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Comparative transcriptomic analysis reveals the gene expression profiles in the liver and spleen of Japanese pufferfish (*Takifugu rubripes*) in response to *Vibrio harveyi* infection

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ABSTRACT

Japanese pufferfish (*Takifugu rubripes*) is one of the main marine aquatic fish species cultured in Asia due to its high nutritional value. In recent years, disease caused by *Vibrio harveyi* infections have led to serious mortality in Japanese pufferfish industry. To understand the complex molecular mechanisms between *V. harveyi* and Japanese pufferfish, we performed a transcriptome analysis of liver and spleen samples from Japanese pufferfish at 1 and 2 day post-infection. Between-group comparisons revealed 922 genes that were significantly differentially expressed. The altered genes emphasized the function in several immune related pathways including MAPK signaling pathway, JAK-STAT signaling pathway, toll-like receptor signaling pathway, cytokine-cytokine receptor interaction and lysosomal pathway. The data generated in this study provided insight into the responses of Japanese pufferfish against *V. harveyi* at the transcriptome level, promoting our comprehensive understanding of immune responses for aquatic animal against *V. harveyi*.

1. Introduction

Japanese pufferfish (*Takifugu rubripes*) is an economically important aquaculture species in Asia, especially in China and Japan. Stocks of wild Japanese pufferfish greatly decreased in the late 1980s because of the higher fishing pressure. Japanese pufferfish are artificially cultured for consumers, and production of the cultured Japanese pufferfish increased rapidly from 2000 to 2005 [1]. Due to the high nutritional and medical value, the annual production of cultured fish has continued to increase in recent years. However, with the rapid development of the Japanese pufferfish aquaculture industry, the production of Japanese pufferfish has encountered tremendous challenges caused by bacteria, including *Vibrio harveyi* [2] and *Vibrio anguillarum* [3]. Therefore, studies concerning the molecular mechanisms of pathogenesis, development of disease prevention and control are essential for the healthy development of the Japanese pufferfish industry.

V. harveyi is a gram-negative, luminous bacterium omnipresent in the marine environment [4], which can cause severe disease outbreaks in many kinds of aquatic animals, such as brill (*Colistium guntheri*) [5], Japanese flounder (*Paralichthys olivaceus*) [6] and seabass (*Lates calcarifer*) [7]. Japanese pufferfish infected by *V. harveyi* could cause skin ulcers, and hyperemia in the liver and spleen tissue [8]. In 2006, a high mortality rate of Japanese pufferfish with clinical signs of a single large and deep ulcer on the lateral or ventral skin was reported to be etiolated by *V. harveyi* in a public aquarium in Dalian, China [8]. Previous reports suggested that immune-related molecules could respond to *V. harveyi* infection in various teleost fishes. In Japanese sea perch (*Lateolabrax japonicus*), the expression levels of the toll-like receptor 5 (membrane form) gene were enhanced in gill, brain, liver, heart, spleen, intestines, muscle and head-kidney against *V. harveyi* infection [9]. The c-type lysozyme gene was highly expressed after *V. harveyi* infection in golden pompano (*Trachinotus ovatus*) [10].

With the rapid development of next generation sequencing technology, transcriptome profiling of infected organisms may discover a cascade of transcripts related to the immune system of the organism. In view of its superiority, a growing number of studies have been conducted at the transcriptome level based on RNA-Seq. Transcriptomic analyses can provides a better understanding of the fish immune responses to bacterial infection, including gene expression, regulation and the intricate biological processes [11]. In addition, RNA-seq has made great contributions to apply transcriptomes annotation, determination of the transcriptional structure of genes, and quantitative analysis of

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transcriptome dynamics during different biological processes [12]. For example, Zhang et al. carried out transcriptome sequencing for *Cynoglossus semilaevis* in liver, head kidney, spleen and intestine tissue in response to *Vibrio anguillarum* and detected a large number of immune-related genes and signaling pathways, such as inflammatory cytokines and receptors, pattern recognition receptors, lysozymes, and members of the complement system [13]. Systematic transcriptome profiling was conducted to discover immune-relevant genes in *Lateolabrax japonicus* during *Vibrio anguillarum* infection by RNA-Seq, 6,909 identified DEGs were clustered by GO and KEGG, and 14 immune-relevant pathways were identified by KEGG enrichment analyses [14]. In spite of classical immune organs, recent studies revealed that the liver is also responsive to immunostimulants [15]. The impact of liver function on metabolism and growth warrants further study to better understand the role of liver immune function during stress in teleosts [16,17]. In addition, the spleen is the major lymphoid organ of teleost fishes [18], and it plays an important role in antigen presentation, the clearance of blood-borne antigens and the immune complex [19,20]. Whole genome sequencing of Japanese pufferfish was completed in 2002. Since then, the Japanese pufferfish has attracted great attention and has been used as a model organism in comparative genomics research [21]. However, no transcriptome analysis has been conducted on Japanese pufferfish revealing molecular mechanism against bacterial infection. Considering the importance of the liver and spleen in fish, transcriptome sequencing was conducted using liver and spleen tissue from Japanese pufferfish, aiming to locate immune-related genes in the liver and spleen after *V. harveyi* infection for 1 d and 2 d. The results would provide valuable resources for further understanding of the antibacterial molecular mechanisms of Japanese pufferfish and new strategies against *V. harveyi* infection.

2. Materials and methods

2.1. Ethics statement

All of the experiments were conducted in accordance with the guidelines and regulations of the Management and Use of Laboratory Animals of Liaoning Province and complied with China's existing laws and regulations for biological research. This study did not involve endangered or protected species.

