5′Adaptor contaminants	0	0.00	0	0.00
Length < 16	3,199,453	14.51	5,631,140	26.26
Length > 30	3,500,123	15.88	1,867,513	8.71
PloyA	258,386	1.17	208,969	0.97
Clean number	13,891,284	63.02	12,561,241	58.58
Unique number	1,323,648	6.00	1,140,082	5.32

Table 2 Summary of Genome blast result

Type of fragments	Fourth-instar day-2 PSG		Fifth-instar day-3 PSG	
	Reads number	Percentage	Reads number	Percentage
Total	1,323,648	100.00	1,140,082	100.00
Mapped	1,210,069	91.42	1,046,397	91.78
Pefect map	762,553	63.02	668,200	63.86
Unpefect map	447,516	36.98	378,197	36.14
Unmapped	113,579	8.58	93,685	8.22

Table 3 Distribution of small RNAs among different categories in fourth-instar day-2 and Fifth-instar day-3 PSGs

RNA types	Fourth-instar day-2 PSG				Fifth-instar day-3 PSG			
	Unique reads	Unique ratio (%)	Total reads	Total ratio (%)	Unique reads	Unique ratio (%)	Total reads	Total ratio (%)
rRNA	223,209	55.21	4,228,078	70.72	194,500	51.54	3,467,893	60.19
tRNA	34,012	8.41	615,730	10.30	36,430	9.65	917,099	15.92
snoRNA	28,718	7.10	184,376	3.08	29,295	7.76	208,359	3.62
snRNA	21,906	5.42	100,626	1.68	20,508	5.43	100,736	1.75
Other	96,438	23.85	849,909	14.22	96,637	25.61	1,067,348	18.53
Total	404,283	100.00	5,978,719	100.00	377,370	100.00	5,761,435	100.00

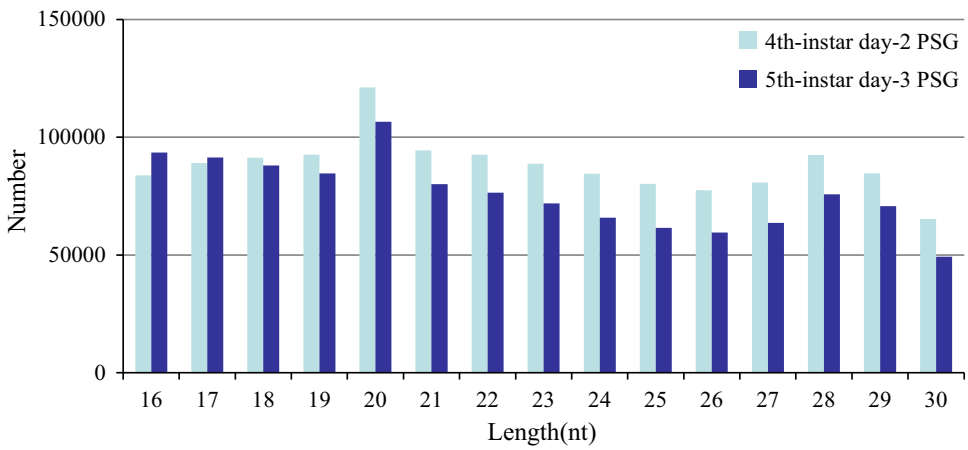

Fig. 1 Length distribution of the unique sequences in fourth-instar day-2 and fifth-instar day-3 PSGs

Table 4 Summary of the known microRNAs

Sample	Mature number	Hairpin number	Family number	Unique number	Total number
Fourth-instar day-2 PSG	548	367	71	10,863	796,397
Fifth-instar day-3 PSG	527	352	72	11,633	949,619

Table 5 Prediction of the novel microRNAs

Sample	Novel_hairpin	Novel_mature	Unique reads	Total reads
Fourth-instar day-2 PSG	16	17	48	274
Fifth-instar day-3 PSG	20	23	69	386

