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Digital gene expression analysis in hemocytes of the white shrimp *Litopenaeus vannamei* in response to low salinity stress

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ABSTRACT

The white shrimp *Litopenaeus vannamei* has been greatly impacted by low salinity stress. To gain knowledge on the immune response in *L. vannamei* under such stress, we investigated digital gene expression (DEG) in *L. vannamei* hemocytes using the deep-sequencing platform Illumina HiSeq 2000. In total, 38,155 high quality unigenes with average length 770 bp were generated; 145 and 79 genes were identified up- or down-regulated, respectively. Functional categorization and pathways of the differentially expressed genes revealed that immune signaling pathways, cellular immunity, humoral immunity, apoptosis, cellular protein synthesis, lipid transport and energy metabolism were the differentially regulated processes occurring during low salinity stress. These results will provide a resource for subsequent gene expression studies regarding environmental stress and a valuable gene information for a better understanding of immune mechanisms of *L. vannamei* under low salinity stress.

1. Introduction

Salinity is one of the most important environmental factors that directly affect the survival, growth, and physiological function of shrimps [1–3]. In recent years, the large-scale outbreaks of diseases in shrimps are related to environment changes and some farming activities, such as heavy rain and a large water change, causing rapid decline of salinity, leading to osmotic imbalance directly and metabolic disorders *in vivo*, inducing outbreaks of disease easily in shrimps [4–7]. Therefore, the research on the strategy of immune response in shrimps under low salinity stress becomes an urgent issue in shrimp aquaculture in domestic and overseas.

The study on immunity of crustaceans under environmental stress has been a hot research field internationally nowadays [3–7]. The cellular and humoral immunity was found decreased after transferred to low salinity environment [8,9]. Extensive articles that described the immune signaling pathway of crustaceans exposed to salinity stress have revealed that the neuroregulators concentration in hemolymph changed rapidly to trigger diverse immune activities via signal transduction pathways [10–12]. A plenty of immune function genes have been identified to date, including immune recognition molecules, oxidative enzymes, non-digestive proteases, protease inhibitors, antimicrobial peptides, heat shock proteins and other immune effectors [13–15]. Gross et al. constructed gene libraries in the *Litopenaeus vannamei* and *Litopenaeus setiferus* via Expressed Sequence Tag (EST) analysis, and 44 immune function genes were identified [16]. These studies revealed much information of immune function. However, the underlying mechanism of immune response to salinity stress has not been fully revealed, especially at the molecular level, including genes and pathways.

Recently, high-throughput next-generation sequencing techniques, the digital gene expression analysis (DGE) have been widely used in gene expression profiling and pathways studies, with several advantages over other expression profiling technologies including higher sensitivity and the ability to detect splicing isoforms and somatic mutations [17]. For it does not require the

Abbreviations: L. vannamei, *Litopenaeus vannamei*; DEG, digital gene expression; EST, Expressed Sequence Tag; RPKM, Reads Per Kilo bases per Million reads; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; QPCR, quantitative real-time PCR; RNA-Seq, RNA-Sequencing; SRA, Sequence Read Archive; CTLD, C type lectin containing domain protein; Crustin, crustin-like antimicrobial peptide; ppA, prophenoloxidase-activating enzyme 1a; PI4K, phosphatidylinositol 4-kinase beta; Dynamin, *Anolis carolinensis* dynamin 1; NLR, NOD-like receptor; ROS, reactive oxygen species; GPx, glutathione peroxidase; proPO, prophenoloxidase; PO, phenoloxidase; MAPK, MAP kinase; PI3K, phosphatidylinositol 3′-kinase; SOD, copper zinc superoxide dismutase; LRP, prolow-density lipoprotein receptor-related protein 1; Cyt b, cytochrome b; PSTPIP, proline-serine-threonine phosphatase interacting protein 1; Cathepsin, cathepsin A; CDKI, cyclin-dependent kinase inhibitor 1B.

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genome reference sequence, the technology has been applied in decoding the genomes of several non-model organisms, which provided valuable information in understanding of gene function, cell responses and evolution [18–20]. For aquatic animals, successful examples have been applied in *Ictalurus punctatus* [21], *Oncorhynchus mykiss* [22], *Villosa lienosa* [23], *Eriocheir sinensis* [24,25], *Fenneropenaeus chinensis* [26], *Macrobrachium nipponense* [27] and *L. vannamei* [28,29]. The countable, nearly digital, nature of DGE data makes them particularly suit for the quantitative analysis of transcript expression levels, which can provide reliable measurements of transcript levels in multiple conditions. However, such investigations of the immune response in low salinity environment of *L. vannamei* have not been reported.

L. vannamei, one of the most productive aquaculture species in the world, is of a great economic value and has excellent properties in breeding. It can tolerate salinity as low as 1‰ [30], and has recently become one of the main aquatic animals cultured in the coastal region of China [31]. In the present study, we conducted DGE using the Illumina's sequencing technology to examine the whole transcriptome responses to low salinity stress of the *L. vannamei* for the first time. Considering individual monitoring of the shrimp responses to low salinity stress, two libraries were established from the hemocytes of shrimps that exposed to natural and low salinity seawater, respectively. The study aimed to gain knowledge on the immune response in *L. vannamei* under low salinity stress and identify related genes of the shrimp. The results of this study provide a preliminary resource for analysis the immune mechanism for crustaceans in low salinity stress.