2.2. Biological materials and tissue collection

Japanese pufferfish fingerlings (average body weight 15.38 ± 1.02 g and average body length 8.61 ± 0.28 cm) were purchased from Dalian Fugu Aquaculture Co. Ltd., China. Prior to experimental challenge, the fish were acclimated in the laboratory for two weeks at a temperature of 20 °C. *V. harveyi* was re-isolated from a symptomatic fish and was identified molecularly before culture. At the beginning of the experiment, fish were randomly divided into three groups, including one control group and two challenge groups, with 30 fish in each tank. The fish in challenge groups were immersed for 2 h in a treatment culture with a final concentration of 1×10^7 CFU/ml *V. harveyi*, meanwhile, control fish were cultured in tank with a continuous supply of water. As the experiment went on, the Japanese pufferfish in the challenge group showed obvious clinical symptoms, including darkened body coloration, loss of balance, skin ulcers, hyperemia and lesions in the liver, spleen and kidney, no food in the intestine. All control fish displayed no external abnormalities and no histopathological changes in the visceral organs. At 1 d and 2 d following the infection, 10 fish were collected from each of the appropriate tank at each timepoint randomly and euthanized with MS-222 (300 mg/L). Liver and spleen samples from each fish were collected and placed in RNAlater solution. Following collection, samples were stored at 4 °C overnight and then transferred to −20 °C prior to RNA extraction.

2.3. Total RNA extraction and cDNA library preparation and sequencing

Prior to RNA extraction, individual samples were ground to a fine powder with mortar and pestle in the presence of liquid nitrogen. Extractions were performed using an RNA Prep Pure Tissue Kit (Tiangen Biotech, Ltd., Beijing) according to the manufacturer's directions. Agarose gel electrophoresis was used to detect the integrity of total RNA. The RNA concentration and purity of each sample were measured on NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA). For each timepoint, equal amounts of RNA from individual fish were pooled together (10 fish) for RNA-seq library construction. NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) was used to generate cDNA libraries with 3 μg of RNA per sample. First-strand cDNA was synthesized using a random hexamer primer and M-MuLV reverse transcriptase (RNase H^-). This step was followed by the synthesis of second-strand cDNA, end repair and ligation of the adaptor. Then, the products enriched by PCR amplification were quantified by Qubit 2.0. Sequencing was performed using the Illumina HiSeq platform, and 125 bp/150 bp paired-end reads were generated.

2.4. Bioinformatics analysis

2.4.1. Quality control of sequencing data

Clean data were obtained from raw data by removing reads that contained adapters, poly-Ns and low-quality reads through in-house perl scripts. In addition, the Q20, Q30, GC content and sequence duplication level of the clean data were calculated. All downstream analyses were analyzed based on the high quality clean data.

2.4.2. Read mapping to the reference genome

The reference genome (Accession Number: GCF_000180615.1) and gene model annotation file were downloaded directly from the NCBI (https://www.ncbi.nlm.nih.gov/assembly/448068). The index of the reference genome was built using Bowtie v2.2.3 [22], and paired-end clean reads were aligned to the reference genome using TopHat v2.0.12 [23].

2.4.3. Differential expression analysis and enrichment analysis

HTSeq v0.6.1 [24] was used to count the read numbers mapped to each gene. Expression profiles of each sample was normalized using TMM normalization method to calculate a normalized RPKM using edgeR [25] software. The fold change between different timepoints and controls were determined based on the normalized RPKM of each sample. Differentially induced genes were defined as at least two-fold change in expression and FDR (false discovery rate) corrected p-value < 0.05. To better understand the functional relevance of the DEGs, all the DEGs were further annotated by GO functional enrichment with corrected P-values less than 0.05 and KEGG pathway analysis using the GOseq R package [26] and KOBAS [27] software.

2.4.4. Experimental validation using RT-qPCR

To validate the gene expression profile obtained by RNA-Seq, ten genes in the Japanese pufferfish liver and spleen transcriptomes were selected for RT-qPCR analysis. RT-qPCR was performed using SYBR® Premix Ex Taq™ (Takara, Dalian) on a 7500 real-time PCR System (Applied Biosystems, USA). The RNA samples used for RT-qPCR amplification were the same as those used to construct the RNA-Seq library mentioned above. Total RNA was subjected to cDNA synthesis using the Takara Primer Script TM RT-PCR kit. The primers were designed using Primer3 software [28] (Table 1), and β-actin was used as reference gene for calculating the relative expression level of the ten genes. The relative fold changes were calculated in the Relative Expression Software Tool-384 v1 [29] based on the cycle threshold (Ct) value generated by RT-qPCR.

Table 1
Primers used in this study.

primer name	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
BPI	TGACATTTGAACCCGACAGA	GCTGCTAACACTGGTGTCCA
IL-8	TCACATCTCTCGTGGTGCTC	GCCGACTCTCTGTCTGGATG
SOCS3	GTGCGACTCCAGCTCTTTCT	TGCTCCCTTTGCTCTGAGAC
IGF1	ACGCTGCAGTTTGTATGTGG	AGCTTTGGAAGCAGCACTCT
LEAP2	CAGGTGCAGAGGAGGATAGC	CGTTGACTGAAGGAGCAGTG
FGA	GACCCCGTTGACAGTTTG	GTACCTGGGAGCTTGGAG
MMP13	GCTGAGCATTGACGCTGTTA	CCACTTTGTTTCGGAGGGAG
IL-6	GGAAAACAAGGTGAGGGA	GGTGGTAGAAGGTGACGG
TRFC	CGCCGTGCTGATCTACCC	CGTTGTTGACCTCCACTCTAA
PRX	CAATGTGCCGAACCCTAT	GCCTTGAATGGCTTACTACC
β-actin	ATCCGTAAGGACCTGTATGC	AGTATTTACGCTCAGGTGGG

3. Results

3.1. Bioinformatics analysis

3.1.1. Sequencing of short expressed reads from Japanese pufferfish liver and spleen samples

Illumina-based RNA sequencing was carried out on liver and spleen samples from 1 to 2 d post-infection (dpi) with a control or *V. harveyi* administration as a treatment. Clean reads with similar Q20 percentages over 97% were obtained from all groups after filtering unusable data from raw reads. All raw data obtained from the transcriptome sequencing were deposited to the sequence read archive (SRA) with the accession numbers SRP178793.