Target genes prediction and function analysis of miRNAs detected from PSG

To further understand the physiological functions and biology processes involved by these miRNAs during silk gland development, target genes prediction was performed based on interaction between miRNAs (466 known and 35 novel miRNAs) and mRNAs by using Miranda software, and 13,383 target genes were obtained for 499 miRNAs. The predicted target genes were classified according to GO and COG function annotations to identify the pathways which the target genes involved in and annotate the target genes. The GO covers three domains: molecular functions, cellular components, biological processes. The GO analysis revealed that most of the target genes were associated to biological processes ontology, including cell proliferation, immune system process, stress response, metabolic processes, biological regulation etc. The COG analysis revealed that the target genes were mainly involved in amino acid transport and metabolism, carbohydrate transport and metabolism, DNA replication, recombination and repair, signal transduction, which is in accordance with fibroin biosynthesis in PSG of *B. mori*, but most targets were annotated to have general function. Thus, these target genes were involved in many metabolic pathways. It indicated that these miRNAs detected from PSG were involved in many metabolic pathways too. Validation of the relationship between miRNAs and the target genes need further more biological experimental evidences.

Differential expression of miRNAs between two PSG libraries

IDEG6 software was used to analyze the differential expression of miRNAs between two PSG libraries, and 29 differential expressed miRNAs were obtained which were all the known miRNAs. Out of the 29 miRNAs, 24 miRNAs were up regulated and the other 5 miRNAs were down-regulated in the PSG of fifth-instar compared with those in PSG of fourth-instar (Table 7). Two of them, bmo-miR-2772a and bmo-miR-277* were the most up regulated ones increased by 17.3-fold and 15.4-fold respectively. And bmo-miR-2772b was the most down-regulated one declined by 16.6-fold.

In these 29 miRNAs, there were 5 pairs of miRNA and miR* delivered from the opposing arm of precursor miRNA, including bmo-let-7/bmo-let-7*, bmo-miR-274/bmo-miR-274*, bmo-miR-308/bmo-miR-308*, bmo-miR-375/bmo-miR-375* and bmo-miR-9c/bmo-miR-9c*. The expression of these 5 miRNAs and their miR* were all up regulated with the similar expression trend (Fig. 2). In the previous reports, miR* species recovered at a lower frequency than that of their partners (Kato et al. 2009). However, in our results, the counts of miR* species exceeded those of their miR species, which is in accordance with Jagadeeswaran's research on *B. mori* (Jagadeeswaran et al. 2010).

Discussion

There are 567 microRNAs identified in the silkworm miRNAs database up to now. Li (Li et al. 2014) found 1229 microRNAs in the PSG at the fifth larval instar aided by sequencing and microarry assay, including 728 novel miRNAs and 110 miRNA/miRNA* duplexes, and these novel miRNAs have to be validated by experiments. And target gene prediction yields 14,222 unique target genes from these miRNAs. In our study, we detected 35 novel miRNAs and 466 known miRNAs (82 % of the known silkworm miRNAs) in PSG of 4–5 instar larvae of *B. mori* by Solexa sequencing. Based on interaction of miRNAs and mRNAs, 13,383 target genes were predicted by Miranda software. These results indicated that there are complicated metabolic activities in PSG, and the miRNAs were likely to play important roles in silk gland development and fibroin biosynthesis.

Researches revealed that each miRNA normally has multiple target genes, on the other hand each gene may be a

