RNA-SEQ ANALYSIS OF THE MYSID CRUSTACEAN TRANSCRIPTOME FOLLOWING EXPOSURE TO NATURALLY OCCURRING 1,3,7-TRIBROMODIBENZO-*P*-DIOXIN

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Introduction

Some of polybrominated dibenzo-*p*-dioxins (PBDDs), less brominated congeners are naturally occurring products and accumulated at high levels in marine biota such as red algae, marine sponge and cyanobacteria[1]. Di-/triBDDs are ubiquitously detected in marine sediments and mussels of the Seto Inland Sea, Japan[2]. It has been shown that in vertebrates some of the naturally occurring lower brominated congeners elicit toxic effects similar to its chlorinated analogues, polychlorinated dibenzo-*p*-dioxins (PCDDs), and induce cytochrome P450 1A (CYP1A) through the aryl hydrocarbon receptor (AhR)[3,4]. The relative potency of TriBDD is two orders of magnitude lower than those of TCDD[5]. On the other hand, toxic effects of less brominated PBDDs on invertebrates remain unclear. Several studies have shown the inability of invertebrate AhR homolog to bind to vertebrate AhR ligands[6,7]. Therefore, it is important to investigate whether naturally occurring PBDDs cause effects in invertebrate species.

Mysid crustaceans are common motile supra-benthic invertebrates[8]. Although the mysid has been put forward as a suitable model of marine crustaceans for assessing the toxicity of environmental contaminants by several agencies including USEPA and OECD[9], a broad spectrum of effects posed by these pollutants, particularly at the molecular level are not fully understood.

The "adverse outcome pathway" concept has recently been introduced as a framework to link multiple levels of biological organization, bridging direct molecular initiating events and adverse outcomes[10]. More recently, Pillai *et al.*[11] has tried to establish a further concept of linking the toxicity and adaptive response pathway. For approach to these frameworks, deep sequencing, known as RNA-seq analysis by next-generation sequencing technologies, provides both the sequence information and the profile of transcriptome in organisms that lack reference genome sequences[12]. In the present study, to supply transcriptome data for studying biological basis in crustaceans, and to understand the mechanism underlying the response to chemical exposure, we conducted RNA-seq analysis of the mysid crustacean transcriptome following exposure to a lower brominated PBDD congener, 1,3,7-tribromodibenzo-*p*-dioxin (1,3,7-TriBDD). We also carried out bioinformatic analyses of the transcriptome to identify genes that were differentially expressed in response to 1,3,7-TriBDD.

Materials and methods

Mysid strain and culture conditions

Mysids (*Americamysis bahia*) were obtained from the Prefectural University of Kumamoto, Kumamoto, Japan[13]. The animals were originally provided from the USEPA and have been maintained for 3 years at the laboratory of Ehime University. Culture medium was prepared using artificial seawater (Marine art BR, osakayakken Co., Ltd., Japan), diluted with aerated deionized tap water to a final salinity of 25 ‰. Culture room was maintained at 25±1 °C, with a photoperiod of 14 h light: 10 h dark. The brine shrimp nauplii (*Artemia* sp., < 24-h after hatching) were fed daily.

Exposure design

In this study, 1,3,7-TriBDD was used for exposure test. Prior to the exposure test, all offspring were removed from a culture of adult mysids and acclimated for 7-days. One hundred fifty juvenile mysids (7-days old) per treatment group were exposed to a single concentration (25.3 µg/L) of 1,3,7-TriBDD for 48 h under static conditions in petri dishes (φ 75×90 mm) containing 150 ml of seawater (Table 1). The solvent control group was exposed to 0.1% (v/v) dimethyl sulfoxide (DMSO) as a solvent carrier. Mysids in each group did not feed any

Table 1. Summary of the transcriptome assembly of libraries from control and 1,3,7-TriBDD-treated mysids.

	DMSO	1,3,7-TriBDD
Nominal concentration (μg/L)	0.10%	25.3
Total number of reads	153,781	255,661
Cut of minimum read	~70	~70
Number of contigs	3,486	2,290
Mean length of contig	115	126

diet during the exposure. Following the exposure, a pooled mysid sample in each group was placed in RNA*later* solution (Ambion), stored at 4 °C overnight, and then maintained at -30 °C until RNA extraction.

Sample preparation and next-generation sequencing

Total RNA samples were extracted from 150 mysids for each exposure group with an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA integrity was verified by using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The qualified total RNA samples were further used for RNA-seq experiments. Ribosomal RNA in each RNA sample was depleted using GeneRead rRNA depletion kit (Qiagen), and mRNA residues were fragmented to the size of an average of 200 nt by RNAse III enzyme. The yield and size distribution of fragmented mRNAs were analyzed by using the Agilent 2100 Bioanalyzer. After RNA oligo ligation at the ends of fragmented mRNAs, RNA sequencing libraries were constructed using the Ion Total RNA-Seq kit v2 (Ion Torrent, Life Technologies) according to the manufacturer's instruction. The constructed library underwent PCR amplification, purification and size selection. The selected PCR products were used for emulsion PCR, followed by positive bead recovery, chip loading and sequencing. Sequencing was carried out using Ion Personal Genome Machine (PGM) sequencing platform (Life Technologies).