3.1.2. Distribution of mapped reads throughout the genome

In liver, a total of 42,284,806, 45,099,918 and 41,554,074 clean reads were obtained from control group, 1 dpi and 2 dpi after cleaning up the raw reads, respectively. Moreover, 84.19%, 82.24% and 85.36% from control group, 1 dpi and 2 dpi were matched to reference genomic sequence, respectively. While in spleen, 56,648,292, 44,455,238 and 49,739,172 high-quality reads were obtained from control, 1 dpi and 2 dpi group. In addition, 85.91%, 85.41% and 85.03% were aligned against the genome of the Japanese pufferfish. The above results revealed that the transcriptome was constructed effectively and could be used for further analysis.

3.1.3. Identification and analysis of differentially expressed genes

A total of 394 and 582 differentially expressed genes were detected from tissue of liver and spleen ($|\log_2$fold change$| \geq 1$, adjusted p-value ≤ 0.05). Between 1 dpi and control in the liver, 264 genes significantly differentially expressed after bacterial infection were identified, among which, 121 genes were significantly upregulated, while 143 genes were significantly downregulated. In the comparison between 2 dpi vs control in the liver, 170 DEGs were identified including 67 upregulated and 103 downregulated genes. In the spleen of 1 dpi vs control, 175 DEGs were identified between the bacterial infection and control group, with 105 DEGs upregulated and 70 DEGs downregulated. The comparison between 2 dpi vs control in the spleen, 489 genes were detected as DEGs after bacterial infection, including 191 upregulated genes and 298 downregulated genes (Fig. 1). The differentially expressed immune related genes were listed in Table 2 categorized based on the literature searching.

3.1.4. GO and KEGG enrichment analysis of DEGs

The GO annotation was performed to analyze the GO term distribution in the differentially expressed genes by Blast2GO. After annotation, the 922 DEGs were assigned to 3,150 GO terms including 1,773 (56.29%) biological process terms, 439 (13.94%) cellular component terms and 938 (29.77%) molecular function terms. Analysis of level 2 GO terms distribution showed that metabolic process (GO:0008152), extracellular region (GO:0005576) and molecular function (GO:0098772) were the most enriched group in three categories. In addition, a total of 62 terms with p-value < 0.05 were considered significantly overrepresented. 30 of the most prominent GO terms were selected in the collection to present in Fig. 2. These GO terms include molecular function (GO:0003674), metabolism process (GO:0008152), catalytic activity (GO:0003824) and single-organism process (GO:0044699). And according to the GO classification, 208, 144, 137 and 108 DEGs were assigned to molecular function (20,153, 89.47%), metabolism process (10,429, 46.30%), catalytic activity (9,244, 41.04%) and single-organism process (8,909, 39.55%), respectively.

The KEGG (http://www.kegg.jp/or http://www.genome.jp/kegg/) as an annotation resource was used to capture knowledge about gene functions [30]. DEGs were annotated into all six categories, including metabolism (55.47%), cellular process (10.16%), environmental information processing (13.28%), genetic information processing (7.03%), human disease (1.56%) and organismal systems (12.5%). In the present study, 992 DEGs were grouped into 128 known pathways and eight pathways with p-value < 0.05 were considered obviously enriched. The largest enriched group is the metabolism pathways containing 47 DEGs. In addition, many immune-related pathways also exhibited DEGs enrichment, such as MAPK signaling pathway, JAK-STAT signaling pathway, PPRA signaling pathway, peroxisome, toll-like receptor signaling pathway and cytokine-cytokine receptor interaction. Immune pathways that interact with each other play an essential role in generating a response to bacterial infection. When bacteria invade the host, recognition of pathogen associated molecular patterns (PAMPs) results in the activation of NOD-like receptor signaling pathway and Toll-like receptor signaling pathway to eradicate pathogen. In this study, the TLR signaling pathway was significantly enriched and some important immune genes in this pathway contributed to the anti-bacterial immunity by upregulating. For the elimination of bacteria, lysosome is an important pathway of the innate immune system to defend against infection with *V. harveyi*. Cytokine-cytokine receptor interaction participated in a complicated inflammatory response in the immune defense against bacterial infection, DEGs were enriched in this pathway to resist bacterial invasion by initiating immune response. The 20 most enriched pathways were shown in Fig. 3.

3.2. Validation of differentially expressed genes by RT-qPCR

To validated the differentially expressed genes identified by RNA-seq, ten genes with different expression patterns were selected for RT-qPCR confirmation. And samples from control, 1 dpi and 2 dpi following infection were used for RT-qPCR. Melting-curve analysis revealed a single product for all tested genes. The relative fold changes from RT-qPCR were compared with the RNA-seq expression analysis results. As shown in Fig. 4, the RT-qPCR results revealed the genes had the same upregulation or downregulation direction with the RNA-seq analysis. In general, the RNA-seq results were confirmed by the RT-qPCR results, suggesting the accuracy and reliability of RNA-seq expression analysis.

4. Discussion

In recent decades, Japanese pufferfish have made considerable headway worldwide as one of the main aquatic species. However, serious disease caused by high-density cultivation has always hindered the healthy development of Japanese pufferfish aquaculture. Therefore, an investigation of the molecular response of Japanese pufferfish to *V. harveyi* infection is vital for understanding the molecular mechanisms and subsequent development of effective measures to combat the disease. The liver and spleen from uninfected or infected Japanese pufferfish were chosen for RNA-Seq analysis due to the significant role they played in immune-related disorders. In this study, 922 DEGs were identified from the liver and spleen of Japanese pufferfish. According to