Table 6 List of the novel microRNAs predicted

miRNA	Mature sequences	5′/3′	Length (nt)	Free energy (kcal/mol)	4th-instar day-2 PSG		4th-instar day-2 PSG	
					Uniq tag	Total tag	Uniq tag	Total tag
Bmo-m0001	ATTTATTTATATAGATAGTACAA	3′	23	−18.10	1	145	1	224
Bmo-m0002	GGTGGTTCGAACGCGGAATT	3′	20	−28.40	1	3	0	0
Bmo-m0003	AATATACTCTATAGGATAACCT	5′	22	−28.30	4	20	6	32
Bmo-m0004	TCGCTGTACAAAAGTCAACATTG	5′	23	−22.60	4	14	0	0
Bmo-m0004*	ATTTGGCTTATGTTCAAGCGATTAT	3′	25	−22.60	1	1	0	0
Bmo-m0005	TTTGCGATTGCTCGTCAACATTAT	3′’	24	−34.90	2	3	0	0
Bmo-m0006	TACTGGCCTGCTAAGTCCCAAG	3′	22	−35.30	2	5	0	0
Bmo-m0007	TACGGTACTGCTTGGACCCATT	3′	22	−29.20	4	17	4	15
Bmo-m0008	CGCATTGTCATAGAAAGAGACGTC	5′	24	−24.82	3	3	4	4
Bmo-m0009	GGCACGAATTTTAATGTAGATGTG	5′	24	−27.50	4	4	0	0
Bmo-m0010	TGACGAAATACATCGATGGT	5’	20	−18.90	2	4	0	0
Bmo-m0011	TAATTCGGGGACTTTAGAAAT	3′	21	−37.30	1	3	0	0
Bmo-m0012	TACTGAATGAAGGCGCCTCT	3′	20	−22.90	3	4	0	0
Bmo-m0013	GTTATCGGAGCCTGCAGTCT	3′	20	−19.00	2	5	0	0
Bmo-m0014	TATCGTAATGCTGGAGAATTTTA	5′	23	−22.90	2	3	0	0
Bmo-m0015	AAAGGATTTAACAGACATGAAGTT	3′	24	−25.80	4	26	7	21
Bmo-m0016	TCTGACGTTAACATGAGGTTGAA	3′	23	−23.50	3	9	0	0
Bmo-m0017	TGACTAGACCCTAAACAGGGTAAT	3′’	24	−31.40	0	0	2	15
Bmo-m0018	GGTGGTTCGAACGCGGAATTT	3′	21	−29.70	0	0	2	4
Bmo-m0019	CCATTAGTCTATGCGAGCTTGA	3′	22	−25.80	0	0	3	4
Bmo-m0020	TTGACAATGTTAATGAATCGCT	3′	22	−24.40	0	0	1	3
Bmo-m0021	TTGGAATTCAATTATGAGTATGA	5′	23	−22.30	0	0	1	3
Bmo-m0022	CACGCATGGTCACTCGCACAAAC	3′	23	−21.50	0	0	5	5
Bmo-m0023	TCACATGTACGCGGTCAAGGCT	5′	22	−47.10	0	0	4	5
Bmo-m0024	TACATTTGGATGGTTTAGTTAGT	5′	23	−19.46	0	0	2	3
Bmo-m0024*	TACACTGTCCAAGTTACTGAT	3′	21	−19.46	0	0	1	1
Bmo-m0025	GGCACGAATTTTAATGTAGATGT	5′	23	−27.50	0	0	5	5
Bmo-m0026	TATCAATGCTTTCAAAACTTGGGA	5′	24	−22.10	0	0	2	3
Bmo-m0026*	TACTTTTTAGCTTGAGAGGGCTGATT	3′	26	−22.10	0	0	1	1
Bmo-m0027	ATGCGGGGATGTAGCTCAGTGGT	5′	23	−32.00	0	0	3	3
Bmo-m0028	CGGCACTGCACGTGATCTCACT	3′	22	−34.60	0	0	2	3
Bmo-m0029	TCTCAGGGCAGGCTGCGCCA	3′	20	−26.80	0	0	2	12
Bmo-m0030	TAAAGTCATCGTTGGTTGGTGTC	3′	23	−27.30	0	0	4	5
Bmo-m0031	CTTAATCTTTGGTCTGGCTTCGG	5′	23	−23.50	0	0	1	1
Bmo-m0031*	TCTGACGTTAACATGAGGTTGAAA	3′	24	−23.50	0	0	3	11

target of multiple miRNAs (Vo et al. 2010; Wu et al. 2010). As shown in this study, a total of 13,383 genes were predicted to be the targets of 499 miRNAs, and each target gene had one or more binding sites for miRNAs. So focusing on any particular target gene may not lead to complete understanding the function of a certain miRNA.

