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Transcriptome analysis of immune response against *Vibrio harveyi* infection in orange-spotted grouper (*Epinephelus coioides*)

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ABSTRACT

Vibrio harveyi is a gram-negative bacterium reported as found in many aquaculture species. To increase knowledge of the immune response against *V. harveyi*, in this study we performed transcriptome analysis of head kidney and spleen in orange-spotted grouper (*Epinephelus coioides*) at 1 and 2 days post-infection (dpi), using the Illumina sequencing platform. After *de novo* assembly, a total of 79,128 unigenes was detected with an N50 of 2511 bp. After alignments with sequences recorded in the major databases (NT, NR, Swiss-Prot COG, KEGG, Interpro and GO), based on sequence similarity, 61,208 (77.4%) of the unigene total could be annotated using at least one database. Comparison of gene expression levels between *V. harveyi* and a control group at each time point revealed differentially expressed genes (DEGs) ($P < 0.05$): a total of 7918 (5536 upregulated and 2282 downregulated genes) from head kidney at 1 day post infection (dpi), 4260 (1444 upregulated and 2816 downregulated genes) from head kidney at 2 dpi, 7887 (4892 upregulated and 2995 downregulated genes) from spleen at 1 dpi, and 8952 (7388 upregulated and 1564 downregulated genes) from spleen at 2 dpi. The DEGs were mainly annotated into signal transduction and immune system categories, based on the KEGG database. The DEGs were enriched in immune-related pathway functions, NOD-like receptor signaling pathways, Toll-like receptor signaling pathways, NF-κB signaling pathways, and Jak-STAT signaling pathways. Additionally, we selected several DEGs and validated their expression level by RT-qPCR. The data generated in this study may provide a valuable resource for further immune response research and offer improved strategies against *V. harveyi* infection in teleost fishes.

1. Introduction

Vibrio harveyi is a luminescent gram-negative bacterium widely reported in the marine environment. This bacterium is a major pathogen responsible for causing fatal vibriosis in aquatic vertebrates and invertebrates. Various teleosts are infected by *V. harveyi*, including Atlantic salmon (*Salmo salar*) [1], common snook (*Centropomus undeeimalis*) [2], olive flounder (*Paralichthys olivaceus*), black rockfish (*Sebastes schlegeli*) [3], gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*) [4], and orange-spotted grouper (*Epinephelus coioides*) [5].

There are many reports that immune-related molecules respond to *V. harveyi* infection in various teleosts. In sea bass (*Lateolabrax maculatus*), the expression levels of heat shock protein 70 genes are enhanced in the head kidney, intestine, and gill by *V. harveyi*

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challenge [6]. The Toll-like receptor 5 (membrane form) gene was highly expressed after *V. harveyi* infection in Japanese sea perch (*Lateolabrax japonicas*) [7]. Japanese pufferfish (*Takifugu rubripes*) infected with *V. harveyi* exhibited elevated expression of nucleotide binding oligomerisation domain-like receptors [8]. In golden pompano (*Trachinotus ovatus*), the expression of the c-type lysozyme gene was enhanced after *V. harveyi* infection [9]. In humphead snapper (*Lutjanus sanguineus*), the expression of lymphocyte cell kinase in the thymus, spleen and head kidney was enhanced after challenge with *V. harveyi* [10]. The interferon gamma gene of the large yellow croaker (*Larimichthys crocea*) was upregulated after injection with *V. harveyi* [11]. Although these reports identified some important genes in teleosts against *V. harveyi* infection, the responses of many immune-regulated genes remain unclear.

Transcriptome profiling using next-generation sequencing (NGS) technologies have recently provided new insights into immune responses against bacterial infections in various aquaculture animals. It has been reported that larval orange-spotted grouper infected with *Vibrio alginolyticus* showed the induction of complement and hepcidin systems [12]. There also have been several reports of immunological reaction to bacteria using transcriptome analysis in teleosts, such as Antarctic notothenioid fish (*Notothenia coriiceps*) [13], Asian seabass (*Lates calcarifer*) [14], barramundi (*Lates calcarifer*) [15], blunt snout bream (*Megalobrama amblycephala*) [16], common carp (*Cyprinus carpio*) [17], Nile tilapia (*Oreochromis niloticus*) [18,19] and Zebrafish (*Danio rerio*) [20]. We have also revealed the transcriptome profiles of differentially-expressed genes in largemouth bass (*Micropterus salmoides*) infected with *Nocardia seriolae* [21] and grey mullet (*Mugil cephalus*) infected with *Lactococcus garvieae* [22].

