Comparative Biochemistry and Physiology, Part D 10 (2014) 30–43

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part D

journal homepage: www.elsevier.com/locate/cbpd

RNA-seq dependent transcriptional analysis unveils gene expression profile in the intestine of sea cucumber *Apostichopus japonicus* during aestivation

Ye Zhao [a,b], Hongsheng Yang [a,*], Kenneth B. Storey [c], Muyan Chen [d,**]

[a] Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, PR China
[b] University of Chinese Academy of Sciences, Beijing 100049, PR China
[c] Institute of Biochemistry, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada
[d] Fisheries College, Ocean University of China, Qingdao 266003, PR China

ARTICLE INFO

Article history:
Received 23 January 2014
Received in revised form 25 February 2014
Accepted 28 February 2014
Available online 27 March 2014

Keywords:
Hypometabolism
Differential gene expression
Aestivation–arousal cycles
Echinoderm
Environmental stress

ABSTRACT

The seasonal marine, the sea cucumber *Apostichopus japonicus* (Selenka, 1867), cycles annually between periods of torpor when water temperature is above about 25 °C in summer and active life when temperature is below about 18 °C. This species is a good candidate model organism for studies of environmentally-induced aestivation in marine invertebrates. Previous studies have examined various aspects of aestivation of *A. japonicus*, however, knowledge of the molecular regulation underpinning these events is still fragmentary. In the present study, we constructed a global gene expression profile of the intestine tissue of *A. japonicus* using RNA-seq to identify transcriptional responses associated with transitions between different states: non-aestivation (NA), deep-aestivation (DA), and arousal from aestivation (AA). The analysis identified 1245 differentially expressed genes (DEGs) between DA vs. NA states, 1338 DEGs between AA vs. DA, and 1321 DEGs between AA vs. NA using the criteria $|\mathrm{Log_2Ratio}| \geq 1$ and FDR ≤ 0.001. Of these, 25 of the most significant DEGs were verified by real-time PCR, showing trends in expression patterns that were almost in full concordance between the two techniques. GO analysis revealed that for DA vs. NA, 24 metabolic associated processes were highly enriched (corrected p value < 0.05) whereas for AA vs. NA, 12 transport and metabolism associated processes were significantly enriched (corrected p value < 0.05). Pathways associated with aestivation were also mined, and indicated that most DEGs were enriched in metabolic and signal transduction pathways in the deep aestivation stage. Two up pathways were significantly enriched at the arousal stage (ribosome and metabolism of xenobiotics by cytochrome P450 pathway). A set of key DEGs was identified that may play vital roles in aestivation; these involved metabolism, detoxification and tissue protection, and energy-expensive processes. Our work presents an overview of dynamic gene expression in torpor–arousal cycles during aestivation of *A. japonicus* and identifies a series of candidate genes and pathways for further research on the molecular mechanisms of aestivation.

1. Introduction

Aestivation is a state of dormancy that is well known to be used by terrestrial animals for surviving environmental conditions of high temperature or aridity (Storey and Storey, 2012). However, aestivation can also occur among marine invertebrate animals although the phenomenon has received little attention to date. The sea cucumber *Apostichopus japonicus* (Selenka, 1867), a seasonal marine aestivator, cycles annually between periods of torpor when water temperature is above about 25 °C in summer and active life when temperature is below about 18 °C. This species has proved to be a good candidate model organism for studies of environmentally-induced aestivation in marine invertebrates. This species has attracted more and more scientific attention, due to its phylogenetic position (an invertebrate deuterostome) and its special physiological characters (Ji et al., 2008; Ortiz-Pineda et al., 2009; García-Arrarás and Dolmatov, 2010; Sun et al., 2013; Chen et al., 2013). *A. japonicus* is also an important aquatic species with high commercial and medicinal values in Japan, Korea and north of China (Liao, 1997). The annual period of aestivation usually lasts more than 4 months with a complete cessation of feeding and locomotion (Li et al., 1996). During this period the intestine degenerates into a very tiny filament associated with obvious hypometabolism of the whole

Abbreviations: NA, non-aestivation; DA, deep-aestivation; AA, arousal from aestivation; DEG, differentially expressed gene; RPKM, reads per kb million reads; FDR, false discovery rate.

* Corresponding author. Tel.: +86 532 82898610 (office).
** Corresponding author. Tel.: +86 15066271208.
E-mail addresses: hsyang@ms.qdio.ac.cn (H. Yang), chenmuyan@gmail.com (M. Chen).

http://dx.doi.org/10.1016/j.cbd.2014.02.002
1744-117X/

animal (Li et al., 1996; Yang et al., 2005; Yang et al., 2006). This undoubtedly shortens the growing cycle of *A. japonicus* and brings economic losses.

The control of gene expression is crucial for any living organism and identification of the underlying mechanisms that allow organisms to switch between torpor and arousal are common central questions in the study of torpor among different organisms (Morin and Storey, 2009). Similar to the situation for mammalian hibernation, the transitions from an active non-aestivating state into deep aestivation and then arousal from prolonged aestivation include complex physiological and biochemical reorganization. Studies of gene expression profiles in response to circannual cycles offer an opportunity to understand the basic parts of this reorganization. Although physiological adaptations supporting sea cucumber aestivation have been well-researched (Yang et al., 2005; Yang et al., 2006; Gao et al., 2009), few attempts have been made to evaluate changes in gene transcript levels, and research on the underlying molecular mechanisms of sea cucumber aestivation is only the beginning. For example, Ji et al. (2008) reported that heat shock protein 70 (Hsp 70) levels decreased during aestivation. Recently, Du et al. (2012) constructed a profile of the differentially expressed genes (DEGs) between the active and aestivating states in sea cucumbers by combining the intestine, respiratory tree and coelomic fluid tissues and applying 454 life sequencing. We sequenced gene expression profile of the intestine from sea cucumbers under different aestivation stage (NA, DA and AA) using Illumina HiSeq™ 2000 (BGI, Shenzhen), which provides a better depth of sequencing compared to 454.