It's helpful to investigate the miRNA expression profiles for better understanding the role of miRNAs in fibroin biosynthesis in PSG of *B. mori*. In this study, bmo-mir-3281, bmo-bantam, bmo-mir-8 and bmo-mir-276-3p showed very high expression abundance both in PSGs of fourth and fifth instar larvae. Compared with the above miRNAs, most of detected miRNAs were expressed at low levels, with less than 100 reads in the two libraries, which was consistent with previous findings (Song et al. 2010; Zhao et al. 2010). These variations in abundance could also reflect differences in the roles of these miRNAs in terms of the regulation of biological processes (Silveri et al. 2006).

The expression approaches of miRNAs are different. Some express in all cells in each developmental stage, while

Table 7 List of differentially expressed miRNAs in the posterior silk glands

miRNA	Fifth-instar day-3 PSG	Fourth-instar day-2 PSG	Chi	Q_value	Regulation
Bmo-miR-100	5729	1410	0	0	Up
Bmo-miR-375	1180	416	0	0	Up
Bmo-miR-34*	130	32	0	0	Up
Bmo-miR-9a*	70	197	0	0	Down
Bmo-miR-2755*	268	90	0	0	Up
Bmo-miR-2766*	5074	1964	0	0	Up
Bmo-miR-317*	532	235	0	0	Up
Bmo-miR-263a*	3783	1595	0	0	Up
Bmo-miR-308*	579	108	0	0	Up
Bmo-let-7*	305	88	0	0	Up
Bmo-miR-305*	87	316	0	0	Down
Bmo-miR-9c	7933	3011	0	0	Up
Bmo-miR-998	312	127	0	0	Up
Bmo-miR-11*	1579	508	0	0	Up
Bmo-miR-277*	649	42	0	0	Up
Bmo-miR-274*	3249	1437	0	0	Up
Bmo-miR-9c*	348	96	0	0	Up
Bmo-miR-2772a	52	3	0	0	Up
Bmo-miR-2772b	3	50	0	0	Down
Bmo-miR-375*	2239	550	0	0	Up
Bmo-miR-7*	403	189	0.000001	7.68E−06	Up
Bmo-miR-2756*	140	44	0.000001	7.68E−06	Up
Bmo-miR-308	139	46	0.000002	1.44E−05	Up
Bmo-miR-282*	14	40	0.000002	1.44E−05	Down
Bmo-miR-3333	309	148	0.000027	0.0001815	Up
Bmo-miR-79*	236	108	0.00006	0.0003924	Up
Bmo-let-7	70	20	0.000136	0.0008439	Up
Bmo-miR-275	2	12	0.00094	0.005687	Down
Bmo-miR-274	45	12	0.001306	0.0077086	Up

others have a more restricted spatial and temporal expression pattern. And the abundance of miRNA in different tissues and different developmental stages were significantly different. In this study, bmo-mir-3281, bmo-bantam, bmo-mir-8 and bmo-mir-276-3p showed very high abundance in both libraries. The previous study revealed bmo-bantam, bmo-mir-8 and bmo-mir-276-3p expressed at all the four stages (feeding larval, spinning larval, pupa and adult) of *B. mori*, bmo-mir-8 and bmo-mir-276-3p expressed in much higher level than bmo-bantam did in the larval period (Jagadeeswaran et al. 2010). But bmo-bantam emerged at specific time-points during embryogenesis. It was up-regulated in late embryos, and robustly expressed from the embryo to adult (Liu et al. 2009). The expression level of bmo-bantam was significantly higher in ASG than that in PSG based on Northern-blot, indicating that miRNAs were unlikely to be transported among different tissues (Liu et al. 2010). Bmo-bantam and bmo-mir-8 expressed in 10 silkworm tissues including head, body wall, ASG, PSG, midgut, fat body, ovary, testis, hemocyte and malpighian tubule, while bmo-mir-276-3p expressed in 9 tissues except testis (Liu et al. 2010), suggesting that bmo-bantam, bmo-mir-8 and bmo-mir-276-3p are ubiquitously and play significant roles in the silkworm development.

