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DIFFERENTIAL EXPRESSION OF microRNA-2b WITH POTENTIAL TARGET CODING P25 IN THE FIFTH INSTAR LARVAE POSTERIOR SILK GLAND OF THE SILKWORM

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MicroRNAs (miRNAs) are a class of non-protein coding small RNAs that regulate a gene expression at the post-transcriptional level. Using *in silico* screening, we found that the 3'-untranslated regions of the *P25* gene mRNA are perfectly complementary to nucleotides 2–8 at the 5' end of the miRNA-2b (miR-2b). The expression of miR-2b and the *P25* gene in posterior silk gland of the fifth instar larval silkworm was investigated using real-time PCR detection method. The results indicated that expression of the *P25* gene was very high in the posterior silk gland during the fifth instar larvae, whereas a level of miR-2b sharply decreased until reaching the lowest one on the 8th day. The expression patterns of miR-2b and *P25* gene indicate that miR-2b might act as a fine-tuning regulator of expression of the *P25* gene at the post-transcriptional level.

Keywords: **microRNA, silkworm, *in silico, P25* gene.**

MicroRNAs (miRNAs) are small non-coding RNAs, which act as important post-transcriptional gene regulators and control the expression of target genes in eukaryotes. miRNAs are expressed as long hairpin-forming precursor RNAs that are cleaved into partially double-stranded RNAs, which are then further processed into mature miRNAs (22 nucleotides) [1–4]. Mature miRNAs recognize their target mRNAs by base-pairing interactions between nucleotides 2–8 of the miRNA (the seed region) and the complementary nucleotides in the 3'-untranslated region (3'-UTR) of the mRNAs. miRNAs inhibit gene expression by targeting mRNAs for translational repression or cleavage [5–7].

The silkworm (*Bombyx mori*) is one of the most economically important insects in many developing countries because of its large-scale cultivation for silk production. Silk fibroin produced by the silkworm consists of a heavy chain (F-H), a light chain (F-L), and P25 (which is a glycoprotein of about 30 kDa) [8–10]. The expression of the three fibroin genes in silkworm is controlled at the transcription level during larval developmental stages [11]. Silk fibroin has been reported to be specifically synthesized in the posterior silk gland (PSG) cells of silkworm and secreted into its lumen in a form of 2.3-MDa protein complex, in which the F-H, F-L, and *P25* are present in 6 : 6 : 1 molar ratios per elementary unit of fibroin [12]. However, molar ratios of corresponding mRNAs greatly differ from the protein composition in the PSG cell [13]. Based on the results, we assumed that expression of the three fibroin genes might be regulated at the post-transcriptional level.

Previous studies demonstrated that the expression of fibroin genes is mainly co-regulated by a series of *trans*-acting factors and the corresponding *cis*-acting elements at the transcriptional level [14]. Recently, it has been reported that at least 4 miRNAs (miR-33, miR-190, miR-276, and miR-7) might play an important role in the regulation of fibroin L-chain transcripts [15]. However, few studies have been reported on miRNAs involved in the regulation of the *P25* gene. Based on the fact mentioned above, we chose the *P25* gene as our research object. *In silico* search for potential miRNAs probably interacting with the 3′-UTR of the *P25* mRNA showed that the miR-2b seed region is perfectly complementary to the target. Thus, miR-2b may be involved in the post-transcriptional regulation of the expression of the *P25* gene. In order to explore the miRNA regulation mechanism at the post-transcriptional level, we compared the expression of miR-2b with that of *P25* gene in the posterior silk gland of the fifth instars larvae using real-time PCR assay. The results indicated that miR-2b was

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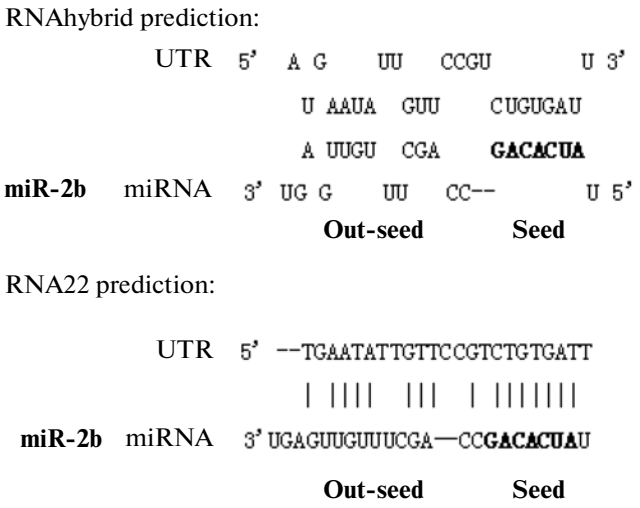


Fig. 1. *In silico* derived base pairing between miR-2b and the 3'-UTR mRNA of *P25* gene. The miR-2b and target *P25* are partitioned into two parts, the seed region and out–seed region. The seed sequence corresponds to seven nucleotides at positions 2–8 of the miRNA sequence. The seed site has perfect complementarity with the miRNA 5'-end that is indicated by black bold letters.

probably to be involved in the post-transcriptional regulation of the *P25* gene.