De novo assembly of transcriptome and analysis of differentially expressed genes

De novo assembly and contig editing were performed with CLC Genomics Workbench v.6.5.1 (CLC Bio, Inc.) using crustacean species RNA database as a reference. For assembly, the reads were uploaded into CLC Genomics as FASTA files and assembled using default parameters. All assembled contigs were compared with crustacea sequences in NCBI non-redundant (nr) protein database for functional annotation by using standalone tBlastx (within the CLC Genomics tool). For gene expression analysis, the expression level of each gene in the control and 1,3,7-TriBDD-treated samples was calculated and normalized to Reads per Kilobase per Million (RPKM)[14]. Two-fold change in the RPKM values of the corresponding transcripts between control and 1,3,7-TriBDD-treated group was regarded as the differential expression.

The genes that were abundant or scarce in 1,3,7-TriBDD-treated group were screened for functional categorization of differentially expressed genes. Gene ontology (GO) analysis of up- and down-regulated genes was performed by using DAVID database[15], and genes were categorized based on enrichment of GO terms into biological process, molecular function and cellular component.

Results and discussion

In order to evaluate the change in gene expression profile by exposure to 1,3,7-TriBDD in invertebrate species, RNA-seq data were obtained for juvenile mysids exposed to this chemical. To prepare enough amounts of transcripts for RNA-seq, pooled samples of 150 juvenile mysids were homogenated. The total RNA concentrations of the lysates obtained from DMSO- and 1,3,7-TriBDD-treated groups were 23 and 47 μg, respectively. The libraries containing RNA transcripts prepared from the lysate samples were sequenced using the Ion PGM sequencing platform.

The high-throughput sequence data using Ion Torrent PGM generated a total of 153,781 and 255,661 reads in DMSO- and 1,3,7-TriBDD-treated groups, respectively. These reads were further analyzed on the commercially available assembly program of CLC Genomics workbench using crustacean RNA database as a reference. Partial

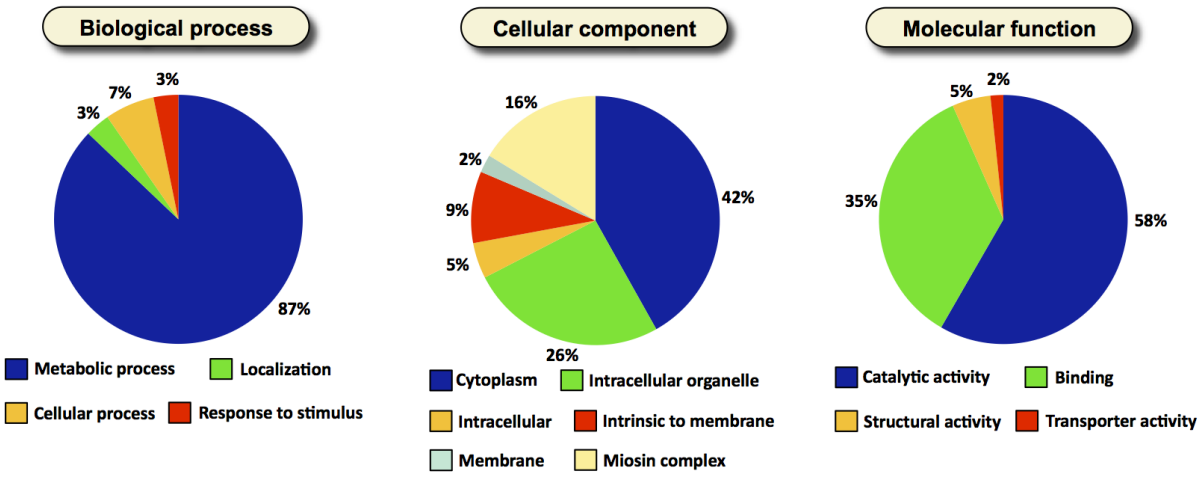

Fig. 1. Results of GO annotation of genes that were up- and down-regulated by exposure to 1,3,7-TriBDD. The classification of the transcriptome was performed according to the gene ontology terms: "Biological process", "Cellular component" and "Molecular function".

assemblies, singletons, tandem repeats, outliers, or less than 70 bp of reads were not used for further analyses. For DMSO- and 1,3,7-TriBDD-treated mysids, 3,486 and 2,290 contigs were assembled with an average length of 115 and 126, respectively (Table 1).

Analysis of differentially expressed genes revealed that the expression of 302 transcripts was altered by treatment with 1,3,7-TriBDD; 184 and 118 genes were up- and down-regulated, respectively. When the differentially expressed contigs were compared, changes in GO categories as an indication of function were observed. The results of this ontology analysis are shown in Fig. 1. The analysis of differentially expressed transcripts in "biological process" category revealed a significant enrichment of metabolic process (87 %). In "cellular component" category, cytoplasm (42%) was the dominant group followed by intracellular organelle (26%). For "molecular function" category, catalytic activity (58%) was the most representative GO term, followed by binding (35%). These well-categorized and annotated resources indicate that 1,3,7-TriBDD may affect the metabolic function in cytoplasm.

In this study, we analyzed a comprehensive transcriptome of mysid *A. bahia* exposed to 1,3,7-TriBDD using next-generation sequencing technology. The genes differentially expressed in mysid following 1,3,7-TriBDD exposure were identified and functionally annotated. This indicates that naturally occurring PBDDs may have a potential to elicit adverse effects in crutaceans as well as other pollutants. The transcriptome data generated in this study provide genetic information on the mysid, and can be useful for better understanding of the biological response to environmental pollutants in crustaceans in future ecotoxicological studies.

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