The miRNA bantam has been studied in detail. Bantam mutant *Drosophila* is smaller than wild type due to a reduction in cell number but not in cell size, and it does not have significant disruption in patterning. Conversely, overexpression of the bantam causes overgrowth of wing and eye tissue (Hipfner et al. 2002). The pro-apoptotic gene hid was a target of bantam miRNA, providing an explanation for bantam's stimulating cell proliferation and preventing apoptosis (Brennecke et al. 2003).

The miR-8 was an inhibitor of Wg signaling in *Drosophila*, in part by directly targeting wntless, a gene required for Wg secretion. In addition, miR-8 inhibits the pathway of Wg signal by repressing CG32767 (another positive regulator of the pathway), which was targeted by

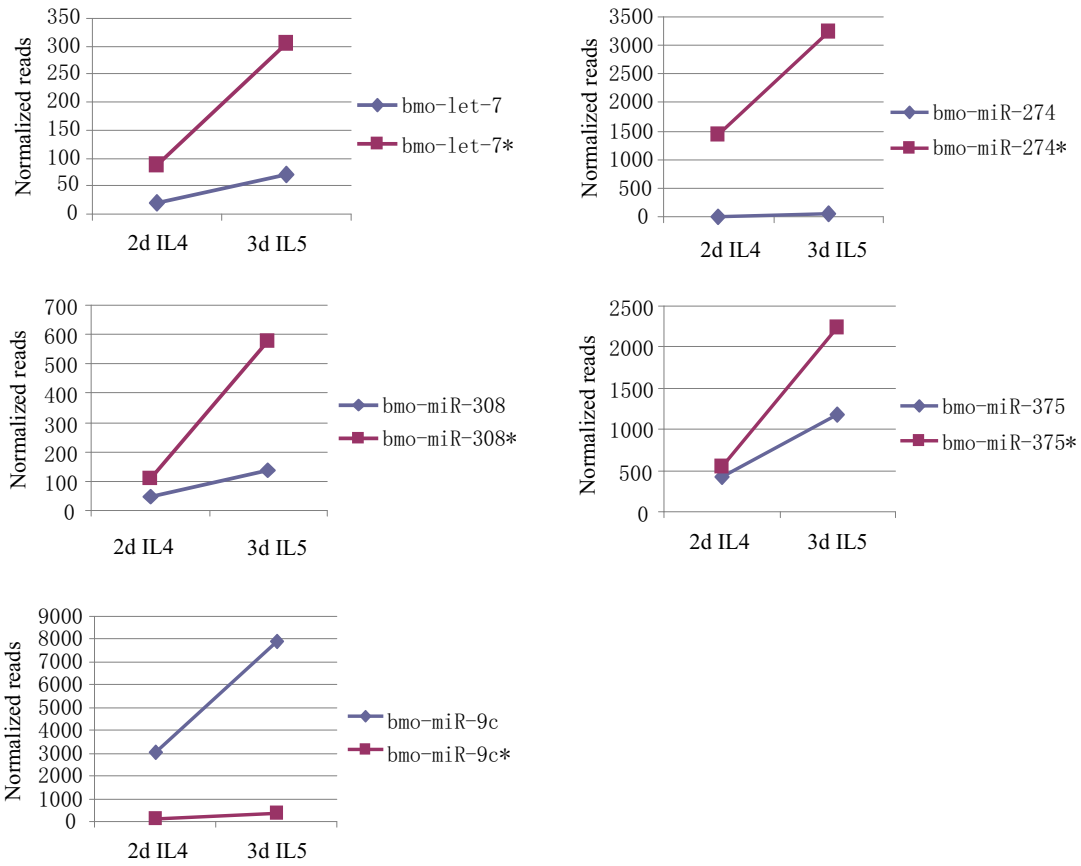

Fig. 2 Plots of expression levels of miRNAs along with their miRNA*. 2d IL4: fourth-instar day-2; 3d IL5: fifth-instar day-3

miR-8 (Kennell et al. 2008). miR-8 and its target, USH, regulated body size in *Drosophila*. miR-8 null flies were smaller in size and defective in insulin signaling in fat body (Hyun et al. 2009).

