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Research paper

Identification of microRNAs involved in cold adaptation of *Litopenaeus vannamei* by high-throughput sequencing

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ABSTRACT

The *Litopenaeus vannamei* (*L. vannamei*) is one of the most widely cultured shrimp species in the world, with low temperature being one of the most serious threats to its growth and survival. To examine the potential regulatory mechanism of cold adaptation, we conducted a microRNAs (miRNAs) analysis on the hepatopancreas of *L. vannamei* under normal temperature 28 °C (M28), cold acclimation 16 °C for 6 days (M16), and recovered under normal temperature (MR). In total 14,754,823, 14,945,246 and 15,880,093 raw reads representing 10,690,259, 8,587,144, and 11,512,941 unique sequences of 18–32 nt length were obtained from the M28, M16 and MR libraries, respectively. After comparing the miRNA sequences with the miRBase database, 68 known mature miRNAs and 47 novel miRNAs were identified. Expression analysis showed that 34 miRNAs were significantly differential expressed in response to cold adaptation. Compared to the M28 library, 21 miRNAs were upregulated and 13 miRNAs were downregulated significantly in the M16 library. After recovery to normal temperature, there are 16 miRNAs upregulated and 15 miRNAs downregulated significantly compared to M28 library.

Then, five significantly differential expressed miRNAs under cold acclimation including three known miRNAs (mja-miR-6491, mja-miR-6494, and Bta-miR-2478) and two newly-identified miRNAs (novel_68 and novel_5) were selected for validation by RT-qPCR in the hepatopancreas and muscle tissues of cold treated shrimps. The expression trend of most the miRNAs from RT-qPCR were consistent with the next-generation sequencing data. Further, the Gene Ontology (GO) annotation showed that the metabolic process GO term was significantly enriched with target genes of the differentially expressed miRNAs. Additionally, KEGG pathway analysis suggested that the fatty acid degradation and glycerolipid metabolism pathways etc. are significantly enriched with the target genes. These findings may contribute to a better understanding of the molecular mechanisms governing the responses to low temperature in *L. vannamei*.

1. Introduction

L. vannamei is one of the most widely-produced shrimp species in the world, and global production of *L. vannamei* reached 3,668,681 tons in 2015, according to FAO data (Ministry of Fisheries and Aquaculture, n.d.). When culturing *L. vannamei*, diverse environmental stresses, including salinity, pH, and temperature changes can result in reduced growth and survive rates, increase disease susceptibility, and even cause death (Ponce-Palafox et al., 1997; Kumlu et al., 2010; Miura and Furumoto, 2013). Despite its tropical origins, *L. vannamei* is often farmed subtropically, where low temperatures present a serious challenge to its survival, growth, and distribution (Peng et al., 2016). So, some researches have focused on the molecular mechanisms governing the responses to low temperature in *L. vannamei* by SSH (Peng et al., 2016) and proteomic analysis (Fan et al., 2015). MiRNAs are short, non-coding RNAs that are able to regulate protein expression. These nucleotide transcripts can bind with full or partial complementarity to the 3′ untranslated regions (UTR) of mRNA targets, leading to the inhibition of translation of the targets (Grimson et al., 2007; Biggar and Storey, 2011). Recently, high-throughput sequencing techniques have been successfully used to identify miRNAs in a variety of organisms. It is predicted that approximately 90% of mRNA

Abbreviations: cDNA, complementary DNA; mRNA, messenger RNA; RT, reverse transcription; qPCR, quantitative polymerase chain reaction; UTR, untranslated regions; h, hour; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes

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transcripts are likely targeted by one or more miRNAs (Perron and Provost, 2008). It is known that miRNAs have significant roles in regulating many eukaryotic cellular processes, including cell differentiation, proliferation, apoptosis, energy metabolism, cancer development, and immune defense, among others (Biggar and Storey, 2016). Since the initial discovery of miRNA in 2001, many researchers have demonstrated that stress-responsive miRNAs are differentially-expressed in response to various environmental stresses in numerous species. These studies have included bat (Biggar and Storey, 2014a) and ground squirrel (Wu et al., 2014) responses to hibernation (Y. Liu et al., 2010; Biggar and Storey, 2014b), aestivation (Biggar et al., 2009) and dehydration in frogs (Wu et al., 2013; Bansal et al., 2016), insect tolerance to freezing (Lyons et al., 2013, 2016; Courteau et al., 2012), and turtle (Biggar and Storey, 2012, 2016) and marine snail anoxia tolerance.

Researches on miRNA expression in response to environmental stresses in aquatic species include zebrafish, *Procambarus clarkii* and others (Yang et al., 2011a; Ji, 2014). Thus far, the researches on miRNAs in *L. vannamei* have largely focused on immune defense (e.g. miRNA expression changes in response to white spot syndrome virus, WSSV, infection) (Sun et al., 2016). Up to date, data on cold-responsive miRNAs in *L. vannamei* are still limited. To examine the potential regulatory capability of miRNAs in the *L. vannamei* cold adaptation, we characterized the miRNAs of the hepatopancreas of *L. vannamei* raised under normal (28 °C), cold-acclimated (16 °C, 6 days), and normal recovery conditions using Solexa sequencing.

