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Differentially expressed genes of the shrimp *Marsupenaeus japonicus* in response to infection by white spot syndrome virus or *Vibrio alginolyticus*

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ABSTRACT

This is the first study to use a next-generation high-throughput DNA sequencing technique, the Illumina Hiseq2000 method, to analyze the transcriptome from hemocytes of *Marsupenaeus japonicus*. A total of 80,929,652 Illumina reads, including 79,525,942 high quality reads were obtained in this study. From these, 40,231 unigenes with a mean length of 1557 bp were assembled using Trinity de novo software and 28,746 cDNA were matched in the NCBI database. Then we compared the transcriptome changes after white spot syndrome virus (WSSV) or *Vibrio alginolyticus* infection. A total of 19,872 putative proteins were classified functionally into 25 molecular families in the cluster of orthologous groups. KEGG pathway analysis identified that the metabolic pathway possessed more unigenes (1358 unigenes), followed by biosynthesis of secondary metabolites, Huntington's disease and RNA transport. Important immune functions like apoptosis, phagocytosis, and lysosomes were in response to WSSV and *V. alginolyticus* early infection. Only 26 transcripts were significantly up-regulated or down-regulated after WSSV infection showed compared with *V. alginolyticus*. Crustin-like protein, endonuclease-reverse transcriptase, putative nuclease HARBI1-like, diphthamide biosynthesis protein 7 and hormone receptor 3 were involved in the immune response to WSSV or *V. alginolyticus* infection. These transcriptome datasets accelerate our understanding of the innate immune mechanisms in *M. japonicus* and other crustaceans.

1. Introduction

The kuruma shrimp (*Marsupenaeus japonicus*) is an economically important commercial farmed species of the *Penaeidae* family of decapod crustaceans in Southeast Asia. White spot syndrome virus (WSSV), an enveloped virus with a large double-stranded genome (~300 kb) [1,2], is a viral pathogen responsible for huge economic losses in the shrimp culture industry [3–5]. Vibriosis has also been implicated as the cause of major mortality in juvenile penaeid shrimp [6]. *Vibrio alginolyticus* is an important bacterial pathogen in the shrimp farming industry. WSSV and *V. alginolyticus* often cause high production losses of farmed shrimp in Southeast Asia.

Crustaceans lack an adaptive immune system and rely totally on the innate immune system to resist pathogen invasion. The innate immune reaction comprises cellular reactions such as phagocytosis, nodule formation and encapsulation performed by hemocytes. Hemocytes are crucial for the immune system in crustaceans as they are the main production site for immune recognition molecules and initiate the humoral reaction like phagocytosis [7,8]. Previous studies have discovered many immune-related genes responding to WSSV infection in crustaceans [9,10].

Transcriptome analysis is widely used to identify novel and differentially expressed genes in shrimp [11–14]. However, the hemocyte transcriptome information for the shrimp response to WSSV and bacterial challenge is still limited, so we constructed and sequenced a cDNA library from WSSV-challenged hemocytes in *M. japonicus*. The objective of this work was to identify and annotate more immune-related genes in shrimp hemocytes and also provide information to elucidate the mechanisms of innate immune response to WSSV and *V. alginolyticus* challenge in crustaceans. In this study, we obtained 40,231 unigenes; most of these were novel for *M. japonicus*. In addition, many genes were found to be related to the innate immune system in other species, such as pattern recognition receptors, antimicrobial peptides, proteases and protease inhibitors, signal transduction proteins, apoptosis-related proteins, and antioxidant proteins.

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Table 1
General information of the transcriptome from health shrimp.

Dataset name	health shrimp
Total raw reads (paired-end)	80929652
Total clean reads	79525942
Q20%	98.94%
N percentage	0%
GC percentage	44.33%
Total unigenes	40231
Total genes	28746
Mean length (bp)	1557.55
N50 (bp)	3208
Min-Max length (bp)	351–18945

2. Methods

2.1. Shrimp culture for challenge experiments

Cultures of *M. japonicus* shrimps, each approximately 10 g and 10 cm in length, were maintained in groups of 20 individuals in 80 L aquariums at 22 °C. Hemolymph from cultured shrimps was subjected to random PCR detection with WSSV-specific primers to ensure that the shrimps were WSSV-free before experimental infection. The WSSV inoculum was prepared from WSSV-infected shrimps according to Zhu et al. [9]. The WSSV-challenged group received 20 μL injections of WSSV (GenBank accession no. AF 332093.1, 10^5 copies/mL). In the *Vibrio* challenge experiment, 30 shrimps were each injected with 20 μL of *V. alginolyticus* (ATCC17749) suspended in PBS solution (10^6 cells/mL). In addition, 30 shrimps were each injected with 20 μL PBS as the control for the challenge experiments. The hemolymph was withdrawn from the ventral sinus located at the first abdominal segment using an equal volume of modified Alsever's solution anticoagulant [15] and subsequently centrifuged for 10 min at 1000 × *g*, 4 °C. Hemocytes was collected at various times after infection (12 and 24 h) and immediately stored in liquid nitrogen for later RNA extraction.