The present study undertakes an in-depth analysis of the transcriptome responses to aestivation by a single specific tissue (intestine) of the sea cucumber. Comparisons are made of three crucial life stages:(1) non-aestivation (NA) animals that have gone through the aestivation period, regenerated their intestinal tissue and returned to active status; (2) deep-aestivation (DA) animals that have stopped feeding and locomotion and the intestine had degenerated into a very tiny string (about 2–3 mm), usually after 15 days of continuous torpor; and (3) arousal from aestivation (AA) after the water temperature was reduced to 18 °C for 2 days and animals showed renewed movement and feeding (feces in the intestine). The Illumina technology used here generated significantly more reads than the previous 454 life sequencing, providing us with much more information; e.g. 1245 DEGs (DA vs. NA), 1338 DEGs (AA vs. DA) and 1321 DEGs (AA vs. NA) were identified as compared with only 446 DEGs identified by Du et al.(2012). Furthermore, we were able to use the most complete reference library of *A. japonicus* to date to help annotate our results; this integrated the transcriptome of the regenerative body wall and the intestine constructed by our team (Sun et al., 2011) and another nine transcriptomes including all the tissues of sea cucumbers at different developmental stages (embryo, larva, white juvenile and black juvenile) and under different physiological conditions (Du et al., 2012). GO and pathway enrichment analysis were also applied to provide an overview of the main functions of the DEGs identified during aestivation. Our results identify the key candidate genes and indicate the potentially significant role of transcriptional regulation in torpor–arousal cycles during aestivation.

2. Materials and methods

2.1. Animals

Adult individuals of the sea cucumber *A. japonicus* (80–120 g body mass) were collected from the coast of Qingdao (Shandong, China) in early March, and acclimated in seawater aquaria at 15 °C for one week before use; sea cucumbers were fed once a day. All sea cucumbers were then divided randomly into either control or experimental groups. Sea cucumbers in the control non-aestivation (NA) group were kept at a constant temperature (about 15 °C) throughout the experiment and individuals were sampled at the same times as their corresponding DA or AA group. Animals in the experimental group were slowly induced to aestivation by increasing the water temperature from 15 °C to 25 °C at a rate of 0.5 °C per day. A temperature of 25 °C was determined to be the threshold for entering aestivation in our previous study (Yang et al., 2006). Animals in deep aestivation (DA) were collected after 15 days at 25 °C. Sea cucumbers had stopped feeding, and their movements, if any, were very limited. Other animals were sampled soon after arousing from torpor, after the water temperature was reduced to 18 °C (at a rate of 0.5 °C per day) and moving and feeding were observed in the experimental animals. In the present study, the AA animals were sampled after 2 days back at 18 °C. The whole sampling process has been described in our previous study (Zhao et al., 2013). At each stage, 15 individuals were sacrificed (NA, DA and AA) and the intestine tissue was rapidly dissected out and frozen in liquid nitrogen for later analysis.

2.2. Preparation of cDNA library for sequencing

Total RNA was isolated from the intestine tissues at the above three stages of the circannual cycle of *A. japonicus* using an RNeasy mini kit including DNase-treatment with an RNase-free DNase (Qiagen Inc., Germany), following the manufacturer's instructions. RNA concentration and quality were determined using an Agilent 2100 bioanalyzer. Total RNA from 6 individuals per stage was pooled. The starting amount of RNA was 1 μg per pool (6 individuals). The following steps were performed at BGI-Shenzhen including mRNA enrichment, fragment interruption, addition of adapters, size selection, PCR amplification, and RNA-Seq. Briefly, mRNA was separated from the total RNA using oligo(dT) magnetic beads and broken into short fragments (approximately 200 bp) as templates for first strand cDNA and second strand cDNA synthesis. Sequencing adaptors were then ligated to the double stranded cDNA after purifying with a QIAquick PCR extraction kit. The required fragments (about 200–700 bp) were screened using agarose gel electrophoresis and amplified as sequencing templates via PCR. The final sequencing of the library products was performed using Illumina HiSeq™ 2000 (BGI, Shenzhen, China).

2.3. Sequence filtering, mapping and assessment

To get clean reads, raw reads were filtered by discarding bad sequences (adaptor reads, >10% unknown bases, and low quality reads) (Fig. A.1). A comparison of clean reads was carried out by SOAP aligner/SOAP2 using the reference databases from sea cucumber large scale transcriptome profiling (Sun et al., 2011; Du et al., 2012) allowing no more than 2 base mismatches. Eventually, sequence quality was validated after a series of data assessments consisting of sequence reads quality assessment, sequencing saturation analysis, distribution of reads on reference genes, and gene coverage analysis. The detailed process has been described in our previous work (Sun et al., 2013). The obtained sequences were submitted to NCBI (accession No. GSE51137).

2.4. Gene expression levels and identification of differentially expressed genes (DEGs)

The number of clean tags exclusively mapped for each gene was counted and normalized by using the RPKM method (reads per kb million reads). Specifically, the computational formula was put forward by Mortazavi et al. (2008) as follows where C is the uniquely aligned number of reads to one gene; N represents the total number of reads which are uniquely aligned to all genes, and L refers to the gene's base number.

$$RPKM = \frac{10^6 C}{NL/10^3}$$

By virtue of this standardization, we can detect the transcription levels without deviation by effectively removing the gene length