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RNA-seq based transcriptional analysis reveals dynamic genes expression profiles and immune-associated regulation under heat stress in *Apostichopus japonicus*

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ABSTRACT

In this study, we explored the gene expression profiles in *Apostichopus japonicus* under continuous heat stress (6 h, 48 h and 192 h) by applying RNA-seq technique. A total of 676, 1010 and 1083 differentially expressed genes were detected at three heat stress groups respectively, which suggested complex regulation of various biological processes. Then we focused on the changing of immune system under HS in sea cucumbers. Key immune-associated genes were involved in heat stress response, which were classified into six groups: heat shock proteins, transferrin superfamily members, effector genes, proteases, complement system, and pattern recognition receptors and signaling. Moreover, the mRNA expression of the immune-associated genes were validated by the real time PCR. Our results showed that an immunological strategy in this species was developed to confront abrupt elevated temperatures in the environment.

1. Introduction

Organisms are exposed to frequent fluctuations of environmental factors, which can cause their various molecular, biochemical, and physiological responses [1]. High temperature is a great threat to marine species due to the climate changing, and many of them have developed regulation strategies to facilitate their survival to some extent [2,3]. Heat shock proteins (HSPs), one of the most highly conserved groups of proteins characterized to date, are essential components in the heat shock response (HSR) [4]. Various HSPs, including HSP100, HSP90, HSP70, HSP60 and some small heat shock proteins (sHSPs), have been characterized and proved to play important roles under heat stress (HS) in kinds of marine species [5–7]. In addition to accumulation of HSPs, many other physiological changes are set into motion to help organisms deal with the HS. It is noteworthy that the immune system is significantly dysregulated under HS by complex interaction of nervous system and endocrine system [8,9]. Therefore, studies about immune responses in marine animals under HS draw more and more attention. These findings suggested that high temperature could impair immune defense and increase probability of diseases. For example, high temperature enhanced the adhesion of bacteria (*Flavobacterium columnare*) to the gills in the carp fish [10]. *Mozambique tilapia* also showed decreased phagocytic activity and pathogen resistance under HS [11]. There is also controversy over the effects of temperature on immune responses. Many humoral innate factors, including lysozyme, C-reactive protein and complement activity, have been observed to be up-regulated in fish under HS, which implied activated immunity [11–13].

The sea cucumber *Apostichopus japonicus* is a temperate species mainly distributed along the coast of eastern Asia. Owing to high value in nutrition and medicine, *A. japonicus* becomes popular food in China [14]. Hence aquaculture of *A. japonicus* is flourishing and expanding industry in China. However, extreme high temperature occurs frequently in summer due to the global warming. Summer mortality, as well as infectious diseases, is becoming a serious problem for *A. japonicus* aquaculture [15,16]. Therefore, it is of vital importance to understand the heat shock responses of *A. japonicus*. In our previous studies, we tracked the expression of HSPs under HS [17,18]. Moreover, a study with iTRAQ-based proteomics has been constructed to investigate the global protein expression profile under an acute short-term (48 h) heat stress from intestine of *A. japonicus* [19]. However, no relevant studies have been reported from *A. japonicus* for understanding their dynamic changes of overall gene expression. Besides, studies focused on the overall changes of immune system under HS are scarcely mentioned though many immune-associated genes have been characterized in this species [20–23].

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The aim of this study is to investigate differentially expressed genes (DEG) in sea cucumbers under HS, examine the dynamic change of DEGs along with exposure time, and particularly focus the regulation of immune system under HS. For these purposes, the gene expression profiles of *A. japonicus* were sequenced in the control, 6 h, 48 h and 192 h groups after 26 °C seawater, using RNA-seq technique. This high-throughput sequencing technique is powerful for obtaining a wide range of information about gene expression profiles [24]. Besides, we analyzed and validated the expression of immune-associated genes, which provides insights of the immunological regulation in *A. japonicus* under HS.

2. Materials and methods

2.1. Animal and sample treatment

A. japonicus (100–120 g) were collected from an aquaculture farm in Weihai (Shandong, China) in April 2016. The temperature of water in the farm was kept about 13–16 °C. These sea cucumbers were transported to our lab in Qingdao and acclimated in tanks for 2 weeks. The acclimation temperature was around 16 °C, and the salinity of seawater was 30‰. During acclimation, *A. japonicus* was fed with regular fodder, and half of water was changed daily.

When acclimation finished, six individuals taken from the tank were regarded as the control group. 26 °C is the temperature stress which sea cucumbers usually experience in summer in most coasts of northern China. Additonally, our previous studies implied HSR of *A. japonicus* was activated at this temperature [3,17,19]. Therefore, we set the HS groups with 26 °C in this study. The methods of heating and sampling were followed by our previous procedures [17,19]. In brief, the rate of heating was about 2 °C/h. The moment water temperature reaching 26 °C was regarded as the initial time. Then water temperature was maintained at 26 ± 0.5 °C during the subsequent experiment. Six individuals were taken randomly after 6 h, 48 h and 192 h stimulation. The intestine tissues of all groups were immediately sampled and frozen in liquid nitrogen. All the intestine samples were stored at −80 °C.