In this study, we used Illumina technology for transcriptome profiling analysis of the orange-spotted grouper (*Epinephelus coioides*) with *V. harveyi* infection. The information of transcriptome sequences and differentially-expressed genes may provide a valuable resource for further research and new strategies against *V. harveyi* infection.

2. Materials and methods

2.1. Experimental animals

We used healthy orange-spotted grouper (*Epinephelus coioides*) without pathogen infection and weighing 100 ± 3 g. The fish were maintained in an indoor facility at a water temperature of 26 °C and acclimatized for two weeks prior to experiments. All experiments were conducted according to the Centre for Research Animal Care and Use Committee of the National Pingtung University of Science and Technology (no# 105-018).

2.2. Vibrio harveyi infection

Twenty fish were anaesthetised with 2-phenoxyethanol and injected intraperitoneally with non-lethal dose of 1.0×10^5 cfu *V. harveyi* ($LD_{50} = 1.7 \times 10^7$ cfu) suspended in 100 μL phosphate-buffered saline (PBS). Another 20 fish were injected with 100 μL PBS as a control. At 1 and 2 days post-infection (dpi), samples of head kidney and spleen were taken from 7 fish (3 fish for RNA-seq and 4 fish for RT-qPCR) each from the infection and control groups.

2.3. Total RNA extraction, cDNA library preparation, and sequencing

Extraction of total RNA was performed using TRIzol® reagent (Invitrogen Corp., Carlsbad, CA, USA). The quality of the extracted RNA was assessed using an Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). TruSeq™ RNA Sample Preparation Kit (Illumina, Inc., San Diego, CA, USA) was used to generate cDNA libraries. Briefly, total RNA (40 μg) was performed to isolate mRNA by poly-T oligo-attached magnetic beads. First-strand cDNA was synthesized using random hexamer primers and Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). This step was followed by synthesis of second-strand cDNA, end repair, and ligation of adaptor. Sequencing was performed using the Illumina HiSeq™ 4000 platform (Illumina, Inc., San Diego, CA, USA) and 150 bp paired-end reads generated at Genomics Bioscience Technology Co., Ltd. (Taipei, Taiwan).

2.4. Filtering of sequencing reads and de novo transcriptome assembly

Clean data were obtained by removing reads which contain adapter, ploy-N and low quality reads from raw data. Q20, Q30 and GC-content of the clean data were calculated. Clean data were stored in FASTQ file format [23]. After read filtering, clean data were used to perform *de novo* assembly using Trinity [24]. Gene family clustering was then performed with TIGR Gene Indices clustering tools (TGICL; version v2.0.6) to establish a final set of Unigenes [25].

2.5. Gene annotation and classification

The assembled transcripts were scanned using the following databases; NCBI nucleotide sequences (NT), NCBI non-redundant protein (NR), Clusters of Orthologous Groups (COGs), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) using BlastP (Version 2.2.25) [26], gene ontology (GO) using Blast2GO (version v2.5.0) [27] and InterPro annotation using InterProScan5 (version v5.17–56.0) [28].

2.6. Differentially-expressed genes and enrichment analysis

Expression data from each library were generated by Bowtie2 software (version v2.2.6) [29], which mapped to the transcriptome assembly. To identify differentially-expressed genes (DEGs), the fragments per kilobase of transcripts per million fragments mapped (FPKM) values were calculated using RSEM (version v1.2.12) [30]. A false discovery rate (FDR) was used to calculate the threshold p-value in multiple tests. Significant enrichment was calculated when FDR was <0.05 and the values of FPKM showed at least a 2-fold difference between the two samples reads.

2.7. Real-time reverse transcription polymerase chain reaction

Total RNA was subjected to cDNA synthesis using iScript™ cDNA synthesis kits, Bio-Rad. Reverse transcriptase real-time PCR (RT-qPCR) was performed using iQ™ SYBR® Green Supermix (Bio-Rad). Gene expression levels were normalized to that of β-actin. The mean threshold cycle was used to determine relative expression levels. Primers used for RT-qPCR were designed using Primer3 software [31]. The sequences of primers were listed in Table S1. Statistics analysis to compare between the two groups were performed using Student's t-test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Transcriptome sequence assembly and functional annotation

RNA-Seq was performed on head kidney and spleen samples from two groups with *V. harveyi* administration or PBS-only injection as a control. After filtering to remove low-quality sequences, a