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# Identification and characterization of differentially expressed genes in hepatopancreas of oriental river prawn *Macrobrachium nipponense* under nitrite stress

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ABSTRACT

The oriental river prawn *Macrobrachium nipponense* is a highly adaptable, tolerant, and fecund freshwater prawn that inhabits a wide range of aquatic environments. The hepatopancreas of crustaceans is not only a site for secretion of digestive enzymes, and also plays important roles in several metabolic processes, such as lipid and carbohydrate metabolism. It is the main organ for the detoxification and immunity. In this study, high-throughput sequencing techniques were used to detect the effect of nitrite stress (10 mg/L nitrite-N for 48 h) on gene expression in the hepatopancreas of *M. nipponense*. A total of 13,769 million reads were harvested, and 94,534 transcripts were de novo assembled using Trinity software and produced 56,054 non-redundant transcripts. A total of 825 differentially expressed genes were obtained comparing 48 h nitrite stress with control group. In the analysis of GO and KEGG database, significant differences were found in 49 pathways. Immune-related pathways under nitrite stress included arginine and proline metabolism, glutamate metabolism, Jak-Stat signaling pathway, endocytosis, wnt signaling pathway, RIG-I-like receptor signaling pathway, TGF-beta signaling pathway, GnRH signaling pathway and phagosome. Apoptosis-related pathway was also significantly altered, such as lysosome and apoptosis. Remarkably, nitrite stress altered the expression patterns of key apoptosis genes (tetraspanins-like protein, *LAMP*, *CD63*, caspase 3C and Caspase 1) and immune genes (Serine proteinase-like protein, C-type lectin, *daf-36*, *SOCS-2*, alpha-2-macroglobulin), confirmed that nitrite-stress induce immune response and eventually even apoptosis. This study provided a new insight into the role of hepatopancreas in crustaceans, and further investigation will continue.

## 1. Introduction

The oriental river prawn *M. nipponense*, a member of the *Palaemonidae* family of decapod *crustaceans*, is widely distributed in freshwater and low-salinity regions of estuaries [1]. *M. nipponense* are especially prone to eutrophication and pollution in the water [2]. Nitrite is one of the most common pollutants in intensified aquaculture or in recirculated water [3]. Nitrite is formed from ammonia and may be accumulated in aquatic systems as a result of imbalances of nitrifying bacterial activity, such as *Nitrosomonas* sp. and *Nitrobacter* sp. [4]. In natural water, nitrite concentration is typically lower than 1 μmol/L [5], whereas it can build up in intensive culture systems especially in recirculation aquaculture systems [6]. The accumulation of nitrite in water is highly toxic to aquatic animals [7,8]. The toxicity of nitrite to crustaceans has been studied by several authors [9–12]. In *crustaceans*, it has been suggested that elevated concentration of nitrite in pond water influences hemocyanin formation, reduce oxyhemocyanin level, impairs the respiratory metabolism, depresses immunologic function, increases susceptibility to bacterial infection and induce apoptosis [10,12–14]. There have been studies on the acute, chronic and sublethal effects of nitrite in prawns. The 96h LC50 of nitrite on *Macrobrachium rosenbergii* was 8.54 mg/L [15]. The 96h LC50 for nitrite was 13.3 mg/L for *M. nipponense* at pH 8.0 [16]. The 96h LC50 values of nitrite were 13.20 mg/L and 145–232 mg/L for the zoeae and juvenile of *Penaeus monodon* [17,18].

It has been reported that nitrite exposure induces expression of apoptosis-related genes in hemocytes of *Litopenaeus vannamei*, and subsequently caused hemocyte apoptosis [13]. A previous *in vitro* study

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demonstrated that extracellular nitrite suppressed cellular functions and induced apoptosis on hemocytes in tiger shrimp *Penaeus monodon* [14]. The results of the histological analysis of *Macrobrachium amazonicum* confirm that the higher the concentrations of total nitrite, the greater the damages caused to the gill structure [19]. The nitrite exposure of *Marsupenaeus japonicus* induced the expression of apoptosis-related proteins displayed both dose- and time-dependent changes [20]. The molecular mechanism of nitrite-induced immune response and apoptosis is unclear. Indeed, much more genes and pathway may participate in this defense process, and need to be identified. High-throughput method of RNA sequencing (RNA-seq) has emerged as a strategy for analyzing the functional complexity of transcriptome, providing insight on gene expression and relevant molecular pathways under specific condition. This technology has been widely used in the study of stress response in aquatic animals [21–23].

In this study, RNA-seq was used to detect the effects of nitrite stress on gene expression, identifying genes and pathways sensitive to nitrite stress in *M. nipponense* hepatopancreas. We compared the transcriptome of a nitrite-challenged group (treatment group) and a non-challenged group (control group). These transcriptomic data provide useful information to reveal putative genes involved in the apoptosis and immune and help identify novel genes.

## 2. Materials and methods

### 2.1. Animals and nitrite exposure

The experimental prawns, *M. nipponense* were collected from Weishan Lake, Shandong Province, China, and acclimated in a 100 L tank at the lab of the Shandong Agricultural University for 1 week. During acclimation periods, prawns were fed commercial particle food for twice daily, and the water temperature, pH, and DO in the tank were 18.5 ± 0.5 °C, 8.2 ± 0.08, and 6.5 ± 0.5 mg/L, respectively. The 200 prawns were divided into two groups and each group was separated to 3 tanks. The nitrite condition was maintained by adding $NaNO_2$ into the tank and the concentration of nitrite-N in the treatment group was 10 mg/L. The control group only contained freshwater and the concentration of nitrite in freshwater was zero. According to previous studies in our lab, the concentration of nitrite was set to 10 mg/L, which is the half-lethal dosage for 48 h nitrite exposure.

After 48 h of nitrite exposure, the hepatopancreas of total six individuals was dissected separately and immediately frozen in liquid nitrogen. The six shrimp in each group were selected based on similar sizes. The hepatopancreas was then stored at −80 °C until RNA extraction for transcriptome sequencing experiments [10,24].

### 2.2. Histopathology

In order to further examine the effect of 10 mg/L nitrite stress treatment on hepatopancreas tissue after 48 h, we performed a section of hepatopancreas tissue. The sampled hepatopancreas from *M. nipponense* in both treatment group and control group were fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for 24 h at room temperature. The fixed hepatopancreas was dehydrated in a series of graded alcohols and cleared with xylene. Then, the hepatopancreas was embedded in paraffin, mounted on a microtome and cut into 5 μm transverse sections. The sections were stained with hematoxylin and eosin and observed under an Olympus microscope.

### 2.3. Total RNA extraction, library construction and sequencing

Total RNA of six individuals were extracted from the hepatopancreas in treatment group/control groups using Trizol Reagent (Invitrogen) according to the manufacture. The RNA quality of each sample was determined by measuring the absorbance at 260/280 nm (A260/A280) using an Eppendorf AG 22331 Hamburg. The extracted RNA was electrophoresed on 1.5% (w/v) agarose gel for integrity analysis.