EXPERIMENTAL

Materials. A bivoltine strain (DaZao) of silkworm was used in the experiments. The posterior silk glands of the fifth instar larvae were separately dissected at 1 day, 3 day, 5 day, 7 day and 8 day, immediately frozen in liquid nitrogen, and then stored at –80°C.

Referenced sequence data. Mature silkworm miRNAs were downloaded from the miRNA database (http://microrna.sanger.ac.uk; Release 14.0, September 2009). The 3'-UTR of *P25* gene mRNA was downloaded from NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene). The potential target sites were detected using RNAhybrid and RNA22 tool. At the 5'-end of the miRNA there is a region called "seed", which is centered on nucleotides 2–8 [16, 17]. miRNA targets were difficult to predict since miRNA : mRNA duplexes often contain several mismatches, gaps and G : U or T: U base pairs in many positions according to different target prediction methods [18]. RNAhybird is an extension of classical RNA secondary structure prediction method [19]. It calculates the energetically most favorable hybridization of a miRNA and its target mRNA rapidly and accurately, which also allowing the user to specify a portion of the miRNA that should form a perfect helix, corresponding to the seed site. RNA22 is distinct form previously reported methods in that it doesn't consider cross-species sequence conservation. It is a pattern-based method for miRNA target prediction [20]. It makes use of miRNA patterns to identify "target islands", regions of the mRNA sequence that have a higher likelihood to contain binding sites for miRNAs.

Total RNA isolation. Total RNA was isolated from posterior silk gland tissues of silkworm fifth instar larvae (see "Materials") using Trizol ("Invitrogen") according to the manufacturer's instructions. Contaminating genomic DNA was removed by DNase I ("Takara", Dalian). The purity of extracted RNA was determined by a UV spectrophotometer NanoDrop™ 1000 ("Thermo Scientific"). Ratios of UV 260/280 were between 1.8 and 2.1 for all of analyzed RNA samples. The total RNA concentration was determined by measuring the absorbance at 260 nm.

Real- time PCR quantification. cDNA was synthesized from a total of 500 ng of RNA in a 20-μl reaction system by using specific stem-loop primes for miRNA obtained from a commercial service ("Takara") and a common prime script™ RT reagent kit ("Takara") for the *P25* gene. The cDNAs were diluted 10 times. Real-time PCR was performed in 20 μl mixture containing 1 μl of cDNA, 800 nM forward and reverse primers, 10 μl of the 2 × SYBR green PCR master mix ("Takara") [21]. Real-time PCR primers used in this experiment are indicated in the table. The reaction was performed in triplicate on an opticon lightcycler ("Bio-Rad Laboratories").The reactions were amplified for 15 s at 95°C and 30 s at 57°C for 40 cycles.

Statistical analysis. Melting curves for each PCR were carefully monitored to avoid nonspecific amplification. Expression levels of the target genes were normalized against the expression level of the 5S rRNA gene by Option monitor analysis software ("MJ Re-

search"). Default threshold settings were used to determine the threshold cycle data. The relative amount of each miRNA to 5S rRNA was described by using the equation $2^{-\Delta Ct}$, where ΔCt = Ct (miRNA) – Ct (5S rRNA) [22, 23]. miRNA expression data were normalized by the levels of 5S rRNA. Data were presented as the mean value ± SD from two or three separate experiments. The *p*-values lower than 0.05 were considered as significant.