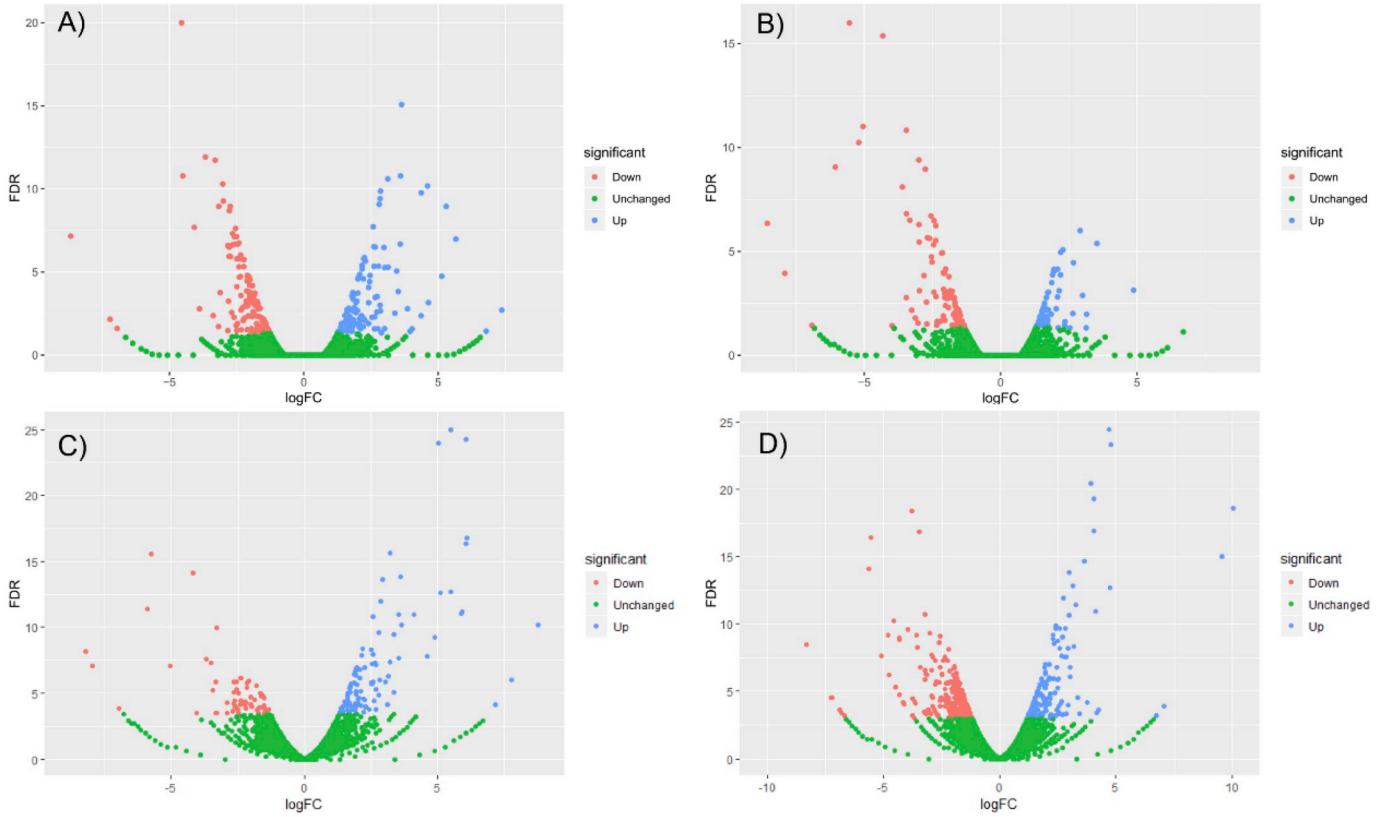

Fig. 1. Volcano plot of Japanese pufferfish DEGs identified from the *Vibrio harveyi* challenge. Liver 1dpi vs control was shown in A, liver 2dpi vs control was shown in B, while spleen 1dpi vs control was displayed in C and spleen 2dpi vs control was displayed in D. The log_2FC value represented the mean expression level of each gene, and each dot represented one gene. The upregulated genes were shown in blue dots, the downregulated genes were shown in red dots, while genes with no differential expression were shown in green dots. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

functional enrichment analyses, 11 immune-related pathways were highlighted, including several inflammatory response pathways such as toll-like receptor signaling pathway, cytokine-cytokine receptor interaction and MAPK signaling pathways, which suggested that the infection of *V. harveyi* induced an inflammatory response in the liver and spleen of Japanese pufferfish.

Toll-like receptors (TLRs) are protective immune recognition receptors that sense pathogen associated molecular patterns including lipopolysaccharide and single-stranded RNA [31]. It has reported that the genes from toll-like receptors signaling pathway in orange-spotted grouper can be triggered by infection with *V. harveyi* [32]. In this study, toll-like receptors signaling pathway member CD40 and inflammatory cytokines including IL-1β, IL-8, IL-6 were significantly upregulated in the liver and spleen of Japanese pufferfish. In mammals, CD40 is known as the “master switch” in the immune response to pathogens. CD40 can regulate cellular and humoral immunity and T cell-mediated inflammatory responses [33]. Although CD40 has been well studied in mammalian immunity, CD40 in fish has only been reported in Atlantic salmon [34], humphead snapper [35] and zebrafish [36]. Upon *V. harveyi* challenge, CD40 was significantly upregulated in the spleen and liver within 2 dpi, implying that CD40, as acute phase proteins, participated in the immune response to bacterial challenge. The same expression pattern was found in humphead snapper post-stimulation with *V. harveyi* [35]. The upregulation of CD40 expression suggested that it might contribute to *V. harveyi* acting as a B cell mitogen that could induce B cell proliferation and activation [37]. Interleukins are the largest group of cytokines in the host innate immune response. To date, the number of ILs has been identified as more than 30 [38]. In this study, the expression of IL-1β was upregulated significantly at 1 dpi in the liver. IL-1β, as a prototypical proinflammatory cytokine, participates in pro- and anti-inflammatory activity [39] and regulates the immune response to immunological challenge [40]. Similar to our results, IL-1β was significantly upregulated following LPS infection in roughskin sculpin (*Trachidermus fasciatus*) [41]. IL-8 is a small cytokine belonging to the CXC chemokine family and functions primarily as an inducer of chemotaxis in neutrophils in response to inflammatory antagonists such as pathogen infection and injury [42]. In teleost, IL-8 also has a series of biological functions that mediate inflammatory and immune responses, such as promoting the proliferation and migration of leukocytes in lymphoid tissue and modulating cytokine responses [43]. As expected, Japanese pufferfish IL-8 tended to be upregulated at 1 dpi but downregulated at 2 d after infection in the liver. The upregulation of IL-8 might be due to the recruitment of leukocytes and the subsequent release of more IL-8 to the inflammatory site. A number of IL-8s from fish, such as those from catfish [44], rainbow trout [45], flounder [46] and zebrafish [47], were also known to be upregulated at the transcription level by bacterial or lipopolysaccharide challenge. These results suggested that IL-8 had a key role in the establishment of inflammation and induction of antimicrobial activities. IL-6 is a pleiotropic cytokine with important immunoregulatory functions. Previous studies indicated that IL-6 can be induced in the immune tissue of several teleost fish after bacterial infection, such as rainbow trout [48], large yellow croaker [49] and seabass [50]. In this study, the expression of IL-6 in the spleen increased significantly at 2 dpi. This indicated that IL-6 might be induced to higher expression by *V. harveyi* infection and involved in bacterial defense activities. The induction of IL-6 expression could be due to a systemic response to injury during infection, the acute phase proteins enhance protection against bacteria and modify inflammatory responses by mediator release and cell trafficking, and their production is controlled mainly by IL-6 and IL-1-type cytokines [51].