A total of 29 differentially expressed miRNAs were predicted in this study. These 29 miRNAs may be involved in the regulation of silk gland development and the generation of silk proteins. The bmo-let-7 was one of the differentially expressed miRNAs, which was up regulated in PSG of fifth-instar day-3 larvae compared with that in PSG of fourth-instar day-2 larvae. The let-7 microRNA were identified to act as developmental switches that control the timing of cell fate determination during the larval transitions in *C. elegans*, and let-7 loss-of-function mutations resulted in severe phenotype defects or death of the embryo (Lee et al. 1993; Reinhart et al. 2000). Gene Cdc34 was a functional target of let-7 and let-7 induced down-regulation of Cdc34, low expression level of Cdc34 protein down-regulated the activity of stem cell growth factor to stabilize the Wee1 kinase, and this would make the primary fibroblasts in G2/M phase (Legesse-Miller et al. 2009). The bmo-let-7 was stage- and tissue-specifically expressed in the silkworm. It was expressed in very low level from the first molt to the early third instar, expressed highly after the third molt, and the most abundant expression was observed after mounting, particularly after pupation (Liu et al. 2007). Microarray analyses showed that bmo-let-7 expressed in all of the 10 tissues of fifth-instar day-3 larvae (head, body wall, ASG, PSG, midgut, fat body, ovary, testis, hemocyte and malpighian tubule) (Liu et al. 2010). All of these studys indicated that bmo-let-7 had been shown to play a critical role in cellular differentiation and tissue development.

In these differentially expressed miRNAs, bmo-miR-9c were up regulated by 2.6-fold. Using miRNA Aarray analysis, the miR-9c was highly expressed in larvae, although it was also present in eggs and pupae. This observation prompted that bmo-miR-9c was involved in regulation of metamorphosis from larvae to pupae (Zhang et al. 2009).

The expression of bmo-miR-274 was associated with silk gland growth and spinning activity. bmo-miR-274 displayed no expression signal during early larval stages, was initially detected in fifth-instar day 2 larvae, and was subsequently up-regulated to peak levels in day 7 larvae (Liu et al. 2009). This showed the same trend with the expression pattern of bmo-miR-274 detected in this study (Table 7).

We detected 29 differentially expressed miRNAs, and lots of target genes were predicted for the 29 miRNAs. Through annotating the most likely target genes (score $\geq$ 180) of these 29 miRNAs by GO and COG, it was found that the target genes of bmo-miR-274 BGIBMGA008477-TA and BGIBMGA011620-TA were involved in translation and structural constituent of ribosome, BGIBMGA005280-TA was involved in protein modification; the target gene of bmo-miR-275 BGIBMGA001081-TA was involved in translational initiation, BGIBMGA011223-TA was involved in protein localization; the target gene of bmo-miR-375 BGIBMGA003418-TA was involved in G-protein coupled receptor signaling pathway. These target genes were related to the process of protein synthesis mostly, so these miRNAs and their target genes might take part in the regulation on the process of silk production. These results provide a solid foundation to investigate the function of miRNAs in vitro or in the silkworm. We have validated the expression profiles of four predicted miRNAs, two known bmo-mir-2755* and bmo-mir-375*, and two novel mirRNAs bmo-mir-0001 and bmo-mir-0015 by RT-PCR. Compared with other tissues such as head, epidermis, fatbody, hemolymph, midgut, trachea, ovary or spermary and malpighian tube, these four miRNAs expressed at much higher level in PSG (data not shown), consistent with the sequencing results.