RESULTS

In silico prediction of the P25 mRNA 3'-UTR's base-pairing with the miRNA 5' seed region

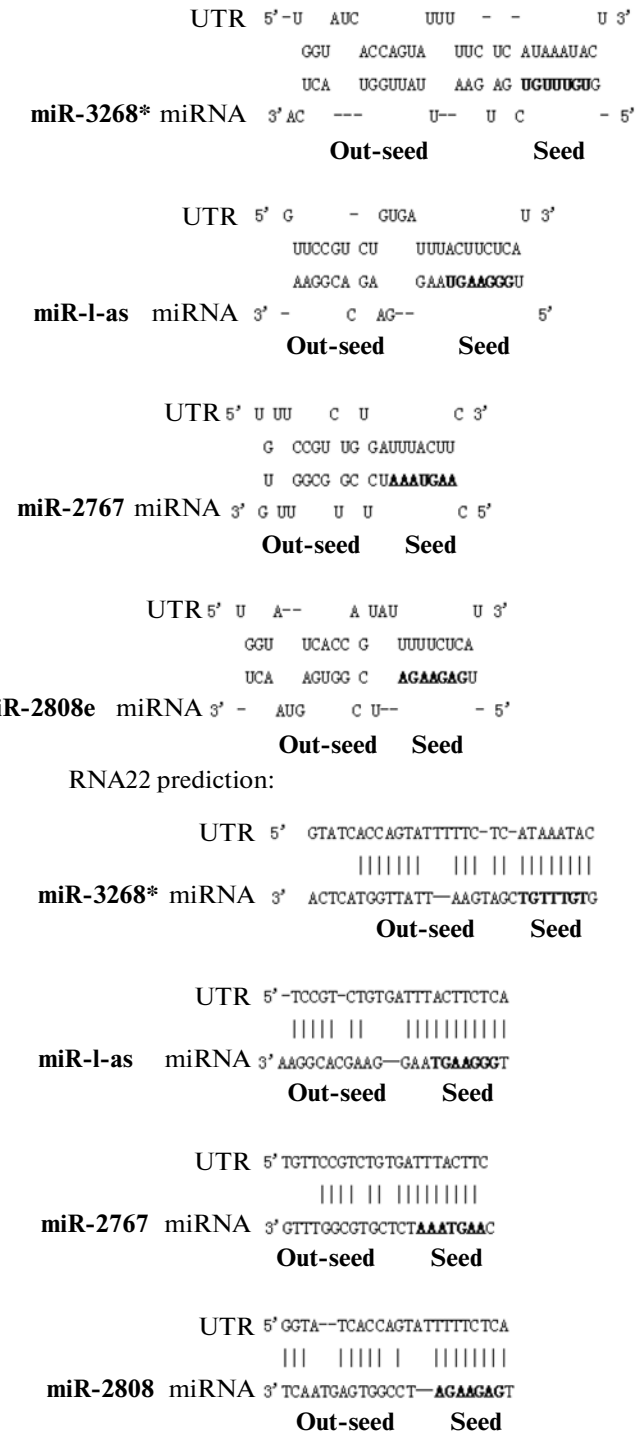
Although animal miRNAs tend to show imperfect base-pairing in the 3'-UTRs of their target transcripts, a 7- or 2–8-nucleotide seed sequence starting from the second nucleotide at the 5'-end of miRNAs with their target mRNA that leads to decreased transcription and/or translation repression [24, 25]. Here, two target prediction tools, the RNAhybrid and RNA22, were used to predict miRNAs through the silkworm miRNA database and to find the seed sites complementary to the 3'-UTR of *P*25 gene mRNA (fig. 1). The results manifested that miR-2b meets the In addition, through searching the new miRNA database (Release 15, April 2010), four miRNAs potentially directed to the 3'-UTR of *P*25 gene mRNA were found (fig. 2). These miRNAs are complimentary to different regions within the 3'-UTR and therefore might affect the expression of *P25* gene.

Expression of miR-2b and P25 gene

An expression level of miR-2b varied across the development cycle of the posterior silk gland in the fifth instar larvae. It was the highest at the first day and then sharply decreased during most of the 8 development days (fig. 3*a*).

Fibroin genes were expressed exclusively highly in the posterior silk gland of the fifth instar larvae [26, 27]. Similarly, real-time PCR analysis revealed a high level of *P25* gene expression in the posterior silk glands of the fifth instar larvae (fig. 3*b*). Its expression was the lowest on the first day; obviously increased at day 3; reached maximal value on the 5 day, and then decreased on the 7 and 8 days. On the base of results obtained we hypothesize that expression of the *P25* gene might be regulated at the transcription level during specific developmental stages in the posterior silk gland of the fifth instar larvae. Thus, we assumed that expression of the *P25* gene might be regulated at the transcription level during specific developmental stages in the posterior silk gland of the fifth instar larvae.

RNAhybrid prediction:



RNA22 prediction:

Fig. 2. *In silico* derived base-pairing between miRNAs and the *P25* 3'-UTR. Black bold letters indicates the 'seed region' of the binding site.