2.2. RNA extraction and processing

Three biological replicates of intestine tissue in each group were prepared for RNA-seq based transcriptional analysis. Total RNA was extracted using Trizol RNA isolation reagent (Invitrogen) following the manufacturer's instructions. The quality and concentration of RNA were measured by NanoDrop 1000 (Thermo Fisher Scientific, USA).

Oligo (dT) magnetic beads are used to select target mRNA with polyA tail. The target RNA was interrupted to ~200 bp fragments by addition of fragmentation buffer. Double-strand cDNA (dscDNA) were formed by reverse transcription. The dscDNA was repaired with phosphate and stickiness A at 5′ and 3′ end respectively, then was ligated with adaptor at 3′ end. Two specific primers are used to amplify the ligation product, and the single strand DNA is cyclized by splint oligo and DNA ligase. The library products were then sequenced via Illumina HiSeqTM 2000 (Beijing Genomics Institute, Shenzhen, China).

2.3. Data processing

Raw reads were filtered before downstream analysis to decrease data noise. The reads with adaptor sequence and with over 10% unknown bases. Low quality reads which contains over 50% low quality bases were also removed. After filtering, the remaining reads are called “clean reads” and stored as FASTQ format. The reference transcriptome used in this work was from a 454 sequence transcriptomic database, which had included data of all tissues, various developmental stages and physiological conditions of *A. japonicus* (NCBI accession NO. SRA020994) [25]. The reference transcriptome database contained 30,622 expressed sequence tags (ESTs).

2.4. Screening of differentially expressed genes (DEGs)

NOISeq method was applied to screen DEGs between two groups, which showed a good performance compared to other differential expression methods [26]. The detailed calculated method was followed as described in Sun et al. (2017) [27]. The default criteria screening DEGs are fold changes ≥ 2 and diverge probability ≥ 0.8.

2.5. Gene ontology and pathway enrichment analysis

Gene Ontology (GO) is an international standardized gene functional classification system, and Kyoto Encyclopedia for Genes and Genomes (KEGG) analysis helps to further understand genes biological functions. We mapped all DEGs to GO terms in the database (http://www.geneontology.org/) using program Blast2GO, and calculated gene numbers. Then the hypergeometric test was used to find enriched GO terms. All DEGs were mapped to KEGG database (http://www.genome.jp/kegg/), and enriched pathways were found. The calculated *P*-value went through Bonferroni Correction, taking corrected *P*-value < 0.05 as a threshold.

2.6. Real-time PCR validation of immune-associated genes

Six immune-associated genes from DEGs were applied with real-time PCR in order to validate their expression profiles. The mRNA extraction was followed by the method mentioned in section 2.2. First-strand cDNA was synthesized using reverse transcriptase (Takara, Japan). The mRNA expression validation were examined by a SYBR Green® real-time PCR assay (SYBR PrimeScrip™ RT-PCR Kit II, Takara) with an Eppendorf Mastercycler® ep realplex (Eppendorf, Hamburg, Germany). The housekeeping gene *β*-actin was widely used as a reference gene for internal standardization under various physiological conditions [28]. The gene *β*-actin was also chosen to be a internal gene in many studies about HSR in *A. japonicus* [17,18,29]. The forward and reverse primers of the genes for RT-PCR were shown in Table 1. The amplification cycling was performed in: (1) 5 s at 95 °C for 1 cycle (2) 10 s at 95 °C, 20 s at 60 °C, 30 s at 72 °C for 40 cycles. The melting curve analysis of the amplification products was performed to demonstrate the specificity of PCR products.

The $2^{-\triangle\triangle CT}$ method was carried out to analyze the comparative mRNA expression levels [30]. Homogeneity of variances (F-test) of the

Table 1
Primers of DEGs and reference genes in RT-PCR validation.

Gene name	F primer (5′-3′)	R primer (5′-3′)
heat shock protein 26	GCGAAATCGGTTGAGTCTTC	AATTGCCGAGACGTGAAATC
major yolk protein 1	TATTGTAGGCTCCCTCAGGTGT	TCAGGGTTCACAAACAGAATTG
annexin	CTTTGATGCAGGAGAAGATCG	ACCCCGACACTCGTCTCTAAT
trypsin-like serine protease	GCCAACCTTCAAACCACACT	GCAATGGTTAGCAGTCAGCA
mannan-binding C-type lectin	TGCTGCTGGACACAAACAA	GAGCCATCGGTCCAACTTT
complement factor H-like	ACGTCTAATGAGGACCCGACT	CATGGCAAAGGTTCACAGATT
β-actin	GGGCCAGCATTCCACTAATA	TGCCACGGGAGGTACTAAA