The above results indicated that in PSG of the silkworm there is a complex miRNA-mediated post-transcriptional regulation system. Analysis of differentially expressed miRNAs in PSG and their target genes provides an insight into regulation of miRNAs on development of the silk gland and synthesis of fibroin. But much experiments have to be done to validate the function of the predicted bmo-mirRNAs.

Acknowledgments This work was partially supported by the National Basic Research (973) Program of China (Grand No. 2012CB114600), the National Natural Science Foundation of China (Grand No. 31172266) and Innovation Foundation for Graduate Students of Jiangsu Province (Grand No. CXZZ12-0726).

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297

Bello B, Couble P (1990) Specific expression of a silk-encoding gene of Bombyx in the anterior salivary gland of Drosophila. Nature 346:480–482

Bino J, Anton JE, Alexei A, Thomas T, Chris S, Debora SM (2004) Human microRNA targets. PLoS Biol 2:e363

Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM (2003) Bantam encodes a developmentaly regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. Cell 113:25–36

Bushati N, Cohen SM (2007) microRNA functions. Annu Rev Cell Dev Biol 23:175–205

Cao J, Tong C, Wu X, Lv J, Yang Z, Jin Y (2008) Identification of conserved microRNAs in *Bombyx mori* (silkworm) and regulation of fibroin L chain production by microRNAs in heterologous system. Insect Biochem Molec 38:1066–1071

Chiara R, Stefania B, Fabio D, Gian AD (2003) IDEG6: a web tool for detection of differentially expressed genes in multiple tag sampling experiments. Physiol Genomics 12:159–162

Guarnieri DJ, DiLeone RJ (2008) MicroRNAs: a new class of gene regulators. Ann Med 40:197–208

Hipfner DR, Weigmann K, Cohen SM (2002) The Bantam gene regulates Drosophila growth. Genetics 161:15–27

Huang Y, Zou Q, Shen XJ, Tang SM, Wang SP, Zhao QL (2011) Differential expression of microRNA-2b with potential target coding P25 in the fifth instar larvae posterior silk gland of the silkworm. Mol Biol 45:576–581

Huang Y, Zou Q, Song F, Wang X, Shen XJ (2012) The regulation of silkworm fibroin L chain production by miRNA-965 and miRNA-1926 in insect cells. Russ J Bioorg Chem 38:477–481

Hui CC, Suzuki Y (1989) Enhancement of teanscription from the Ad2 major late promoter by upstream elements of the fibroin-and sericin-1-encoding genes in silk gland extracts. Gene 85:403–411

Hyun S, Lee JH, Jin H, Nam J, Namkoong B, Lee G, Chung J, Kim VN (2009) Conserved microRNA miR-8/miR-200 and its target USH/FOG2 control growth by regulating PI3K. Cell 139:1096–1108

Inoue S, Tanaka K, Arisaka F, Kimura S, Ohtomo K, Mizuno S (2000) Silk fibroin of Bombyx mori is secreted, assembling a highmolecular mass elementary unitconsisting of H-chain, L-chain, and P25, with a 6:6:1 molar ratio. J Biol Chem 275:40517–40528

Jagadeeswaran G, Zheng Y, Sumathipala N, Jiang H, Arrese EL, Soulages JL, Zhang W, Sunkar R (2010) Deep sequencing of small RNA libraries reveals dynamic regulation of conserved and novel microRNAs and microRNA-stars during silkworm development. BMC Genom 11:52

Kato M, de Lencastre A, Pincus Z, Slack FJ (2009) Dynamic expression of small non-coding RNAs, including novel microRNAs and piRNAs/21U-RNAs, during *Caenorhabditis elegans* development. Genome Biol 10:R54

Kennell JA, Gerin I, MacDougald OA, Cadigan KM (2008) The microRNA miR-8 is a conserved negative regulator of Wnt signaling. Proc Natl Acad Sci USA 105:15417–15422