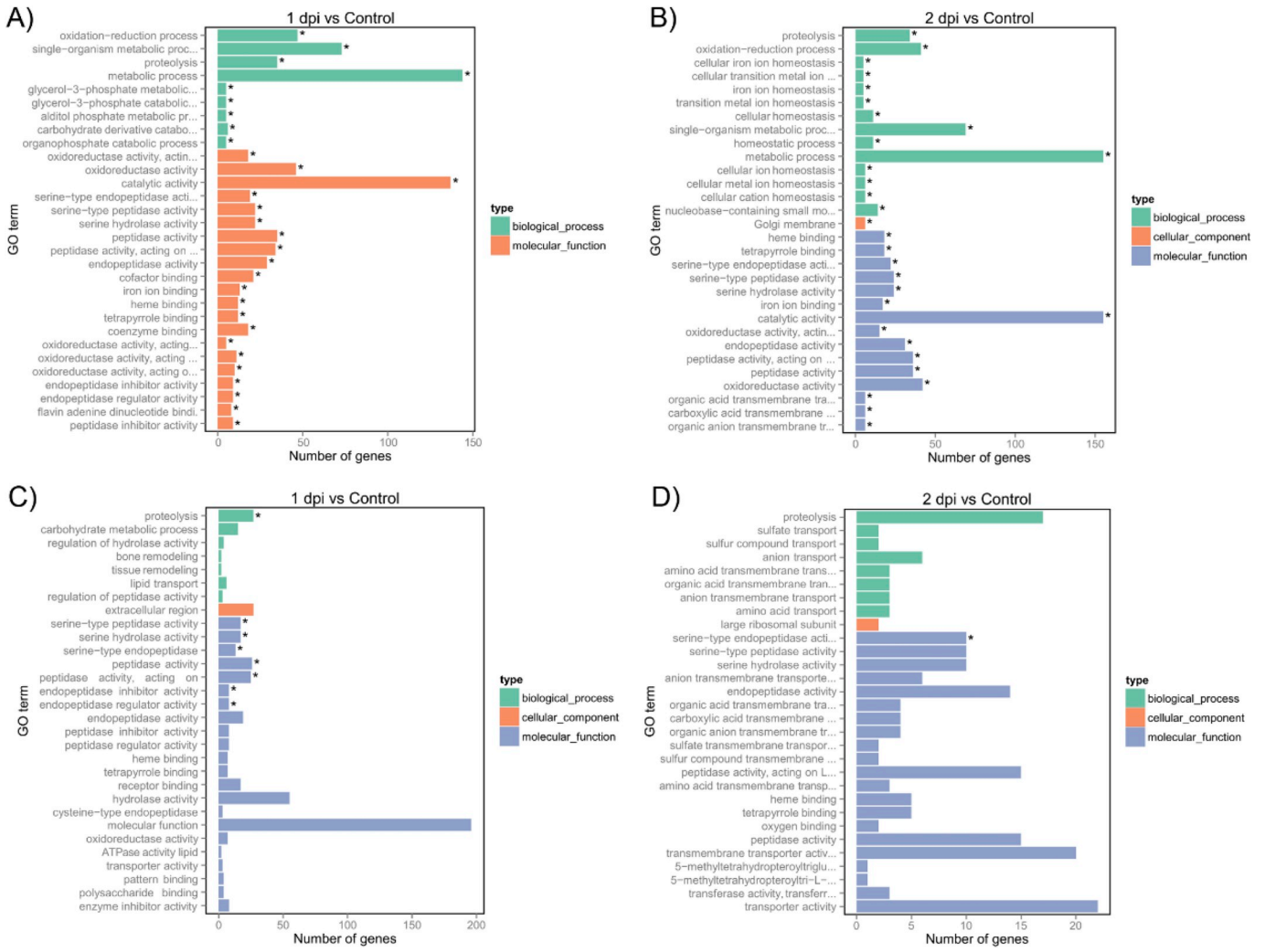

Fig. 2. Japanese pufferfish DEGs in GO distribution after *V. harveyi* infection. Liver 1dpi vs control was shown in A, liver 2dpi vs control was shown in B, while spleen 1dpi vs control was displayed in C and spleen 2dpi vs control was displayed in D. Ordinate represented the number of enriched genes, abscissa represented GO terms.

Hence, pleiotropic interleukins are considered molecular markers that regulate the immune response after infection.

MAPK signaling pathways play an important role in regulating gene expression by phosphorylating transcription factors in response to extracellular signals [52]. Three genes in MAPK signaling pathway were altered after infection with *V. harveyi* that are involved in the innate immune response, including IL-1β, HSPA1L and HSP70. HSPs are ubiquitously essential molecular chaperones that maintain cellular protein homeostasis under normal and stress conditions, and the crucial roles of heat shock proteins in resistance to stress in aquaculture have been observed. HSP70 is one of the most heat inducible and protective proteins, and some studies have reported that HSP70 is involved in the immune response in vertebrates from nonspecific immune responses to adaptive immune responses [53]. In this study, the expression level of HSP70 was increased significantly at 1 dpi in the liver and then decreased to normal levels. Wei et al. [54] reported that the HSP70 mRNA expression levels in the miiuy croaker significantly increased at 1 dpi and then decreased between 1 and 2 dpi. Similarly, the HSP70 mRNA expression levels in the hemocytes of mussels infected with *V. anguillarum* increased at first and then decreased [55]. This result indicated that HSP70 was also involved in the Japanese pufferfish response to *V. harveyi* infection, and played a role in modulating the cellular immune response and protected the organisms from pathogenic stress.

