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Transcriptomic responses of *Perna viridis* embryo to Benzo(a)pyrene exposure elucidated by RNA sequencing

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HIGHLIGHTS

- Transcriptome responses to BaP exposure of *Perna viridis* embryos were examined.
- Genes involved in stress, infectious disease and innate immunity were altered.
- Gene Ontology and Kyoto Encyclopaedia of Genes and Genomes analysis were conducted.
- Six stress response-related genes were selected for qPCR analysis for validation.

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ABSTRACT

The green mussel *Perna viridis* is an ideal biomonitor to evaluate marine environmental pollution. Benzo(a)pyrene (BaP) is a typical polycyclic aromatic hydrocarbon (PAH), which is well known for the mutagenic and carcinogenic characteristics. However, the toxicological effects of BaP on *Perna viridis* embryo are still unclear. In this study, we investigated the embryo transcriptomic profile of *Perna viridis* treated with BaP via digital gene expression analysis. A total of 92,362,742 reads were produced from two groups (control and BaP exposure) by whole transcriptome sequencing (RNA-Seq). Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis were used on all genes to determine the biological functions and processes. Genes involved in various molecular pathways of toxicological effects were enriched further. The differential expression genes (DEGs) were related to stress response, infectious disease and innate immunity. Quantitative real-time PCR (qRT-PCR) measured expressional levels of six genes confirmed through the DGE analysis. This study reveals that RNA-seq for transcriptome profiling of *P. viridis* embryo can better understand the embryo toxic effects of BaP. Furthermore, it also suggests that RNA-seq is a superior tool for generating novel and valuable information for revealing the toxic effects caused by BaP at transcriptional level.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large group of substances ranging from two-ring benzene structures to other more complex structures containing up to 10 rings. United Nations Environment Program (UNEP) classified PAHs as Persistent Organic Pollutants (POPs). Most of them comes from pyrolysis and inadequate combustion of organics, such as fossil fuel emissions (Menzie et al., 1992). They have lower water solubility and refractory that leads to bio-accumulation and bio-amplification in the food cycle (Deribe et al., 2011). Due to their carcinogenic, teratogenic and mutagenic features, PAHs have been paid much more attention in ecotoxicology. Extensive researches have indicated that these chemicals can induce deterious effects such as fatal ischemic heart disease (IHD), low birth weight, immune system deficiency, and neural tube defects (Burstyn et al., 2005; Choi et al., 2006; Goodale, 2013). Studies on the PAH toxicity show that high doses led to very

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high fish embryo mortality and decreased growth, leading to malformation, oedema and difficulty swimming (Barron et al., 2004). Furthermore, PAHs can cause oxidative damage (Sureda et al., 2013), and decrease lysosome integrity, inhibit phagocytosis and have negatively affect on the cellular immunity (Grundy et al., 1996).

BaP is a typical PAHs compound that is ubiquitous in marine environment. The toxicological effects of BaP have been tested in various marine animals (Wiebel et al., 1971; Gibson et al., 1975; Payne and Penrose, 1975; Fossi et al., 2000; Juhasz and Naidu, 2000; Zhang et al., 2011a,b). Because BaP has genotoxic and non-genotoxic properties, many transcriptomic responses of marine animal exposed to BaP have been performed (Van Delft et al., 2012), including bivalves (Brown et al., 2006). Changes in microRNA expression have been detected in mice after BaP exposure (Halappanavar et al., 2011). Proteomics approaches have also been used to study the molecular mechanism of BaP toxicity (Song et al., 2016). Due to the research methods are very limited previously. Thus, the molecular toxicological mechanism of BaP still remains unclear in marine bivalves (Deng et al., 2014).

With the development of system biology, the -omic techniques have been widely used in toxicology (Xu et al., 2016; Ji et al., 2013a; Liu et al., 2011; Liao et al., 2007; Wu et al., 2005a,b). The transcriptomic approach based on the next generation sequencing (NGS) technique for high-throughput RNA-sequencing (RNA-seq) is superior to other methods, including higher sensitivity, greater gene coverage, and better reproducibility of highly complex data sets (Wang et al., 2009; Ekblom and Galindo, 2011). Digital gene expression (DGE) profile is based on the transcriptome sequencing method, which can determine gene expression levels relative to other expression profiles, in spite of a potential bias (Qin et al., 2011; Ye et al., 2011). This type of result could help to analyse the whole transcriptional changes after BaP exposure.