The extracted RNA from hepatopancreas was used to isolate mRNA with a mRNA-Seq Sample Prep Kit (Illumina) for constructing a non-directional Illumina RNA-Seq library [25]. The libraries were loaded onto flow cell channels for sequencing using an Illumina Genome Analyzer at Beijing biomarker Co., Ltd. (Beijing, China) in 2016 [26]. The cDNA library was sequenced on the Illumina Hi-Seq TM 2500 sequencing platform. Both ends of the library were sequenced. Trinity software was used for de novo transcriptome assembly. Firstly, overlaps generated from assembling of clean reads were connected into longer fragments called contigs. Reads were used for gap-filling of these scaffolds to generate final scaffold sequences. The RNA-seq data was deposited into NCBI SRA database, and its accession is SRP149589.

### 2.4. Transcriptome annotation and gene ontology analysis

All transcripts were compared with the NCBI Non-redundant (Nr) protein database, Gene Ontology (GO) database, Clusters of Orthologous Groups of Proteins (COG) database, and Kyoto Encyclopedia of Genes and Genomes (KEGG) database for functional annotation using BLAST software with an e-value cutoff of $1 \times e^{-5}$. Functional annotation was performed with GO terms (www.geneontology.org) that were analyzed using Blast 2 GO software (http://www.blast2go.com/b2ghome) [27]. The COG and KEGG pathway annotations were performed using Blast software against the COG and KEGG databases.

### 2.5. Differentially expressed genes

To obtain expression levels of every transcript, cleaned reads were first mapped to all transcripts using Bowtie software [28,29], then the FPKM (fragments per kilobase per million mapped reads) value of every transcript was obtained using RSEM (RNASeq by expectation maximization (http://deweylab.biostat.wisc.edu/rsem/)), software. Differentially expressed genes were identified using edgeR (empirical analysis of digital gene expression data in R) software. For this analysis, the filtering threshold was set as an FDR (false discovery rate) $< 0.01$.

### 2.6. Real-time quantitative PCR (RT-qPCR) validation and statistical analysis

RT-qPCR was used to validate the partial differential expressed genes identified using RNA-Seq. The primer sequences are listed in http://www.sciencedirect.com/science/article/pii/S0378111913014315, Table 1. RT-qPCR reactions were carried out using the STRATAGENE MX3000 Real-time System (USA) and the β-actin gene was used as reference gene. The PCR was performed using SYBR Premix Ex Taq kit (TaKaRa, Dalian, China), and the PCR reaction systems (20 μl) consists of 10 μl SYBR Premix Ex Taq (2 × ), 0.4 μl of each gene specific primer (10 nmol), 2 μl cDNA, 0.4 μl ROX reference dye II. RT-qPCR was performed in a total volume of 20 mL, and cycling conditions were 95 °C for 1 min and followed by 40 cycles of 94 °C for 5s, 59 °C for 15s and 72 °C for 20s. Each sample was run in triplicates. At the end of each PCR reaction, the dissociation curve analysis of the amplification products was done. The relative copy number of the gene was calculated by the $2^{-\Delta\Delta CT}$ comparative CT method. The data were subjected to one-way ANOVA using SPSS 16.0, and the p-values less than 0.05 were considered statistically significant.

**Table 1**
Primers used for the qRT-PCR analysis.

| Gene | Description | Sequence (5′-3′) | Amplicon Size (bp) | Annealing temperature (°C) | UP or DOWN |
|---|---|---|---|---|---|
| c15194.graph_c0 | tachylectin | **F: TCACACTTCACTTTCTCGAACAG**<br>R: CCAACTAGAACGGCATGATTAGA | 102 | 57 | UP |
| c4928.graph.c0 | monocarboxylate transporter 1-like | **F: GTATGGTTATGGAGAGGCAGGTA**<br>R: GAAGGAAGGAGGAGGAGGAATC | 116 | 58 | UP |
| c18244.graph.c0 | RING-type zinc-finger, LisH dimerization motif protein | **F: TCAACATGGAGAGTGAAAGAGG**<br>R: CACACGCTGCTTGGATACC | 110 | 58 | UP |
| c19877.graph.c0 | vegetative cell wall protein gp1-like | **F: TGGCTCTGGTTCCTCTCCT**<br>R: CACCGCTCTCACCTTCCTT | 167 | 58 | UP |
| c4748.graph.c0 | hypothetical protein | **F: GAATATCCTGGTGCTTGTGGTT**<br>R: CATCCTCAGTGTAGCCTTCCT | 211 | 56 | UP |
| c14893.graph.c0 | gonadotropin-releasing hormone receptor | **F: ACATTCAAGGCTACAGAGATACTC**<br>R: TCACGTAGTTGTGGATGTTCTT | 125 | 55 | UP |
| c5529.graph.c0 | Anaphase-promoting complex | **F: CGGTCTTCTTCTTGAACTGTATGT**<br>R: CGTCTGGTCTGTGTCCTCTTAT | 131 | 57 | UP |
| c10252.graph.c0 | hypothetical protein | **F:CTTACAGCATATCGCAGACACAA**<br>R:TCCTCACATTCAGCCAATTCTATG | 196 | 56 | UP |
| c16113.graph.c0 | Pleckstrin homology domain | **F:CAGACCGAGGAGAAGAAGGAA**<br>R:AACAATAAGGCGAGAGTGAAGG | 256 | 57 | UP |
| c25990.graph.c0 | Glutathione S-transferase, C-terminal domain L-lactate dehydrogenase | **F:CCTGTTACGCAATCTCTGTCTC**<br>R:GCCGAGTTCTGGAGTTGGT | 154 | 58 | UP |
| c28251.graph.c0 | E3 ubiquitin-protein ligase HECTD1 | **F:CATCCAGCAATACAGCAACCA**<br>R:TCAGCACTCGCAACACTCA | 274 | 56 | UP |
| c25793.graph.c0 | Malic enzyme, NAD binding domain | **F:AGCAAGACGCACGATCCAA**<br>R:AATAGCAGCAGCACCAATAAGAG | 182 | 56 | UP |
| c23463.graph.c0 | Major Facilitator Superfamily | **F:CCCAAACAGACTCGTTACAGAA**<br>R:CCACATTCTATTTCTCGGAGGTAT | 121 | 56 | DOWN |
| c 17,155.graph.c0 | G-protein alpha subunit | **F:TCCACAACGGTGGATGTCCCCGG**<br>R:CCAGAGGACTTGCACGAGTGGGC | 192 | 57 | UP |
| c20648.graph.c0 | Lysosome-associated membrane glycoprotein (LAMP) | **F:GCGTGTGGGTCAGTCATTGGC**<br>R:CCAGAGGACTTGCACGAGATGG | 168 | 57 | UP |

## 3. Results

### 3.1. Histological changes of M. nipponense hepatopancreas after nitrite stress

To analyze the effects of nitrite stress on hepatopancreas morphology, the hepatopancreas of *M. nipponense* from control and treatment groups were used for histopathological slice. The results showed that glandular epithelial cells have different degrees of loss (Fig. 1). The gap between the adjacent glands becomes larger. We also found four kinds of cells (secretory cells, B cell; fibroblasts, F cell; rest zellen cell, R cell) [30] in *M. nipponense* hepatopancreas, which were badly injured or partially shed after nitrite stress. The rough endoplasmic reticulum of ribosomal particles began dropping off. Serious edema in the endoplasmic reticulum was found. There was partial collapse inside the mitochondria, and the double membrane of the mitochondria dissolved. Electronic transparent banded appear and some tend to collapse. The microvilli were deformed and a few were broken. These phenomena indicate that the action of nitrite on the hepatopancreas constituted a greater damage and destruction.