The JAK/STAT signaling pathway plays a significant role in cellular immune response, cell proliferation, apoptosis and immune regulation [56]. In the present study, five genes were enriched into this pathway and three of them were investigated their immune functions towards *V. harveyi* infection, including IL-6, IL-1β and SOCS3. Members of the suppressor of cytokine signaling (SOCS) family are crucial for controlling signal transduction pathways that are involved in the immunity of organisms and are key regulators of immunological homeostasis [57,58]. SOCS3 was initially identified using a functional screen for inhibitors of cytokine signaling, and it played a key role in the negative regulation of interleukins by inhibiting the JAK/STAT signal pathway in mammals and teleost fish [59]. Previous studies have shown that fish SOCS3 genes are involved in regulating the fish immune system. In yellow perch, expression of SOCS3 mRNA was significantly increased in multiple tissues including gill, head kidney, liver, kidney and spleen following LPS injection. The maximum mRNA expression level occurred at 6 h post-injection, and then began to decreased by 12 post LPS-injection [60]. The SOCS3 was triggered to reduce STAT3 activation, thus induced apoptosis and enhanced sensitivity to inflammatory damage during the immune defense against pathogens. Similar expression tendency was also observed in this study, the expression SOCS3 was significantly upregulated after infection at 1dpi in the liver while downregulated at 2dpi. Moreover, in Nile tilapia, the mRNA expression of SOCS3 in response to LPS challenge was significantly induced in the gill and liver *in vivo*, as well as in the hepatocyte culture *in vitro* [61].

Table 2
Enriched differentially expressed immune-related genes from Japanese pufferfish following *Vibrio harveyi* infection. Bold values indicate significant fold change relative to control ($p \leq 0.05$).

Accession	Gene Name	1 dpi (spleen)	2 dpi (spleen)	1 dpi (liver)	2 dpi (liver)
Immune Component					
XM_011607736.1	C–C chemokine receptor type 3-like	1.74	**7.89**	−2.02	−30.99
NM_001280054.1	c-c motif chemokine 13-like	3.14	2.20	**6.31**	1.86
NM_001197250.1	chemokine (C–C motif) ligand 19	1.67	**2.30**	**2.90**	**−4.38**
NM_001032742.1	chicken-type lysozyme	**59.47**	−8.31	−1.53	1.53
NM_001032608.1	coagulation factor VIIb precursor	3.97	−1.62	−2.27	**2.49**
XM_011604158.1	complement C1q-like protein 2	1.62	**2.72**	1.09	−1.79
XM_011612315.1	complement C3-like	**6.86**	−2.79	−1.03	−1.30
XM_011615298.1	complement component 5	2.19	**2.81**	−1.25	1.06
XM_011611635.1	complement factor B-like	**11.68**	−1.07	1.04	−1.72
XM_011616627.1	complement factor H-related protein 1-like	**10.19**	1.50	−1.05	−1.31
XM_011620411.1	complement factor I	**5.63**	−1.55	−1.18	1.38
XM_011616713.1	C-X-C chemokine receptor type 4-like	−1.15	1.38	**39.42**	4.35
XM_011620596.1	ES1 protein homolog, mitochondrial-like	**3.44**	**−7.46**	1.56	−1.22
XM_011610296.1	glucagon	−1.81	**−13.77**	3.79	**11.56**
XM_011604179.1	integrin beta-3	1.69	**2.37**	−1.06	1.00
XM_011615826.1	interleukin 1, beta	1.50	**6.95**	**11.44**	−30.99
XM_011620686.1	interleukin-1 receptor-like 1	2.85	**3.54**	−1.04	−23.49
NM_001032722.1	interleukin-6	1.91	**7.81**	1.51	−1.31
NM_001032587.1	interleukin 8	1.01	1.22	**4.50**	**−10.98**
XM_011614295.1	interleukin 10	**3.19**	2.38	1.82	−8.50
XM_011613467.1	interleukin 17 receptor E-like	1.53	**7.36**	1.49	−2.45
XM_011619255.1	interleukin-31 receptor subunit alpha-like	−1.11	−1.96	**3.29**	1.00
XM_011606617.1	liver-expressed antimicrobial peptide 2-like	6.10	1.20	**−6.82**	**3.76**
XM_011615962.1	nitric oxide synthase 1	1.71	**5.27**	1.65	−1.37
XM_011616660.1	transferrin receptor	**2.48**	**2.85**	1.34	2.29
XM_011605611.1	trypsin-3-like	**3.88**	−1.85	**3.05**	**3.32**
XM_011602727.1	tumor necrosis factor receptor superfamily member 4-like	1.89	**2.87**	1.27	−1.98
Patheogen Recognition					
XM_011611856.1	alpha-1-antitrypsin homolog	**65.92**	4.59	**−3.09**	1.85
XM_011617824.1	alpha-1-microglobulin/bikunin precursor	**4.32**	−1.50	−1.44	1.10
XM_011614460.1	alpha-2-HS-glycoprotein-like	**68.46**	1.20	**−2.37**	1.54
XM_011614357.1	bactericidal permeability-increasing protein-like	**2.59**	**2.71**	**7.25**	**−6.77**
NM_001145654.1	CD40 molecule	1.12	1.04	**2.66**	1.22
XM_011621902.1	CD59B glycoprotein-like	**45.03**	7.43	−1.26	−1.96
XM_011617897.1	cell adhesion molecule 3-like	−1.01	−1.25	**−5.01**	−1.70
XM_011608667.1	claudin-3-like	**3.85**	**3.62**	1.59	−1.66
XM_011620896.1	C-type lectin domain family 12 member B-like	2.84	**3.91**	−1.53	−2.45
XM_011617756.1	fibrinogen alpha chain	**66.27**	**5.92**	−1.62	−1.39
XM_011617902.1	fibrinogen beta chain	**4.21**	1.04	−1.58	−1.06
XM_011612765.1	fibrinogen gamma chain	**5.72**	1.05	−1.98	−1.56
XM_011606128.1	midline 1	−1.78	**−2.66**	1.42	1.52
XM_011616937.1	septin 3	1.95	**2.33**	−1.06	2.03
NM_001078628.1	suppressor of cytokine signaling 3	1.29	1.94	**3.47**	**−5.43**
XM_011605484.1	tumor necrosis factor receptor superfamily member 11b	1.50	**2.16**	**2.39**	−2.04
XM_011617995.1	vascular cell adhesion protein 1-like	1.00	**1061.88**	1.03	−1.13
Oxidative Stress/Apoptosis					
XM_011611887.1	alpha-1-antiproteinase-like	10.43	1.00	**−2.52**	1.32
XM_011609259.1	BCL2/adenovirus E1B 19 kDa protein-interacting protein 2-like	**4.68**	**7.63**	1.41	1.25
XM_011611574.1	calpain-5-like	−1.73	**−5.15**	2.02	1.71
XM_011617267.1	caspase recruitment domain family, member 14	2.29	−1.07	**4.11**	1.02
XM_011615947.1	cathepsin L	2.05	**2.44**	1.82	1.07
XM_011614247.1	cytidine deaminase-like	**60.65**	11.83	−2.23	1.77
XM_011618261.1	cytochrome P450 2D10-like	1.00	1.00	−3.98	**5.08**
XR_965373.1	cytochrome P450 2J2-like	−1.33	−2.25	**−5.96**	**2.68**
XM_011620325.1	dnaJ homolog subfamily B member 5-like	2.54	2.30	**7.16**	−1.22
XM_011613737.1	dnaJ homolog subfamily B member 9-like	1.99	**3.41**	1.43	1.97
XM_011617117.1	glutathione S-transferase zeta 1	−1.21	−1.49	**−2.44**	1.29
XM_011621198.1	heat shock 70 kDa protein-like	−1.16	−1.44	**3.59**	1.32
XM_011617315.1	heat shock 70 kDa protein 1-like	1.72	1.90	**4.21**	−1.25
XM_011612561.1	keratin 222, type II	**3.73**	−1.14	−1.46	2.41
NM_001280035.1	matrix metallopeptidase 13	1.81	**16.30**	13.80	−8.50