Lee RC, Feinbaum RL, Ambros V (1993) The *Caenorhabditis elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843–854

Legesse-Miller A, Elemento O, Pfau SJ, Forman JJ, Tavazoie S, Coller HA (2009) let-7 overexpression leads to an increased fraction of cells in G2/M, direct down-regulation of Cdc34, and stabilization of Wee1 kinase in primary fibroblasts. J Biol Chem 284:6605–6609

Li JS, Cai YM, Ye LP, Wang SH, Che JQ, You ZY, Yu J, Zhong BX (2014) MicroRNA expression profiling of the fifth-instar posterior silk gland of *Bombyx mori*. BMC Genom 15:410–419

Liu S, Xia Q, Zhao P, Cheng T, Hong K, Xiang Z (2007) Characterization and expression patterns of let-7 microRNA in the silkworm (*Bombyx mori*). BMC Dev Biol 7:88

Liu S, Zhang L, Li Q, Zhao P, Duan J, Cheng D, Xiang Z, Xia Q (2009) MicroRNA expression profiling during the life cycle of the silkworm (*Bombyx mori*). BMC Genom 10:455

Liu S, Gao S, Zhang D, Yin J, Xiang ZH, Xia QY (2010) MicroRNAs show diverse and dynamic expression patterns in multiple tissues of *Bombyx mori*. BMC Genom 11:85

Ma M, Huang Y, Gong Z, Zhuang L, Li C, Yang H, Tong Y, Liu W, Cao W (2011) Discovery of DNA viruses in wild-caught mosquitoes using small RNA high throughput sequencing. PLoS One 6:e24758

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G (2000) The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. Nature 403:901–906

Silveri L, Tillly G, Vilotte JL, Le PF (2006) MicroRNA involvement in mammary gland development and breast cancer. Reprod Nutr Dev 46:549–556

Song C, Wang C, Zhang C, Korir NK, Yu H, Ma Z, Fang J (2010) Deep sequencing discovery of novel and conserved microRNAs in trifoliate orange (Citrus trifoliata). BMC Genomies 11:431

Suzuki Y, Suzuki E (1974) Quantitative measurements of fibroin messenger RNA synthesis in the posterior silk gland of normal and mutant *Bombyx mori*. J Mol Biol 88:393–407

Suzuki Y, Gage LP, Brown DD (1972) The genes for silk fibroin in *Bombyx mori*. J Mol Biol 70:637–649

Takiya S, Kokubo H, Suzuki Y (1997) Transcriptional regulatory elements in the upstream and intron of the fibroin gene bind three specific factors POU-M1, Bm Fkh and FMBP-1. Biochem J 321:645–653

Vo NK, Dalton RP, Liu N, Olson EN, Goodman RH (2010) Affinity purification of microRNA-133a with the cardiac transcription factor, Hand2. Proc Natl Acad Sci USA 107:19231–19236

Wu S, Huang S, Ding J, Zhao Y, Liang L, Liu T, Zhan R, He X (2010) Multiple microRNAs modulate p21Cip1/Waf1 expression by directly targeting its 3′ untranslated region. Oncogene 29:2302–2308

Yue L, Zhuo Z, Feng L, Vongsangnak W, Jing Q, Shen B (2012) Performance comparison and evaluation of software tools for microRNA deep-sequencing data analysis. Nucleic Acids Res 40:4298–4305

Zhang Y, Zhou X, Ge X, Jiang J, Li M, Jia S, Yang X, Kan Y, Miao X, Zhao G, Li F, Huang Y (2009) Insect-specific microRNA involved in the development of the silkworm *Bombyx mori*. PLoS One 4:e4677

Zhao CZ, Xia H, Frazier TP, Yao YY, Bi YP, Li AQ, Li MJ, Li CS, Zhang BH, Wang XJ (2010) Deep sequencing identifies novel and conserved microRNAs in peanuts (*Arachis hypogaea L.*). BMC Plant Biol 10:3