### 3.2. Overview of the sequencing data and de novo assembly

Across all six samples, a total of 34.68 Gb Clean Data were obtained, with Q30 > 86.33%. The clean reads were assembled by Trinity software. After splicing and removing redundancy of the assembled contigs, a total of 94534 transcripts and 56054 unigenes were obtained, and the N50 of transcripts and unigenes were 3310 and 2055, respectively. The size distribution of all unigenes is shown in Fig. S1. The main statistical data of the six samples were described in Tables 2 and 3. The variation between individual hepatopancreas samples was evaluated through pairwise correlation. Except for one sample (T1), a strong correlation among other five samples were found (Spearman correlation coefficient across all genes ranging from 0.850 to 0.985), therefore, the T1 sample was excluded from further analysis.

### 3.3. Annotation of the assembly and classification of unigenes

This project get 19022 unigene annotated when comparing to the known sequences in major databases including nr, nt, SWISSPROT, GO, COG, and KEGG (Table 4). The GO and KEGG enrichment analysis were performed to gain insights into the biological implications of the identified unigenes.

GO classification describes their properties by assigning these unigenes to biological processes, cellular components and molecular functions. The GO analysis showed that the differentially expressed genes (DEGs) clustered in molecular functions, biological processes, and cellular components (Fig. 2). They are highly represented in GO analysis (Fig. 3). The metabolic process (4050 unigenes), cellular process (3305 unigenes), and single-organism process (2504 unigenes) represented the majority category of biology process. The cell part (2065 unigenes), cell (2047 unigenes), organelle (1390 unigenes) and membrane (1033 unigenes) represented the majorities of cellular component. Catalytic activity (3510 unigenes) and binding (3071 unigenes) showed the highest percentages in the molecular function category.

Functional classification and pathway assignment were based on a KEGG analysis. The unigenes were also mapped into the KEGG pathway database to predict the significantly enriched pathways. The most abundant 20 KEGG pathways were shown in Fig. S2. The top 5 KEGG pathways were lysosome, purine metabolism, ubiquitin mediated proteolysis, endocytosis and RIG-I-Like receptor signaling pathway. Some unigenes were mapped to several pathways related to reproduction, growth and development, such as TGF-beta signaling pathway, regulation of autophagy, Gap junction, Jak-Stat signaling pathway and apoptosis.

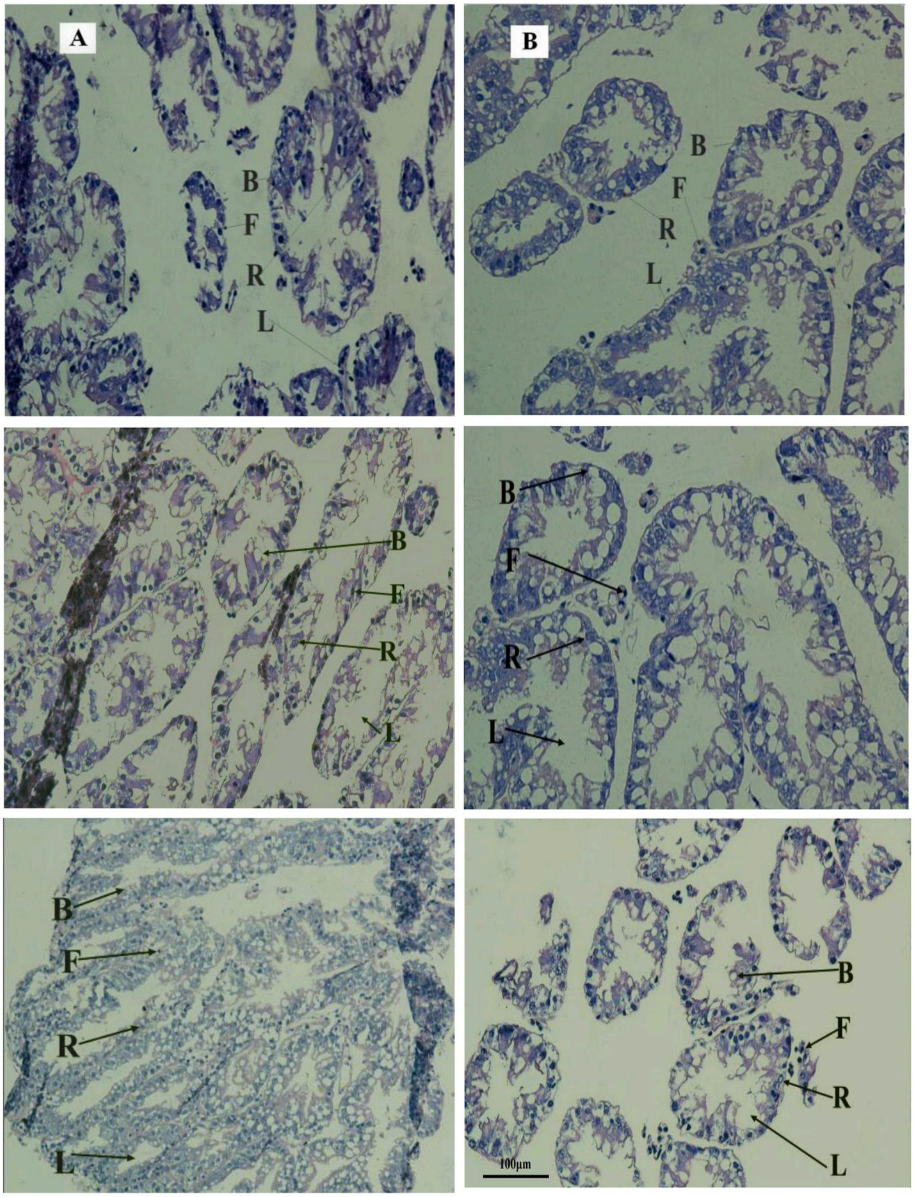

**Fig. 1.** The hepatopancreas morphological changes after 48h nitrite stress. A: hepatopancreas morphology of *M. nipponense* after 48h nitrite stress. B: hepatopancreas morphology of *M. nipponense* in control group. B: B-cells; F: F-cells; L: star shape of the lumen; R: R-cells.