2.2. RNA isolation and illumina sequencing

Total RNAs were isolated from the hemocytes of the infected and non-infected shrimps at different times after infection using Unizol reagent (UnionGene, China) and treated with DNase I. The mRNAs were purified from the total RNAs using the PolyATtract mRNA isolation system (Promega, Madison, USA) following the manufacturer's instructions. The quantity and purity of total RNAs were monitored using a NanoDrop ND-1000 spectrophotometer (NanoDrop, DE, USA).

cDNA libraries were generated using Illumina TruSeq™ RNA Sample Preparation Kit (Illumia, USA) following manufacturer's instructions and the index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in Illumina proprietary fragmentation buffer. First strand cDNA was synthesized using random oligonucleotides and SuperScript II. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities and enzymes were removed. After adenylation of 3′ ends of

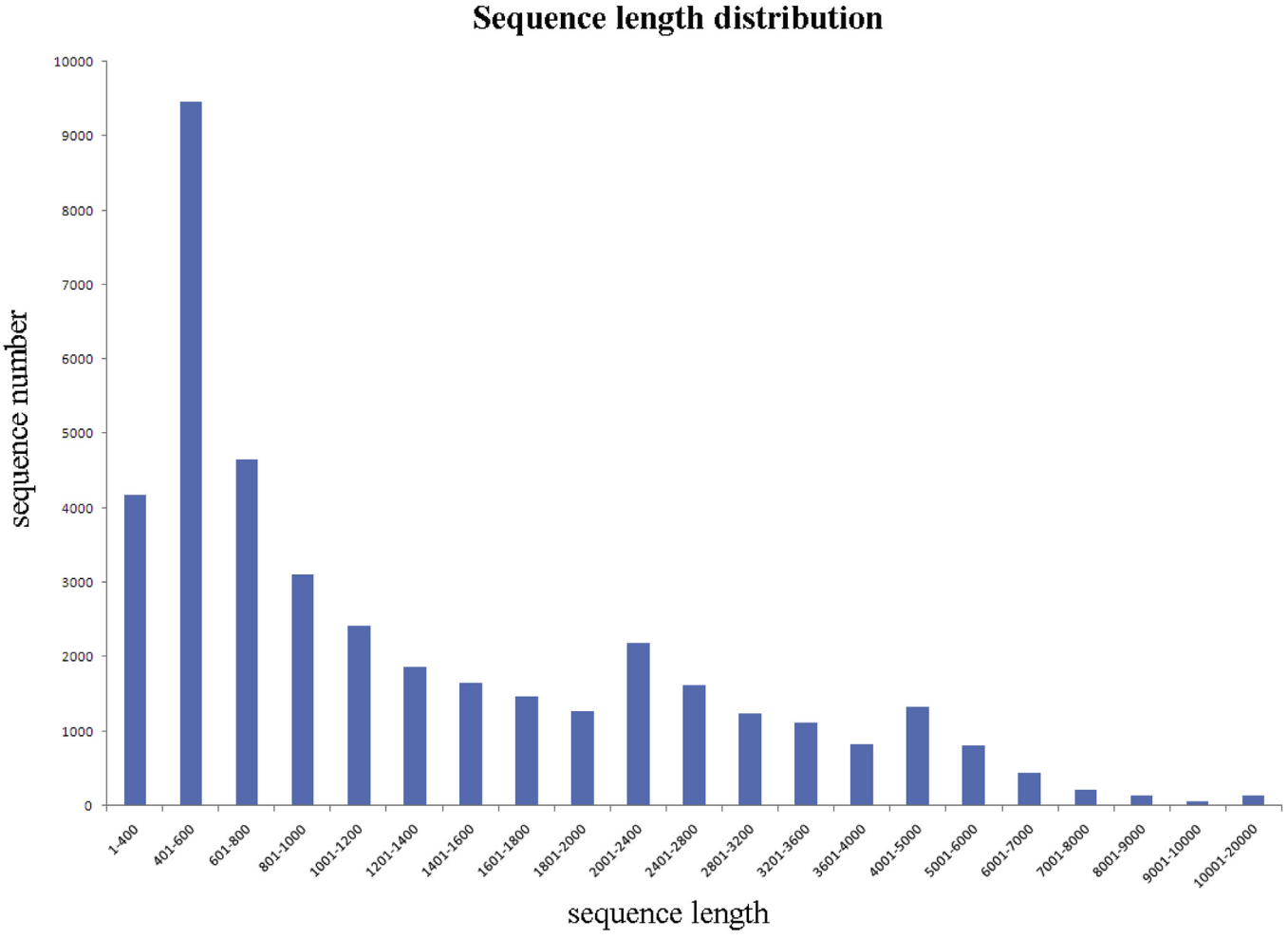

Fig. 1. Contig length distribution of *M. japonicus* transcriptomic ESTs.

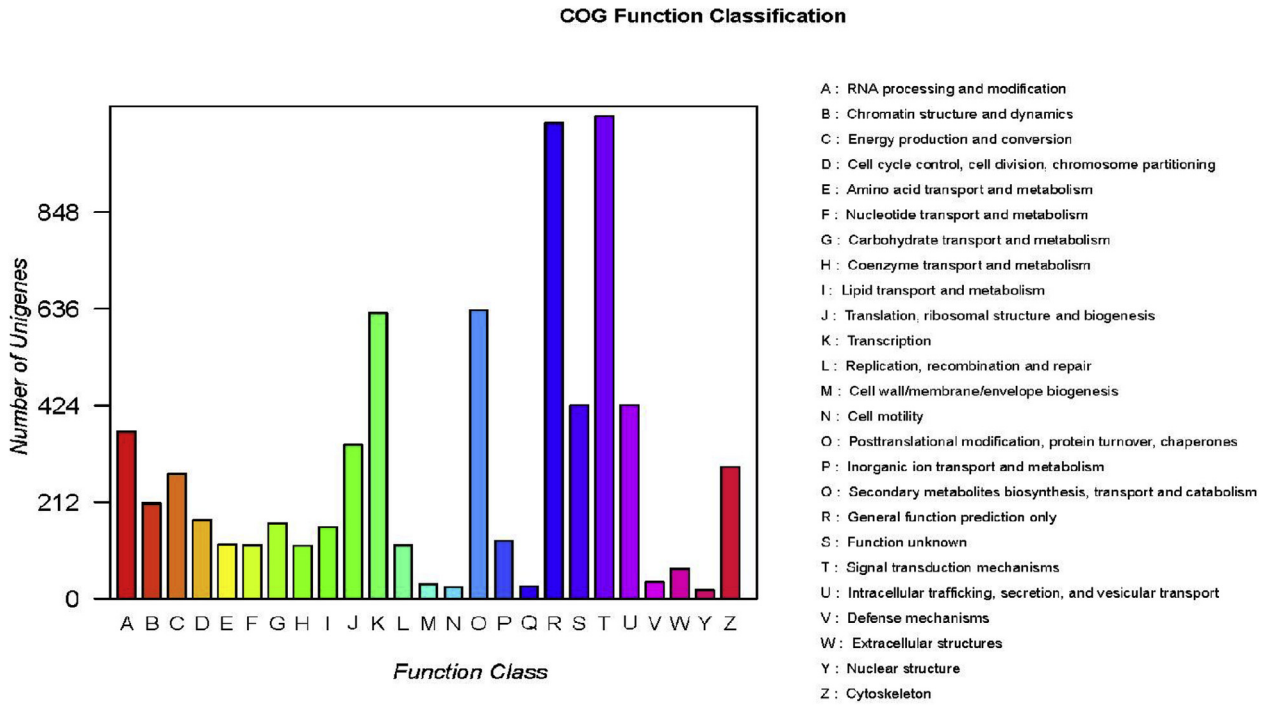

Fig. 2. Cluster of orthologous groups (COG) function classification in the transcriptome of hemocytes in healthy *M. japonicus*.