2. Materials and methods

2.1. Ethics statement

All procedures involving the treatment of *L. vannamei* used in this study were conducted with the approval of the Animal Care and Use Committee of the Guangxi Academy of Fishery Sciences, Nanning, China.

2.2. Low-temperature treatment and collection of samples

The experiments were conducted at 2 different temperatures, viz., 28 °C (control) and 16 °C (cold-acclimated). A total of 54 shrimps (3 months old, weight of 10–15 g) obtained from the Fangchenggang aquaculture base were reared at the Guangxi Academy of Fishery Science and adapted to conditions of 28 °C, 30–35% salinity for one week prior to experimentation. The experimental groups were cooled at a rate of 1 °C per 2 h until reaching 16 °C in an incubator and the shrimps were maintained at 16 °C for six days. Samples for the 16 °C (M16) group were collected at time 0, 24, 72, and 144 h (6 days) after exposure to the low temperature. The normal recovery (MR) group was maintained for 24 h after recovered to the normal temperature (28 °C) and sampled. The control (M28) group was maintained at 28 °C, and sampled at the same timepoint as the M16 group and the MR group. For each sampled shrimp, the hepatopancreas and muscle tissue were dissected and immediately immersed in liquid nitrogen or RNAlater solution and stored at −80 °C until RNA isolation. The total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.3. Library preparation for small RNA sequencing

The pooled hepatopancreas RNA (three RNA samples from each group were pooled equally) samples from the M28, M16 and MR group shrimps were prepared for the generation of three small RNA libraries. A total of 3 μg of RNA per sample was used as input material for the small RNA library. Sequencing libraries were generated using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA). Briefly, the NEB 3′ SR adaptor was ligated to the 3′ end of the miRNA, siRNA and piRNA. Following the 3′ ligation reaction, the SR RT primer was hybridized to the excess of the 3′ SR adaptor, transforming the single-stranded DNA adaptor into a double-stranded DNA molecule. Then, the 5′ end adapter was ligated to 5′ends of miRNAs, siRNA and piRNA. Next, first strand cDNA was synthesized using M-MuLV Reverse Transcriptase (RNase H). PCR amplification was performed using SR Primer for Illumina and index (X) primer. The PCR products were purified on an 8% polyacrylamide gel (100 V, 80 min). Finally, the libraries quality was assessed using the Agilent Bioanalyzer 2100 system with DNA High Sensitivity Chips, then the libraries were sequenced by using Hiseq2000 technology.

2.4. Bioinformatic analysis

After adaptor trimming, removal of single-read sequences and low-quality sequences, reads of 18–32 nt in length were kept for further bioinformatic analysis. Then the remaining reads were mapped to the tanscriptome reference sequence (data not published) by bowtie. Subsequently, by BLAST against the Rfam database (11.0, http://Rfam.sanger.ac.uk/), the reads mapped to the *L. vannamei* tanscriptome reference sequence were analyzed to discard the rRNA, tRNA, snRNA and snoRNA sequences from the small sequences. After that, the remaining sequences were used for identify the conserved miRNAs in *L. vannamei* by BLAST against the miRBase (version18.0, http://www.mirbase.org/).The software miREvo (Wen et al., 2010) and mirdeep2 (Friedlander et al., 2011) were used to predict novel miRNAs by exploring the secondary structure. The expression amount of conserved and novel miRNA in each library was statistically analyzed and the expression level was normalized using TPM. Before difference analysis with DEGseq (http://www.bioconductor.org/packages/release/bioc/html/DEGseq.html), the readcount data was inputted and normalized using TMM (Wang et al., 2010). MiRNA target genes were predicted with miRanda (Enright et al., 2003). Gene Ontology (GO) enrichment analysis was used on the target genes of differentially expressed miRNAs. KEGG is a database resource that is useful for understanding high-level functions and utilities of a biological system, especially for large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/) (Kanehisa et al., 2008). We also applied KOBAS (Mao et al., 2005) software to evaluate the statistical significance of the target gene candidates identified by KEGG pathway analysis.

2.5. Stem-loop RT-qPCR

To validate the differentially expressed miRNAs identified from the sequencing data, the relative expression of the 5 selected miRNAs was analyzed by stem-loop RT-qPCR, using samples collected from the M28, M16 and MR group shrimps. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed to cDNA using the total RNA ($\leq$1 μg) with a reverse transcription kit (Takara, RR047). Quantitative real-time PCR was performed on the ABI 7500 PCR system (Applied Biosystems, Foster City, CA) using qPCR/Real-Time PCR Reagents (TakaRa, RR820A). The primer sequences are presented in the Table 1. The PCR program was as follows: 20 s at 95 °C, followed by 40 cycles of 10 s at 95 °C, and 30 s at 65 °C. Each group included three shrimp samples for biological replicate, and all samples were tested in triplicate. Finally, agarose gel electrophoresis and melting curve analysis were performed to confirm amplification specificity. *β-Actin* was used as an internal control, and the relative expression levels were normalized to the *β-Actin* transcript levels in the M28 group hepatopancreas samples using the comparative Ct method (2-[delta][delta]Ct method). The data were analyzed by the ANOVA using SPSS 23.0. A P-value of < 0.05 was considered to be significant.