### 3.4. Functional annotation and analysis of differentially expressed genes

A total of 825 significantly differential genes (In the screening process, FDR $< 0.01$ and FC (Fold Change) fold difference over 2 as screening criteria.) were achieved between the treatment group and the control group, which included 762 up-regulated genes and 63 down-regulated genes. A heatmap that depicted the majority of DEGs was presented in Fig. 4.

**Table 3**
The samples comparing to transcriptome.

| Length Range | Contig | Transcript | Unigene |
|---|---|---|---|
| Total Number | 1,006,291 | 94,534 | 56,054 |
| Total Length | 103,467,045 | 153,673,014 | 54,045,419 |
| N50 Length | 365 | 3310 | 2055 |
| Mean Length | 102.82 | 1625.58 | 964.17 |

**Table 2**
The RNA-Seq data of six samples.

| Samples | Read Number | Base Number | GC Content | %≥Q30 | Clean Reads | Mapped Reads | Mapped Ratio |
|---|---|---|---|---|---|---|---|
| T1 | 24,063,953 | 6,061,881,739 | 44.85% | 86.86% | 24,063,953 | 20,193,825 | 83.92% |
| T2 | 23,812,034 | 5,998,408,493 | 47.70% | 86.59% | 23,812,034 | 20,576,261 | 86.41% |
| T3 | 24,592,099 | 6,194,354,104 | 45.98% | 86.99% | 24,592,099 | 21,042,617 | 85.57% |
| T4 | 22,444,177 | 5,653,016,289 | 46.49% | 86.33% | 22,444,177 | 19,024,538 | 84.76% |
| T5 | 21,451,873 | 5,404,787,017 | 44.86% | 86.55% | 21,451,873 | 17,928,472 | 83.58% |
| T6 | 21,326,451 | 5,371,542,127 | 45.90% | 86.45% | 21,326,451 | 17,935,923 | 84.10% |

**Table 4**
Unigene annotations.

| Annotated databases | Unigene | ≥300 nt | ≥1000 nt |
|---|---|---|---|
| COG | 6449 | 5584 | 3864 |
| GO | 6807 | 5682 | 3509 |
| KEGG | 5692 | 5107 | 3669 |
| KOG | 12,398 | 10,795 | 7370 |
| Pfam | 13,100 | 11,662 | 8194 |
| Swiss-Prot | 12,434 | 11,096 | 7591 |
| nr | 18,186 | 15,638 | 9806 |
| All | 19,022 | 16,076 | 9906 |

Therefore, the expression patterns of immunity and apoptosis related DEGs were examined in this study by the comparison between treatment group and control group. Up-regulated immunity-related genes included cyclin B, serine proteinase-like protein, masquerade-like protein (*Sb*), mannose-binding protein, viral A-type inclusion protein (DDB_G0290503), polehole-like protein, Homeobox protein otx5-B (*OTX*), S-antigen protein (PF10_0343), Chlorophyllide a oxygenase (*daf-36*), ficolin-like protein 2, C-type lectin, reverse transcriptase, alpha-2-macroglobulin, tachylectin, suppressor of cytokine signaling-2 like protein (*SOCS-2*) and Protein neuralized (*neur*), etc. These immune-related genes have obvious up-regulation expression at 48 h of nitrite stress (Fig. 5).

Indirectly or directly, these genes with obvious differential expression have a certain response to nitrite stress, which can promote the body's immune defense mechanism on prawn and cope with external environmental stress. Up-regulated apoptosis-related genes included tetraspanins-like protein, lysosome-associated membrane glycoprotein (*LAMP*), Beta-hexosaminidase A (*hex-1*), sialin-like, *CD63*, caspase 3C and Caspase-1, etc (Fig. 6). These genes will be the focus of our further research (Table 5).

DEGs were further analyzed using GO and KEGG annotations to identify their potential functions and metabolic pathways. Fig. 3 showed the GO classifications of the DEGs. The biological process, metabolic process (56), cellular process (41) and single-organism process (34) had the most abundant GO function items while cell (22), cell part (22) and membrane (13) were most abundant in cellular component. In molecular function, catalytic activity (59) and binding (43) had more GO items than the others.

The DEGs were mapped to 60 pathways. Based on the threshold of P value $< 0.05$, the top 10 KEGG significant pathways were list in Table S1. They are lysosome, purine metabolism, ubiquitin mediated, proteolysis Endocytosis, RIG-I-Like receptor signaling pathway, TGF-beta signaling pathway, regulation of autophagy, Gap junction, Jak-Stat signaling pathway and apoptosis. Among those pathways, some closely related to immunity and apoptosis, metabolism and Cell.

### 3.5. qRT-PCR verification of the gene expression

To validate the veracity and reliability of differentially expressed genes identified by RNA-Seq, we randomly selected 15 genes for qRT-PCR validation from those with different expression patterns based on functional enrichment and pathway results. Primer set was designed based on each identified gene sequence of transcriptome library by Primer 5.0 (Table 1). The P $< 0.05$ was considered as statistical significance. The expression patterns of the 15 genes were consistent with the RNA-seq results (Fig. 7), which suggest that the results of the RNA-seq experiments were accurate and reliable.

## 4. Discussion

To better understand how organisms adapt to nitrite stress at the molecular level, detailed transcriptome was used to analyze the systemic gene expression and regulatory mechanisms about the nitrite tolerance of *M. nipponense*. The corresponding key genes and complex pathway were identified. In this study, we used the RNA-Seq platform