2. Materials and methods

2.1. Experimental animals

Adult shrimps of *L. vannamei* with an average body length of 9.5 ± 0.5 cm were obtained from a commercial farm in Shazikou, Qingdao, China. The shrimp were acclimated in tanks (40 cm × 50 cm × 60 cm) containing aerated natural seawater (salinity 31‰, pH 8.1) with an air-lift at 24 ± 0.5 °C for 7 days prior to experiments. Apparently healthy animal at the intermolt stage were chosen for the following experiment. The molt stage was discerned by observing partial retraction of the epidermis in uropoda. Half of the tank water was renewed twice daily. During the acclimation period, the shrimp were fed with a formulated commercial diet daily (Haiyue Company, Qingdao).

2.2. Experimental design

Before the experiment, no significant difference was observed in the shrimp weight among different treatments. At the beginning of the experiment, animals were randomly allocated to the experimental group and the control group with three replicates, and each replicate contained 12 shrimps. The shrimps of experimental group were transferred from the natural seawater with salinity 31‰ (as control) to 16‰, and then sacrificed rapidly with those in the control group at 24 h. During the experimental period, the water condition was kept the same as that for the acclimation (24 ± 0.5 °C; pH 8.1 ± 0.1). The salinity grade 16‰ was achieved by mixing seawater with insolated tap water. The hemocyte samples were prepared according to procedures described by Sung [32]. A hemolymph sample (0.6 mL) was drawn from the first abdominal segment of each shrimp with a 25-gauge needle containing 0.3 mL of an anticoagulant [33] (450 mmol/L NaCl, 10 mmol/L KCl, 10 mmol/L EDTA-Na_2 and 10 mmol/L HEPES, pH 7.45) with an osmolarity of 780 mOsm/kg. 6 mL hemolymph sample was placed into 10 mL RNase-free tubes and centrifuged at 700× *g* for 10 min at 4 °C. The pellet was suspended in 1 mL Trizol reagent (TransGen, China) and stored at −80 °C until for total RNA extraction.

2.3. Library preparation and sequencing

Total RNA was extracted using Trizol reagent at a concentration of 1 mL/6 mL of hemolymph according to preliminary experiment and treated with RNase-free DNase I (TaKaRa, Dalian, China). The quality and quantity of the purified RNA were determined by measuring the absorbance at 260 nm/280 nm (A260/A280) using NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA integrity was monitored on 1% agarose gels. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. Magnetic beads with poly T oligos attached were used for purifying the mRNA from the total RNA. Fragmentation was carried out using divalent cations under elevated temperature. First strand cDNA and Second strand cDNA were synthesized. cDNA fragments of 150~200bp and 3 μl USER Enzyme (NEB, USA) were used for running PCR with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The library preparations were sequenced on an Illumina Hiseq 2000 platform and 100 bp/50 bp single-end reads were generated.

2.4. Differential gene expression analysis

For the raw data, the clean reads were obtained by removing the reads containing adapter, ploy-N and low quality reads. The transcriptome library of *L. vannamei* (SRR346404), which contains 64,589 unigenes, was immediately used to link the expressed sequences to known genes from the hemocyte of *L. vannamei*. RPKM (Reads Per Kilo bases per Million reads) was used for a relative assessment of gene expression levels [34], DEGs were identified by the DESeq R package (1.10.1), corrected *P*-value<0.005 and the absolute value of $\log_2$ ratio≥1 were set as the threshold for significantly differential expression. For pathway enrichment analysis, all differentially expressed genes were mapped to the terms in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg), the statistical enrichment of differential expression genes in KEGG pathways was tested by KOBAS software (http://kobas.cbi.pku.edu.cn). Gene Ontology (GO) enrichment analysis of functional significance was applied using the GOseq R package, corrected *P*-value <0.05 were considered significantly enriched by differential expressed genes.

2.5. Quantitative analysis by quantitative real-time PCR (QPCR)

Eleven candidate genes were randomly selected for validation by QPCR. Total RNA was extracted from hemocytes of shrimps in control and low salinity groups at 24 h using Trizol method (TransGen, China), and the RNA sample was reverse-transcribed using SMARTer™ PCR cDNA Synthesis Kit (Clontech, USA), which allowed the amplification of cDNA from total RNA without the isolation of mRNA. Oligonucleotide primers (Table 1) specific to five target genes and the housekeeping gene (β-actin) were designed with the Primer Premier 5.0 software and synthesized by BGI. Each pair of primers was examined by RT-PCR to ensure their availability for QPCR. The QPCR was carried out using SYBR® PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China), performing with a PikoReal 96 Real-Time PCR System (Thermo Scientific) in final volumes of 10 μl, each containing 1 μL of cDNA, 5 μl 2 × SYBR premix Ex taq™ (Takara, Shiga, Japan), 0.2 μL each of forward and reverse primers (10 μM), and 3.6 μL sterile water. The PCR program for crustin-like