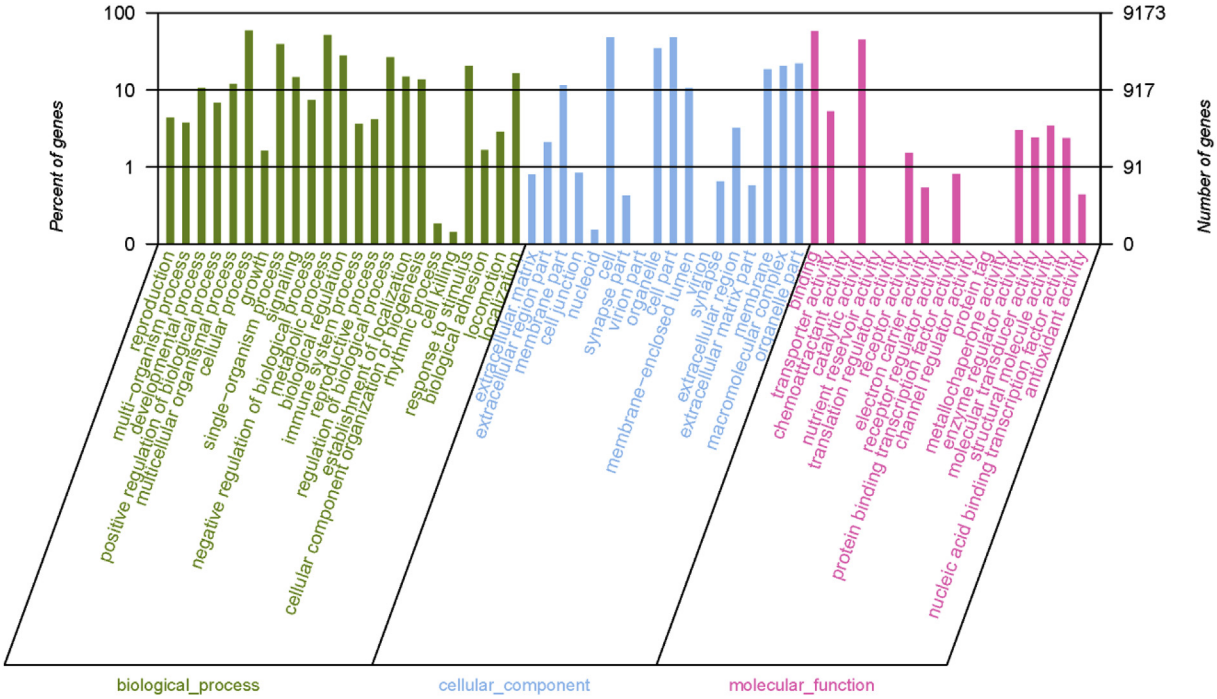

Fig. 3. Gene ontology classification of the hemocyte transcriptome in healthy *M. japonicus*. The left y-axis indicates the percentage of a specific category of genes existing in the main category, whereas the right y-axis indicates the number of a specific category of genes existing in the main category.

DNA fragments, Illumina PE adapter oligonucleotides were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 300 bp, the library fragments were purified with AMPure XP system (Beckman Coulter, USA). DNA fragments with ligated adaptor molecules on both ends were selectively enriched using Illumina PCR Primer Cocktail in a 10 cycle PCR reaction. Products were purified (AMPure XP system) and quantified using the Agilent high sensitivity DNA assay on the Agilent Bioanalyzer 2100 system.

2.3. De novo assembly and functional annotation

Raw sequencing reads were quality trimmed, and adaptor sequences were removed before the assembly. The processed reads were assembled using Trinity software with default parameters [16]. The derived unigene sequences were searched against the NCBI nonredundant protein sequence database for annotation, with an e-value cutoff of 1×10^{-10}. For Gene Ontology (GO) annotation, InterproScan was used for obtaining GO terms (http://nar.oxfordjournals.org/content/43/D1/