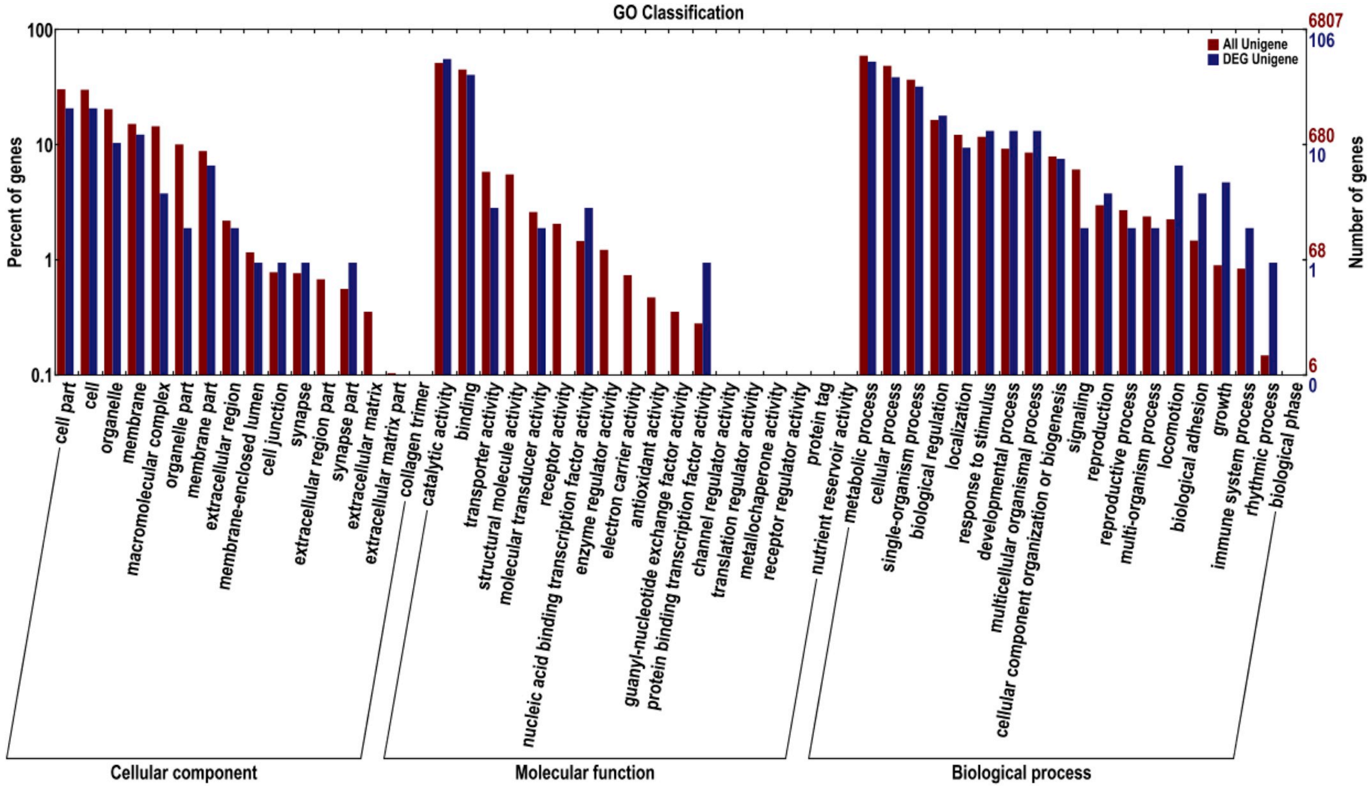

**Fig. 2.** GO analysis of DEGs between the different samples. The DEGs are classified into three categories: cellular component, molecular function, and biological process. The percentage of genes in each category and the number of genes are shown above.

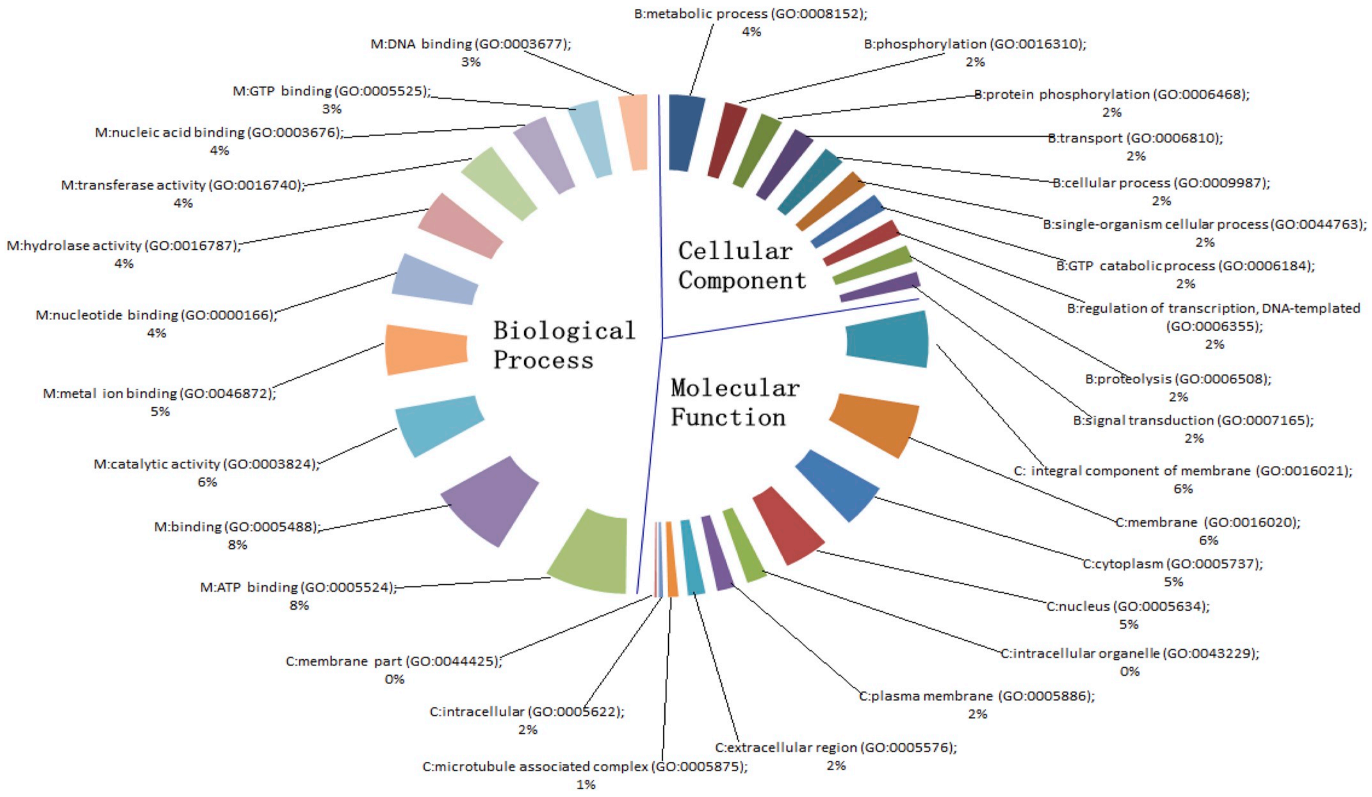

**Fig. 3.** Annotation of the *M. nipponense* hepatopancreas unique sequences according to GO. GO terms were derived based on the similarity search with web-based DAVID database. The top ten GO terms in the cellular component, molecular function, and biological process groups are displayed.

and bioinformatic analysis to investigate the transcriptomic response of the *M. nipponense* to nitrite stress. The study generated 34.68 Gb clean data. The map ration to reference genome ranged from 83.58% to 86.41%, meeting the requirements for the subsequent analysis. 825 genes were shown to be significantly differentially expressed between the two groups. The GO annotation and KEGG pathways analysis showed that the DEGs were involved in single-organism process, cellular process and metabolic process of biological process, the cell part, cell and membrane of cellular component, and the binding and catalytic activity of molecular function. Numerous key genes and numbers of important pathways that may be related to the immunity and apoptosis were obtained in the current transcriptome data.