(*continued on next page*)

Table 2 (*continued*)

Accession	Gene Name	1 dpi (spleen)	2 dpi (spleen)	1 dpi (liver)	2 dpi (liver)
XM_011616175.1	matrix metalloproteinase-16-like	−1.54	**−4.18**	16.68	9.58
XM_011616249.1	matrix metallopeptidase 21	**3.82**	**5.26**	1.09	1.95
XM_011617907.1	platelet glycoprotein Ib alpha chain-like	−1.18	**2.29**	1.90	2.02
XM_011619989.1	protein phosphatase 2, regulatory subunit B′ epsilon	29.29	**107.09**	−1.04	−15.99
XM_011615968.1	selenoprotein M-like	**3.66**	−1.91	1.21	**2.73**

Cytokines are soluble extracellular proteins signaling molecules that involve in systemic immune response, mainly including interleukins and tumor necrosis factor families [62]. In this study, cytokine-cytokine receptor interaction pathway was significantly enriched. Furthermore, proinflammatory cytokines and corresponding receptors, such as IL-8, IL-6 and TNFRSF11B significantly upregulated, which indicated that cytokines participated in process of immune defense against *V. harveyi* infection in the liver and spleen of Japanese pufferfish. TNF receptor super family (TNFRSF) proteins are a class of cell-surface receptors that are critically involved in the maintenance of immune system homeostasis [63]. Here, we identified a TNFRSF-encoding gene that was upregulated in Japanese pufferfish infected by *V. harveyi*. In mitogen-stimulated PBL expression analysis, TNFRSF11B was strongly expressed in black rockfish [64]. TNFRSF11B played an important role in

