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Differential expression of microRNAs in hemocytes from white shrimp *Litopenaeus vannamei* under copper stress

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ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNAs that regulate diverse cellular processes, including organismal stress response, through posttranscriptional repression of gene transcripts. They are known to have antiviral functions in aquatic crustacean species, but little is known about the role of miRNAs against environmental stress caused by Cu, a common chemical contaminant in aquatic environment. We performed small RNA sequencing to characterize the differentially expressed microRNAs in Cu exposed shrimp. A total of 4524 known miRNAs and 73 novel miRNAs were significantly ($P < .05$) differentially expressed after Cu exposure. The peak size of miRNAs was 22 nt. Among them, 218 miRNAs were conserved across 115 species. The validation of 12 miRNAs by stem-loop quantitative RT-PCR were found to be coherent with the expression profile of deep sequencing data as evaluated with Pearson's correlation coefficient ($r = 0.707$). Target genes of these differentially expressed miRNAs related to immune defense, apoptosis, and xenobiotics metabolism also showed significant changes in expression under Cu stress. The present study provides the first characterization of *L. vannamei* miRNAs and some target genes expression in response to Cu stress, and the findings support the hypothesis that certain miRNAs along with their target genes might be essential in the intricate adaptive response regulation networks. Our current study will provide valuable information to take an insight into molecular mechanism of *L. vannamei* against environmental stress.

1. Introduction

Litopenaeus vannamei is the most important farmed shrimp species and is economically valuable. With the development of industrialization and intensive aquaculture, aquatic pollution has become a severe and growing problem. Among various environmental pollutants, heavy metals have been of great concern because of their inherent toxicity, non-degradability and persistence [1]. In aquaculture practices, copper sulfate is commonly used to eradicate filamentous algae and phytoplankton in shrimp farms. The excess application of copper sulfate in pond management may deteriorate the aquatic environment due to Cu accumulation in the pond sediments [2,3].

In crustaceans, hemocytes in the circulating hemolymph have vital roles in immune responses against pathogens, such as phagocytosis, coagulation, encapsulation, nodulation, production of antimicrobial peptides (AMPs) and proPO activity [4,5]. Consequently, toxic effects on hemocytes potentially affect the survival of these animals [6]. Cu has been reported to decrease the total hemocyte count (THC), respiratory burst activity and phenoloxidase activity of hemocytes in *L. vannamei* [7]. In our previous study, exposure of *L. vannamei* to Cu induced hemocyte apoptosis and the expression of antioxidant biomarker genes, apoptosis-related genes and a specific biomarker gene of heavy metal pollution [8]. However, the molecular mechanism of Cu stress in shrimp remains largely unclear.

Biological systems use a variety of mechanisms to maintain their functions in the face of environmental and genetic perturbations [9]. MicroRNAs (miRNAs), a class of small noncoding RNAs with 20–24 nt in length, can regulate gene expression at the post-transcriptional level [10] and play crucial regulatory roles in a large variety of biological processes, such as development, cell differentiation, apoptosis, oncogenesis, immune and stress response in various organisms [11–14]. In animals, miRNAs regulate gene expression through imperfect sequence-specific binding to the 3′-untranslated regions (3′UTR) of the target mRNAs and usually cause translational repression [15]. A growing

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number of miRNAs were discovered and studied in variety of organisms since the first miRNA lin-4 has been discovered in *Caenorhabditis elegans*. To date, over 28 thousand miRNAs have been discovered across 223 species (miRBase, release 21.0; June 2014).

Initially, miRNAs were cloned and identified by a traditional method in *M. japonicus* [16]. However, PCR cloning followed by traditional sequencing remains very labor- and cost-intensive with a limited dynamic range to detect and define relative miRNA expression [17]. Recently, next-generation sequencing (NGS) technologies, including the Illumina Genome Analyzer (GA), Applied Biosystems SOLiD System, and 454 Life Sciences (Roche) FLX instruments, have emerged as well-established approaches for miRNA profiling, which can rapidly produce millions of sequence reads simultaneously with lower cost than Sanger sequencing and allows for the identification of low abundance miRNAs and genome-wide discovery of novel tissue-specific miRNAs with high specificity and sensitivity [18–20]. Deep-sequencing technologies have facilitated a sharp rise in the rate of novel microRNA discovery [21]. In recent years, miRNA studies have been performed using NGS technologies in crustaceans with limited genomic information [22–24].

Although hundreds of miRNAs have been identified, only a small number of shrimp miRNAs have been discovered and functionally identified in *Marsupenaeus japonicus* [22], *Penaeus monodon* [25] and *L. vannamei* [23] that responded to *Vibrio alginolyticus* or white spot syndrome virus infection. The investigation of miRNAs that responds to copper stress has not yet been performed. The objective of this study was to identify and characterize the differentially expressed miRNAs in shrimp *L. vannamei* in response to copper stress. The results will extend the knowledge of crustacean miRNA regulation and provide information for further research on shrimp response against environmental stress.