In our study, immune-related pathways including arginine and proline metabolism, taurine and hypotaurine metabolism, alanine, aspartate and glutamate metabolism, Jak-Stat signaling pathway, endocytosis, wnt signaling pathway, RIG-I-like receptor signaling pathway, TGF-beta signaling pathway, GnRH signaling pathway and phagosome under the nitrite stress were up-regulated in this study. The arginine and proline metabolic pathway are associated with immune diseases. The central metabolites and the final products of this pathway (polyamines, dimethylarginine, hydroxyproline, creatinine) were found to have stress induced by reactive oxygen species (ROS) as well as some certain protective effects [31,32], activation of NMDA glutamate ion channel receptors [33–35] and induced autophagy [36]. In our study the glutamine synthetase gene is an important gene whose expression is up-regulated in the arginine and proline metabolic pathway under nitrite stress. Under stress, the increase of glutamine synthetase gene expression has a positive effect on the expression of positive regulatory arginine and proline metabolic pathway. Its specific role in the pathway requires further research. The Jak-Stat signaling pathway is a pathway for most cytokines and plays an important role in the regulation of the innate immune responses [37,38]. JAK is a cytosolic tyrosine kinase involved in the signaling of a range of cell surface receptors and particularly members of the cytokine receptor superfamily. To date, four have been discovered: JAK1, JAK2, JAK3, and Tyk2 [39]. JAK binds to the conserved regions BOX1 and BOX2 of cytokine receptor to recognize the cytokine receptor membrane proximal region motif, then undergoes a series of phosphorylation under the stimulation of the receptor and its ligand and selectively activates its downstream substrate STAT. It is translocated to the nucleus and binds to specific nuclear DNA regulators to direct transcription, the Jak-Stat signaling pathway. STATs (signal transducer and activator of transcription) are cytoplasmic protein families that bind to the target gene regulatory region DNA. It is coupled with a tyrosine phosphorylation signal to exert transcriptional regulation. STAT is an important transcription factor in cells and an important substrate for JAK [40]. In our study, the differentially expressed gene *SOCS-2* on the Jak-Stat signaling pathway was up-regulated under nitrite stress. The SOCS family is a group of Jak-Stat inhibitors discovered in recent years, and consisting of *CIS* (*CIS-1a*, *CIS-1b*), *SOCS-1*, *2*, *3*, *4*, *5*, *6*, and *7* [41]. Recently, it is clear that there are at least three different types inhibitory proteins are involved in the negative regulation of cytokine signaling: *SOCS*, *PTP* and *PIAS* [42]. The SOCS family (suppressor of cytokine signaling) has been discovered since 1997 as a negative regulator of cytokine-induced and feedback-blocking cytokine signaling. SOCS family members modulate signal transduction through several mechanisms including JAKs deactivation, hindering the proximity of STATs to receptor binding sites, and signal transduction [43,44]. There are also some reports on the key components and functions of the Jak-Stat signaling pathway in shrimp. Studies have shown that LvDome of *Litopenaeus vannamei* promotes the WSSV069 expression of WSSV, and JAK plays a very important role in anti-WSSV immunity [45]. The *SOCS-2* of *Litopenaeus vannamei* affects the Jak-Stat signaling pathway to resist WSSV invasion [46]. In addition, other studies have shown that SOCS and STAT of shrimp are up-regulated when stimulated by PGN [47]. *SOCS-2* of *Marsupenaeus japonicus* is up-regulated by *V. anguillarum* stimulation and regulates the expression of AMPs by affecting nuclear translocation and phosphorylation of STAT [48]. Overall, our results suggest that, in addition to

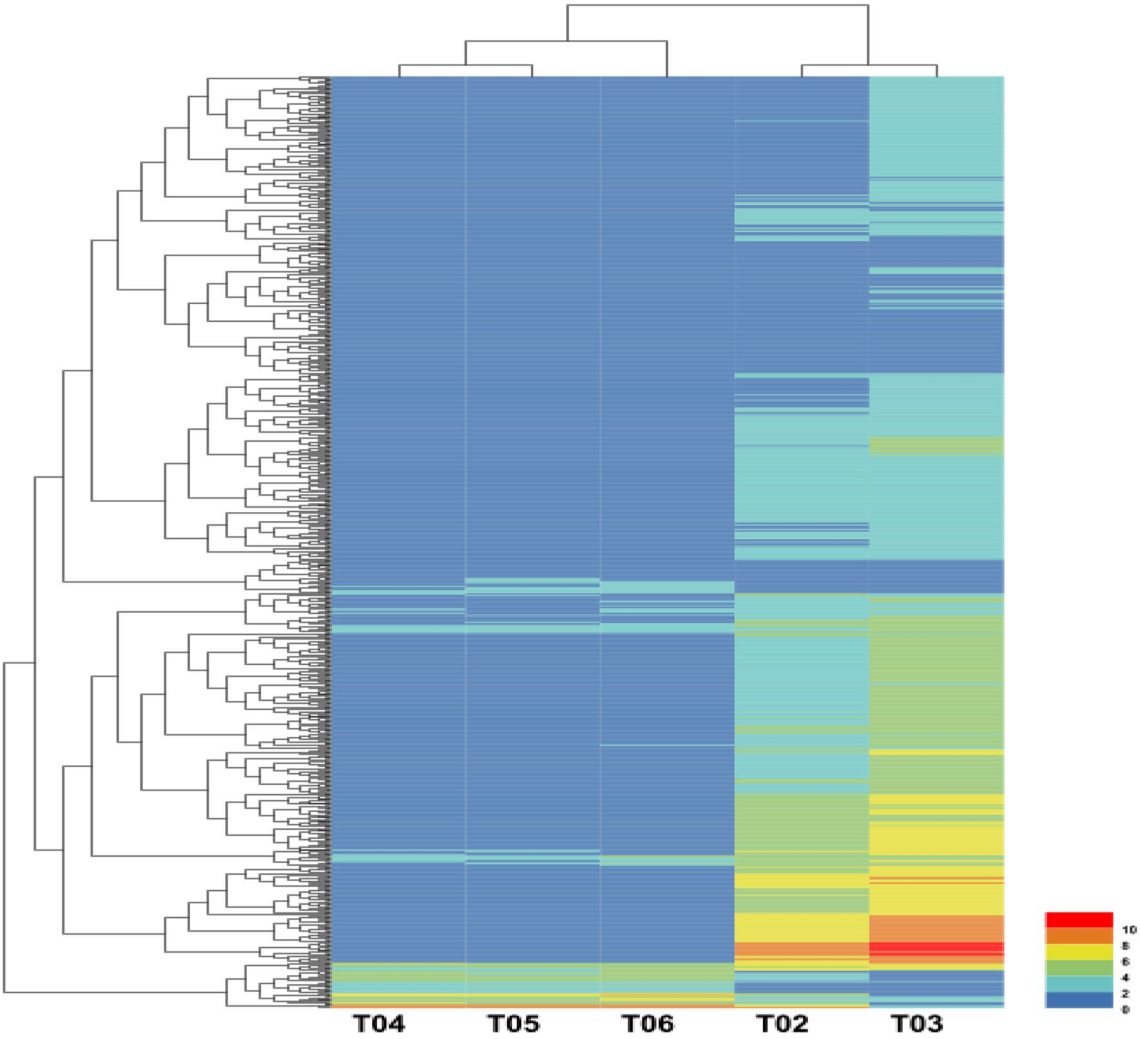

**Fig. 4.** Hierarchical clustering for the differentially expressed genes between Treatment group and Control group. Different columns in the figure represent different samples, and different rows represent different genes (Treatment group:T02, T03. Control group: T04, T05, T06). The color represents the logarithm of the expression level of the gene in the sample FPKM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