In marine ecosystems, green mussel *Perna viridis* is widely distributed in Asian coastal waters from oceanic waters to estuarine systems. It is also a commercially important species (Yap et al., 2004). Furthermore, *P. viridis* plays a crucial role in the food chain as a biomonitor of environmental pollution in coastal areas. *P. viridis* has a fixed lifestyle near campsites and retain filtered food particles that can increase pollutant bio-concentrations (Skarphedinsdottir et al., 2010; Deribe et al., 2011). In order to better understand the embryo molecular toxicological mechanism of BaP-induced, we utilized RNA-Seq to measure the genome-wide transcriptional responses in *Perna viridis* embryo exposed to BaP.

2. Materials and methods

2.1. Mussel embryo and BaP exposure

Healthy adult *Perna viridis* (both male and female; shell length: 6.0–7.0 cm) were collected from a mussel farm in Lingshui County (Hainan, China). These green mussels were acclimated in vessels containing aerated sand-filtered natural seawater (salinity, 32‰; temperature, 25 ± 2 °C; pH, 8.2 ± 0.1). After acclimating, the green mussels were artificially inseminated. Fertilized eggs were randomly allocated into two groups (BaP exposure and control) with three replicates with a number about 100 for each treatment. At the 2-cell stage, embryos were treated with seawater containing 400 μg/L of BaP for 24 h. The experimental concentration of BaP was designed according to the pre-experiment and the report of Wang et al. (2012). Dimethylsulfoxide (DMSO) is a cosolvent used to increase the solubility of BaP. In order to remove the toxic effects of DMSO to embryo, the control group green mussel embryos were exposed to seawater in DMSO for 24 h. The final DMSO concentration was 0.001% in all beakers including control. All other conditions remained the same as the acclimation period.

2.2. RNA extraction

Embryos from three replicates (treated and control groups) were resuspended in 1 ml of Trizol reagent (Invitrogen) to extract the total RNA in accordance with the manufacturer instructions. And then, we utilized the NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer to detect the total RNA concentration and purity.

2.3. cDNA library preparation and DGE sequencing

In briefly, poly-dT oligo attached to magnetic beads was used to purify mRNA from total RNA samples (3 μg), and then fragmented at an elevated temperature. cDNA was synthesized through random oligonucleotides and SuperScript II (Invitrogen). Second-strand cDNA was synthesized by DNA polymerase I, and the previous RNA template was digested using RNase H. Ends were repaired and 3′ single nucleotide A (adenine) was added. Fragment sizes of 200 bp were used for PCR. The quality and quantity of sample library were detected by Agilent 2100 Bioanalyzer and ABI StepOnePlus qPCR System respectively. Library products were further sequenced by Illumina HiSeqTM2000.

2.4. Sequence reads mapping and assembly

Reads mapped to each gene were measured by RESM method (Li and Dewey, 2011; Hu et al., 2015). Afterwards, the Reads Per Kilobase per Million mapped reads (RPKM) of each transcript was counted upon the transcript length and the read count mapped to a transcript. RPKM simultaneously take into account the transcript length for the reads count and the effect of sequencing (Mortazavi et al., 2008).

2.5. Analysis of DEGs

DEGs were screened among samples, and GO functional enrichment analysis and KEGG pathway enrichment analysis were used for these DEGs (Audic and Claverie, 1997). DEGs were idenfied by a strict algorithm between two samples. False Discovery Rate (FDR) was used as the threshold of the *p*-value in multiple tests (Benjamini and Yekutieli, 2001). FDR $\leq$0.001 and |log2 Ratio| $\geq$ 1 were set as the thresholds to evaluate the significance of gene expression difference.

2.6. GO and KEGG analysis

At present, GO is a commonly standard gene functional classification system in the world. GO terms can exactly describe the properties of genes and their products for all organism. The Blast2GO program retrieves GO annotations of DEGs annotated Nr. We then used WEGO software to classify the GO categories for DEGs and help to comprehend the distribution of gene functions from the species at macro level (Ye et al., 2006). GO terms with corrected *p*-value < 0.05 were considered significant.

Because gene products interact with each other, pathway analysis provides understanding of significant genes for additional biological functions. KEGG is a major database resource about pathway analysis. Q-values < 0.05 were defined as significant in a given pathway for differentially expressed genes. We identified significant metabolic pathways or signal transduction pathways in DEGs analysis via pathway enrichment analysis.