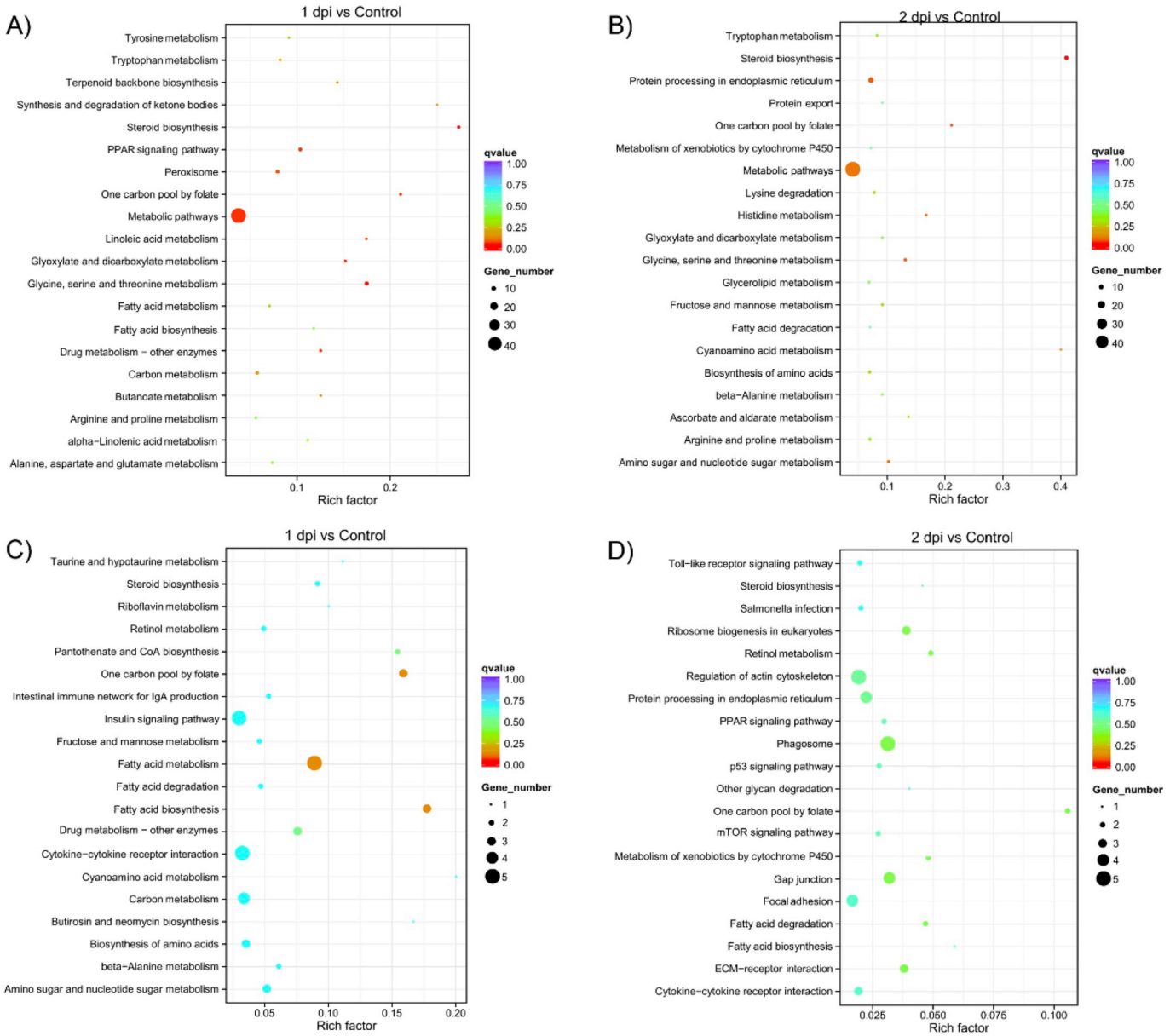

Fig. 3. Japanese pufferfish DEGs in KEGG classification after *V. harveyi* infection. Liver 1dpi vs control was shown in A, liver 2dpi vs control was shown in B, while spleen 1dpi vs control was displayed in C and spleen 2dpi vs control was displayed in D.

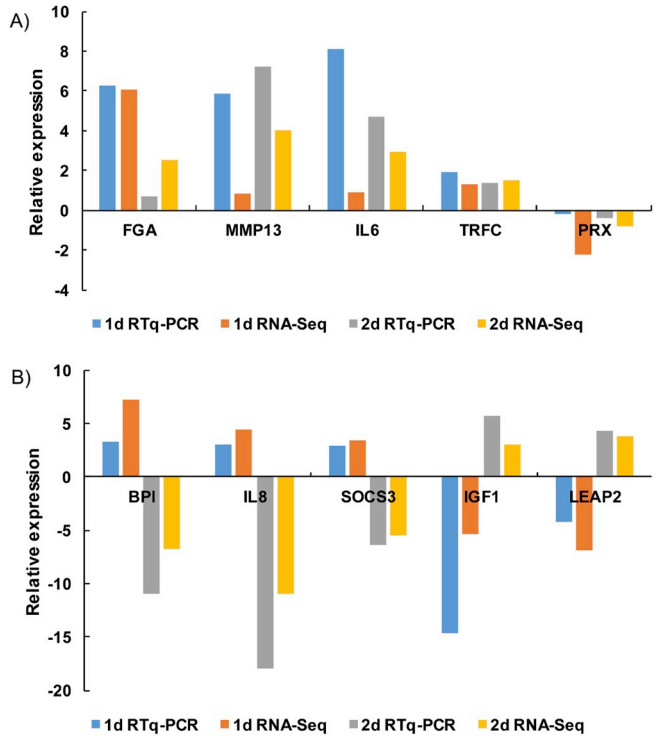

Fig. 4. Gene expression analysis from RNA-seq and RT-qPCR of Japanese pufferfish infected with *V. harveyi*. A showed the genes selected and validated from the spleen, while B showed the genes selected and validated from the liver. The expression of target genes was normalized to β-actin as a reference gene. Statistically significant differences compared to controls were presented, with *p < 0.05.

enhancing macrophage survival and restricting bacterial growth in infected macrophages.

Phagocytosis in macrophages are essential during immune response, lysosomes are membrane-delimited organelles serving as the cell's main digestive compartment for degradation, they are the final steps of phagocytosis for clearance of the microorganisms [65]. Many proteases of lysosomes, such as cathepsins, participate in host defense against phagocytized microorganisms [66]. Cathepsin L (CTSL) is a lysosomal cysteine protease that involves in different levels of immune responses, including apoptosis, antigen processing and inflammation [67,68]. In the present study, as an important effector gene in the lysosomal pathway, CTSL is significantly upregulated in the spleen. Similar to our study, Zhu et al. performed transcriptomic analyses between tilapia with different susceptibility to *S. agalactiae* infection, their comparison analysis revealed CTSL was highly expressed in resistant group than that in susceptible group after challenge [69]. In addition, two CTSL genes were also characterized in channel catfish, both of which were significantly induced following *Edwardsiella ictaluri* and *Flavobacterium columnare* infection at most timepoints in mucosal tissues [67].Moreover, Western blot and proteinase activity analysis conducted in Pacific abalone revealed that the expression and enzyme activity of CTSL were significantly up-regulated in hepatopancreas at 8 h following *V. parahaemolyticus* infection, demonstrating its role in the innate immune system [70].

5. Conclusion

Transcriptional changes in the liver and spleen of Japanese pufferfish in response to infection with *V. harveyi* were detected by gene expression profiling using RNA-seq. 922 DEGs were identified through a set of bioinformatics analyses. A number of various genes relevant to innate immunity were located, and the functions of several candidate genes notably induced by *V. harveyi* deserve further investigation. Our study first presented a large scale transcriptional resource for immune response research of Japanese pufferfish against *V. harveyi*, promoting our comprehensive understanding of the host's defense against disease and infection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.304.

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