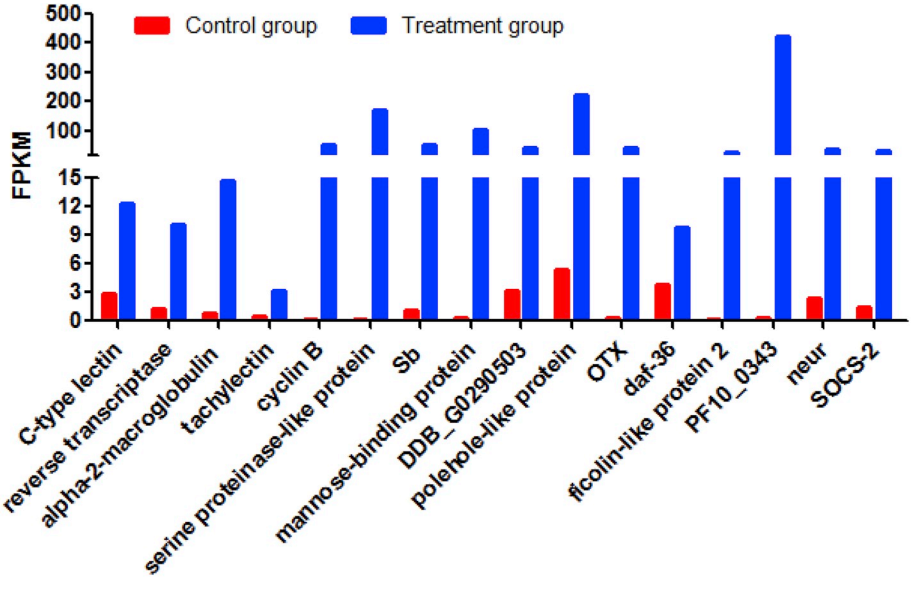

**Fig. 5.** Expression profiles of 16 genes involved in immune, displayed on the basis of their FPKM values from RNA-seq data from the control–treatment comparison. The FDR values of all comparisons are < 0.05.

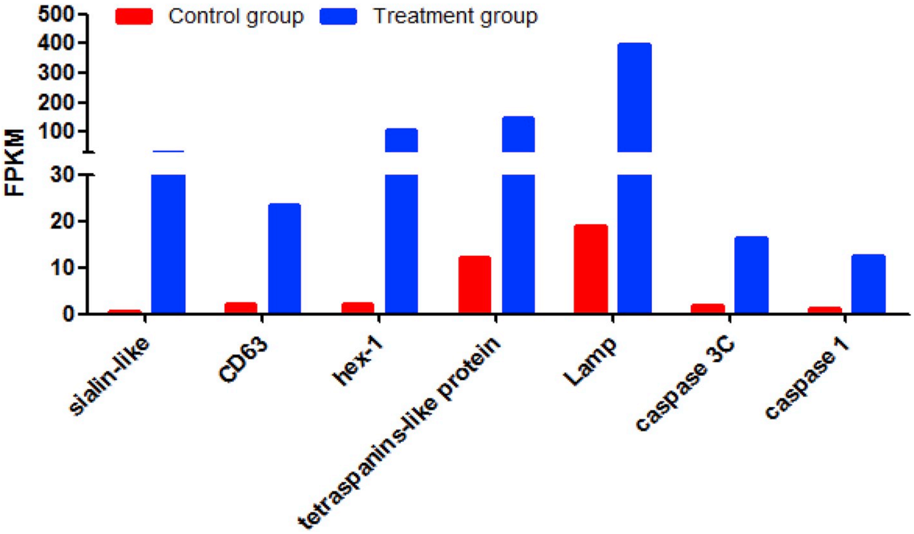

**Fig. 6.** Expression profiles of 7 genes involved in apoptosis, displayed on the basis of their FPKM values from RNA-seq data from the control–treatment comparison. The FDR values of all comparisons are < 0.05.

**Table 5**
Twenty-five DEGs related to immunity and apoptosis.

| Gene ID | Gene Name | Up or Down |
|---|---|---|
| c29559.graph_c0 | cyclin B | up |
| c10662.graph_c0 | serine proteinase-like protein | up |
| c25572.graph_c0 | serine proteinase inhibitor | up |
| c20214.graph_c0 | masquerade-like protein | up |
| c5943.graph_c0 | thread matrix protein 2E, partial | up |
| c30395.graph_c0 | mannose-binding protein | up |
| c30163.graph_c0 | clip domain serine protease 3 precursor | up |
| c21966.graph_c0 | viral A-type inclusion protein | up |
| c5550.graph_c0 | PREDICTED: protein PXR1-like | up |
| c25485.graph_c0 | tetraspanins-like protein | up |
| c24962.graph_c0 | vitellogenin receptor | up |
| c15978.graph_c0 | serine proteinase | up |
| c20808.graph_c0 | polehole-like protein | up |
| c27423.graph_c1 | Homeobox protein otx5-B | up |
| c23041.graph_c0 | Chlorophyllide a oxygenase | up |
| c20935.graph_c0 | ficolin-like protein 2 | up |
| c27760.graph_c0 | C-type lectin | up |
| c32745.graph_c0 | reverse transcriptase | up |
| c23050.graph_c0 | alpha-2-macroglobulin | up |
| c26525.graph_c0 | tachylectin | up |
| c25298.graph_c1 | suppressor of cytokine signaling-2 like protein | up |
| c30373.graph_c0 | tetraspanins-like protein CD63 | up |
| c24666.graph_c0 | crustacyanin-like lipocalin | down |
| c26907.graph_c0 | beta-1,3-glucan-binding protein precursor | down |
| c26459.graph_c0 | chitin binding-like protein | down |

pathogens, environmental stressors such as nitrite can also trigger innate immunity by activating the Jak-Stat signaling pathway.