DISCUSSION

At present, there are certain characteristics of the interactions between miRNAs and their targets which

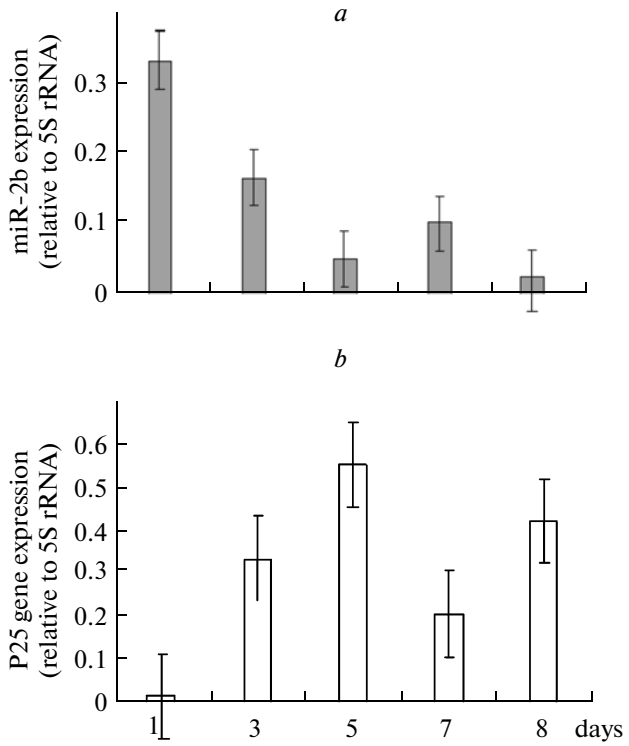


Fig. 3. Expression of miR-2b (*a*) and *P25* gene (*b*) in the posterior silk gland of the fifth instar larvae. Data are represented as the mean value ± SD from three independent experiments.

current algorithms generally use: (1) the complementarity between miRNAs and their target sites exists; (2) the 5'-end of a miRNA binds to the target mRNA stronger than the 3'-end does. In addition to these basic principles, different prediction methods impose different restrictions on the algorithms to achieve optimization in accordance with their respective rules [28–31]. In order to identify the miRNA targets in the 3'-UTR of *P25* gene mRNA for evaluating miRNA-target association, two computational target-prediction approaches were used. The results revealed that the 2–8 nucleotides of miR-2b seed region were full complimentary to the identically located sequence within *P25* mRNA 3'-UTR (fig. 1). The outcome strongly indicates that miR-2b might be involved in the post-transcriptional regulation of the *P25* gene expression. Meanwhile, among the new identified silkworm miRNAs there are more possible miRNAs which may regulate the expression of the *P25* gene (fig. 2). These prediction results are required further test and verification *in vivo* and *in vitro* experiments using methods of miRNA over-expression and knocked-down.

It was shown that some miRNAs could up-regulate translation besides their down-regulating role [32–34]. In this paper, we used real-time RT-PCR method for evaluating the *P25* gene and miR-2b expression. As one can see from the data presented in fig. 3*a* and fig. 3*b,* expression of *P25* gene increased significantly from day 1

Sequences of stem-loop RT primers, forward primers, and reverse primers for real-time PCR

Gene Name	Primer	Sequence
miRNA-2b	RT	5'-GCGTGGTCCCGACCACCACAGCCGCCACGACCACGCACTCAA-3'
	Forward	5'-GCGTATCACAGCCAGCTTTG-3'
	Reverse	5'-TCCCGACCACCACAGCC-3'
5S	RT	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAGCCTACGCTCTGGAATACCGGGTGC-3'
	Forward	5'-GCTCTGGAATACCGGGTGC-3'
	Reverse	5'-TCCCGACCACCACAGCC-3'
P25	Forward	5'-CCCTGCTACTTGGACGATT-3'
	Reverse	5'-GATTATGGTCGACGTAGGTG-3'

to day 3, and reached the highest value at the 5 day, whereas the expression of miR-2b, on the contrary, decreased at that time interval. Based on the results we suppose that there might be a certain regulation relationship of expression between miR-2b and the *P25* gene. Obviously, our hypothesis will further need to be verified *in vivo* experiments. It is known that miRNAs are often expressed in a strict tissue-specific manner during development stages [35–37]. As one can see in fig. 3*b*, the expression of *P25* gene displayed obviously the down-regulation from day 7 probably because a lot of fibroin genes would be activated and translated into fibroin proteins in this development stage.

To sum up, through combination with two methodologies, the computational prediction for a search of *P25* 3'-UTR-targeted miRNAs and real-time RT-PCR for testifying model obtained, we conclude that miR-2b might play a biological role in regulating the *P25* gene expression.

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