2. Materials and methods

2.1. Animals

The experimental shrimp *L. vannamei* (4.94 ± 0.50 g) were obtained from a commercial farm in Zhanjiang (Guangdong, China), and acclimated for 2 weeks prior to experiment in cycling-filtered plastic tanks with aerated seawater at 26 ± 2 °C and a salinity of 5‰. During the acclimation period, shrimp were fed twice daily with shrimp diet (40% protein, 5.0% fat, 5.0% fiber and 16% ash, supplied by a commercial diet, China) until 24 h before the experimental treatments began. Only shrimp apparently healthy and in the intermolt stage were used for the study. The molt stage was determined by examining the uropoda in which partial retraction of the epidermis could be distinguished [26].

2.2. Cu exposure

Exposure experiment was conducted in triplicate with thirty shrimps in plastic tank with 180 L water (26 ± 2 °C, pH 7.9–8.0, and salinity 5‰). Each tank was aerated continuously using an aeration stone. Normal and experimental concentrations were 0 and 5 mg L^{-1} according to previous study [7]. Experimental Cu concentrations were prepared by adding copper sulfate to 5‰ seawater until the desired concentration was attained.

2.3. RNA extraction, library construction and sequencing

At the beginning (0 h) and after 3 h of Cu exposure, three shrimp were randomly sampled from each tank (n = 9). Hemolymph (200 μL) was withdrawn from the ventral sinus of each shrimp by a 1 mL sterile syringe (25 gauge) containing an equal volume of ice-cold anticoagulant (glucose 20.5 g L^{-1}, sodium citrate 8 g L^{-1}, sodium chloride 4.2 g L^{-1}, pH 7.5). The hemolymph from each shrimp was transferred into an individual eppendorf tube held on ice, and then samples of hemolymph from three replicates were pooled for RNA extraction.

The total RNAs of hemocytes were extracted using miRNA isolation kit (Ambion, USA) according to the manufacturer's protocol. The integrity and concentration were assessed using the Agilent Bioanalyzer 2100 System (Agilent Technologies, USA). The average RIN (RNA integrity number) value of samples was 8.9. Subsequently, the small RNAs ranging from 18 to 30 nt were separated by denaturing 15% polyacrylamide gel. After recovery by ethanol precipitation, the small RNAs were ligated sequentially to a pair of RNA adapters both in 5′ and 3′ ends by combing the reagents and incubating on the preheated thermal cycler according to the manufacturer's instructions. The reverse transcription and polymerase chain reaction (PCR) amplification were preformed after the ligation. The last products were sequenced using the Illumina Genome Analyzer (Illumina, San Diego, CA, USA) following the manufacturer's protocol and instructions.

2.4. MiRNA identification and expression analysis

The raw reads obtained from HiSeq were processed by removing low quality reads. After removal of the reads with proportion of N (N means unable to determine the base information) is greater than 10, 5′ primer contaminants and without the insert fragments reads, without 3′ primer reads, poly A reads and reads shorter than 18 nt, the remaining reads were regarded as clean reads and were blasted against the Rfam database (http://www.sanger. ac.uk/software/Rfam) and the GenBank noncoding RNA database (http://blast.ncbi.nlm.nih.gov/) to identify rRNA, tRNA, snRNA, snoRNA, mRNA, and repeat sequences. Those sequences were discarded assuming that they represent degradation and other undesired products. As there was no genome data available for *L.vannamei,* the remaining sequences were searched against the miRNAs from all the animals in miRBase 21.0 (http://www.mirbase.org/) using BLAST to identify known miRNAs. The steps are as following: (1) considering the difference among species, align clean data to the miRNA precursor/mature miRNA of all animals in miRBase allowing two mismatches and free gaps; (2) choose the highest expression miRNA for each mature miRNA family which is regarded as a temporary miRNA database; (3) align clean data to the above temporary miRNA database and the expression of miRNA is generated by summing the count of reads which can align to the temporary miRNA database within two mismatches; (4) predict the precursor of the identified miRNA, and those that cannot fold stem-loop hairpin structure will be regarded as pseudo-miRNA. The feasibility of the result can be greatly improved by this verification. The novel miRNA candidates were predicted using miRdeep software [27] with *L. vannamei* transcriptome deep sequencing data [28] based on hairpin-like secondary structure pattern.

2.5. Differential expression analysis

To find out the differentially expressed miRNAs between two libraries, the expression of miRNA in 0 h and 3 h libraries were normalized to obtain the expression of transcripts per million (TPM) [29] using the following formula: Normalized expression = (Actual miRNA count/Total count of clean reads)*1,000,000. Then, the fold-change (Fold change = $\log_2$(3 h/0 h) and P-value [30] were calculated from the normalized expression. Identification of differentially expressed miRNAs between control and exposed samples was performed by pairwise comparisons across 0 h and 3 h samples using the DESeq R package (http://bioinfo.au.tsinghua.edu.cn/software/degseq/), and a corrected P-value < .05 and a threshold absolute $|\log_2(3\,h/0\,h)| \geq 1$ were set as the threshold for significantly differential expression [31,32].