The study found that in addition to immune-related genes and pathways, apoptosis-related genes were also screened out, after 48 h exposure. This also showed that the nitrite stress can indeed cause the decline of various functions of the individual by destroying the normal morphological structure of the cells of the tissue, and lead to illness or death. We also find similar findings in the study of *Litopenaeus vannamei* [49] and *Marsupenaeus japonicus* [50]. Apoptosis-related pathways included lysosome and apoptosis. Apoptosis is a physiological cell death, which is accompanied by a series of morphological changes such as cell shrinkage, chromatin condensation and formation of apoptotic bodies [51]. Since the concept of apoptosis was established in 1972 [52], research efforts have identified hundreds of genes that control the initiation, execution, and regulation of apoptosis in some species [53]. Nitrite stress is one of an important induced factor for apoptosis on various cells including shrimp hemocytes and hepatopancreas [54,55]. It is reported that the expression of apoptosis related genes in *Litopenaeus vannamei* was induced by nitrite stress [56]. In vitro nitrite stress can inhibit the cell function and induce apoptosis of blood cells of the tiger shrimp and *Penaeus monodon*. The nitrite in the environment can induce the overproduction of ROS and result in DNA damage and apoptosis, and thus the total haemocyte count (THC) decreases [16]. The lysosomal pathway is involved in apoptosis. Lysosomes require many special proteins to maintain their role in cells. The lysosomal membrane contains up to 100 LMPs, of which about 25 have been studied and all of these may be critical to the biosynthesis and function of lysosomes [57,58]. Lysosomal metabolic pathways were involved in cell apoptosis. Many proteases of lysosomes, such as Cathepsins and *LAMP*, are involved in cell apoptosis [59]. Lysosomal integration membrane protein-2 (*LIMP-2*), encoded by gene SCARB2, has been shown to be an important participant in this kind of protein in lysosomes [60,61]. In our study the tetraspanins-like protein gene is an important effector gene on *LIMP* in the lysosomal pathway, which is up-regulated. The tetraspanins-like protein gene is the gene of the *LIMP* protein in the lysosomal pathway, with structural homologies to tetraspanins. Tetraspanins belongs to the transmembrane 4 superfamily (*TM4SF*) family, which is a group of proteins with four transmembrane spanning domains [62]. The tetraspanins special four-transmembrane structure can link intramembrane, transmembrane and extramembrane proteins, and function as a channel linking signals inside and outside the membrane, to facilitate the interaction of proteins in tetraspanins signaling networks [63]. Tetraspanins are involved in different cell functions like cell development, adhesion, motility and differentiation probably, which play critical roles in multiple physiological and pathological processes [64–66]. In this study, the up-regulation of the tetraspanins-like protein gene demonstrated that this gene has a protective effect on hepatopancreas cell apoptosis under nitrite stress. This also provided us with a new direction for the study of the nitrite tolerance of *M. nipponensis*. The study of tetraspanins-like protein gene and lysosomal pathways in aquatic animals is rare, and the tetraspanins-like protein genes could be used as a new molecular immune indicator in crustaceans during nitrite stress. The specific function of tetraspanins-like protein gene is still unclear and needs to be further verified by experiments.

Thus we hypothesize that the stress of nitrite leading to *M. nipponense* unhealthy or death was due to the destruction of the body tissue cells. Paraffin sections of hepatopancreas tissue after nitrite stress demonstrated this view. Observations on paraffin sections showed the following results. With the *M. nipponense* survival in the nitrite

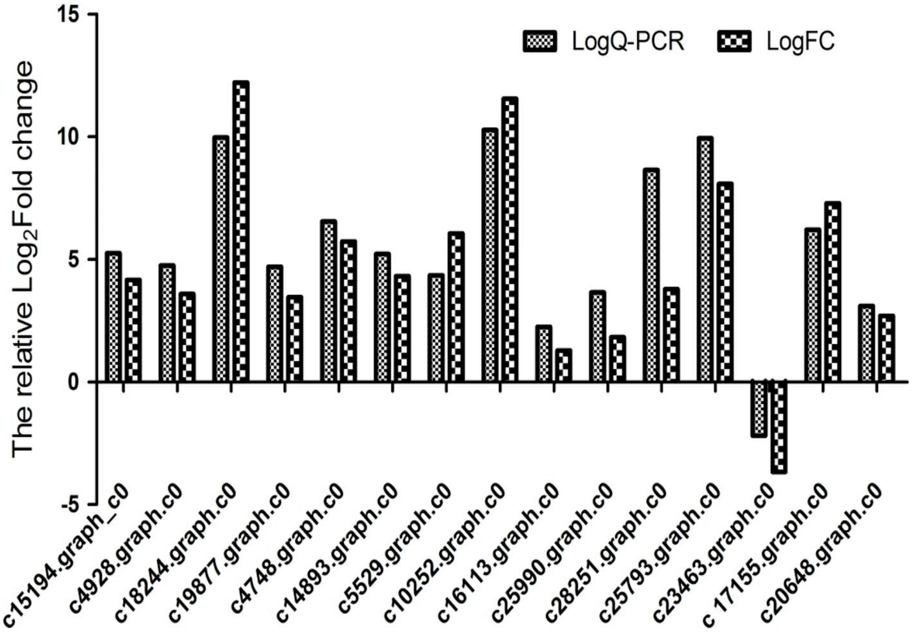

**Fig. 7.** Candidate unigene expression levels revealed by qRT-PCR and RNA-seq. They are showing the same expression trend.

environment, hepatopancreas cells will appear rupture phenomenon (separation between cell wall and membrane). It shows that the nitrite stress destroyed the hepatopancreas organizational structure, which may affect the normal physiological function of hepatopancreas. Loss and damage of *M. nipponense* hepatopancreas may depress the immune ability and even induce death. Hepatopancreas plays a vital role in the immune and physiological processes of shrimp, which may be seriously affected by the nitrite stress.

## 5. Conclusions

In this study, we performed a genomic transcriptional analysis of *M. nipponense* under nitrite stress to explore nitrite-related genes and pathways. It is well known that aquatic animals suffer a greater risk of nitrite poisoning because nitrite can pass through their sacral epithelium and accumulate in their body fluids to very high concentrations. A total of 825 unigenes changed significantly after 48 h of nitrite exposure. Immunization, cellular activity, metabolism, signaling, and genetic material-related transcripts are clearly detected. We identified a variety of pathways and genes involved in immune defense, apoptosis, and the like, which includes arginine and proline metabolism, taurine and hypotaurine metabolism, alanine, aspartate and glutamate metabolism, Jak-Stat signaling pathway, endocytosis, wnt signaling pathway, RIG-I-like receptor signaling pathway, TGF-beta signaling pathway, GnRH signaling pathway and phagosome. These molecular responses to nitrite stress confirmed that these stress proteins and pathways play an important role in the protective mechanism of nitrite stress. These genes and pathways are involved in defense responses under nitrite stress, and these DEGs can be used as molecular immunological indicators under crustacean nitrite stress. Transcriptome analysis also produced a large number of new transcripts that laid the foundation for further genomic research in the future. Well-categorized and annotated *M. nipponense* transcriptome could serve as important and valuable resources for gene identification and functional analysis.

## Conflicts of interest

The authors declare that they have no conflicts of interest.

## Authors' contributions

The first authors Jielun Yu, Xiangshan Ji and Xuepeng Wang contributed equally to this work.

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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.2018.12.075.

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