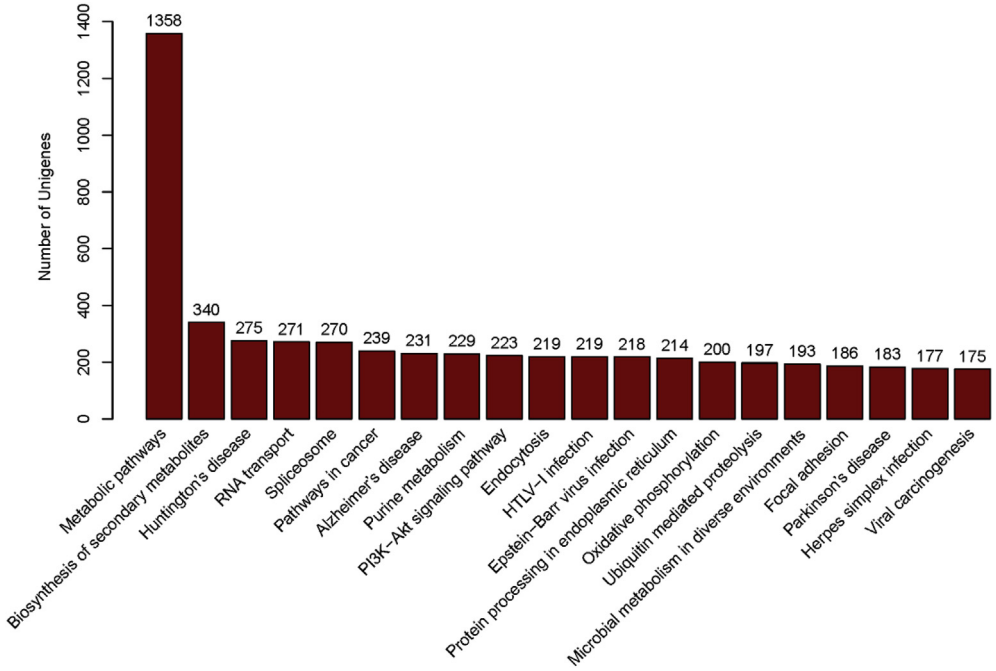

Fig. 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification of hemocytes transcriptome in healthy *M. japonicus*.

Table 2
Candidate genes involved in the immune response of M. *japonicus* at 12 h after challenged with WSSV or VA.

Gene ID	Fold change		Homologous function	Species
	WSSV	VA		
comp16935_c0	2.97	23.41	crustin-like protein	*Litopenaeus vannamei*
comp6853_c0	2.5	0	conserved hypothetical protein	*Tribolium castaneum*
comp150797_c0	6.55	0.36	hormone receptor 3 isoform B2	*Blattella germanica*
comp2737_c0	6.34	0.38	diphthamide biosynthesis protein 7	*Callorhinchus milii*
comp87977_c0	1.57	0	putative nuclease HARBI1-like	*Amphimedon queenslandica*
comp163681_c0	0	4.12	hypothetical protein DAPPUDRAFT_303826	*Daphnia pulex*
comp208166_c0	5.59	0.34	hormone receptor 3	*Tribolium castaneum*
comp295508_c0	0.18	19.51	uncharacterized protein LOC101485176	*Maylandia zebra*
comp298067_c0	6.51	0.11	endonuclease-reverse transcriptase	*Bombyx mori*
comp16658_c0	5.96	0.31	interferon alpha-inducible protein 27-like protein 2-like	*Cricetulus griseus*
comp1883_c0	0.11	2.79	putative H23L24.5	*Danaus plexippus*
comp304295_c0	2.36	0	Unknown	
comp115129_c0	3.49	0.19	Unknown	
comp11890_c0	5.76	0	Unknown	
comp16340_c0	2.68	0.22	Unknown	
comp17608_c0	13.09	0.96	Unknown	
comp19544_c0	2.09	18.5	Unknown	
comp19254_c0	13.78	1.39	Unknown	
comp308819_c0	0.23	4.36	Unknown	
comp114591_c0	0.17	17.97	Unknown	
comp10817_c0	17.6	1.08	Unknown	
comp9461_c0	3.46	0.19	Unknown	
comp15328_c0	31.11	2.88	Unknown	
comp247791_c0	10.65	0.09	Unknown	
comp135892_c0	1.55	0	Unknown	
comp144955_c0	5.52	0.06	Unknown	

FC indicates the fold change; VA indicates *V. alginolyticus*.

D213), and WEGO was used for plotting GO annotation results [17]. The determination of the eukaryotic complete genome (KOGs) type followed a described method [18]. The KEGG pathways were annotated using the KEGG Automatic Annotation Server (http://nar.oxfordjournals.org/cgi/content/full/35/suppl_2/W182). The expression abundances and identification of differentially expressed genes were estimated using the Cuffdiff program in the software package Cufflinks with default parameters (http://cole-trapnell-lab.github.io/cufflinks/).

2.4. Expression analysis by real-time PCR

The expression levels of a gene in multiple organs of both healthy and pathogen-challenged crabs were analyzed by real-time quantitative PCR using SYBR Green qRT-PCR assay (Promega). The procedure was performed according to a method described previously [19]. Total RNA of hemocytes was extracted by Easy spin tissue/cell RNA extra kit (Aidlab, China) following the protocol of manufactures. Experiments were performed in triplicates and at least three shrimps were analyzed

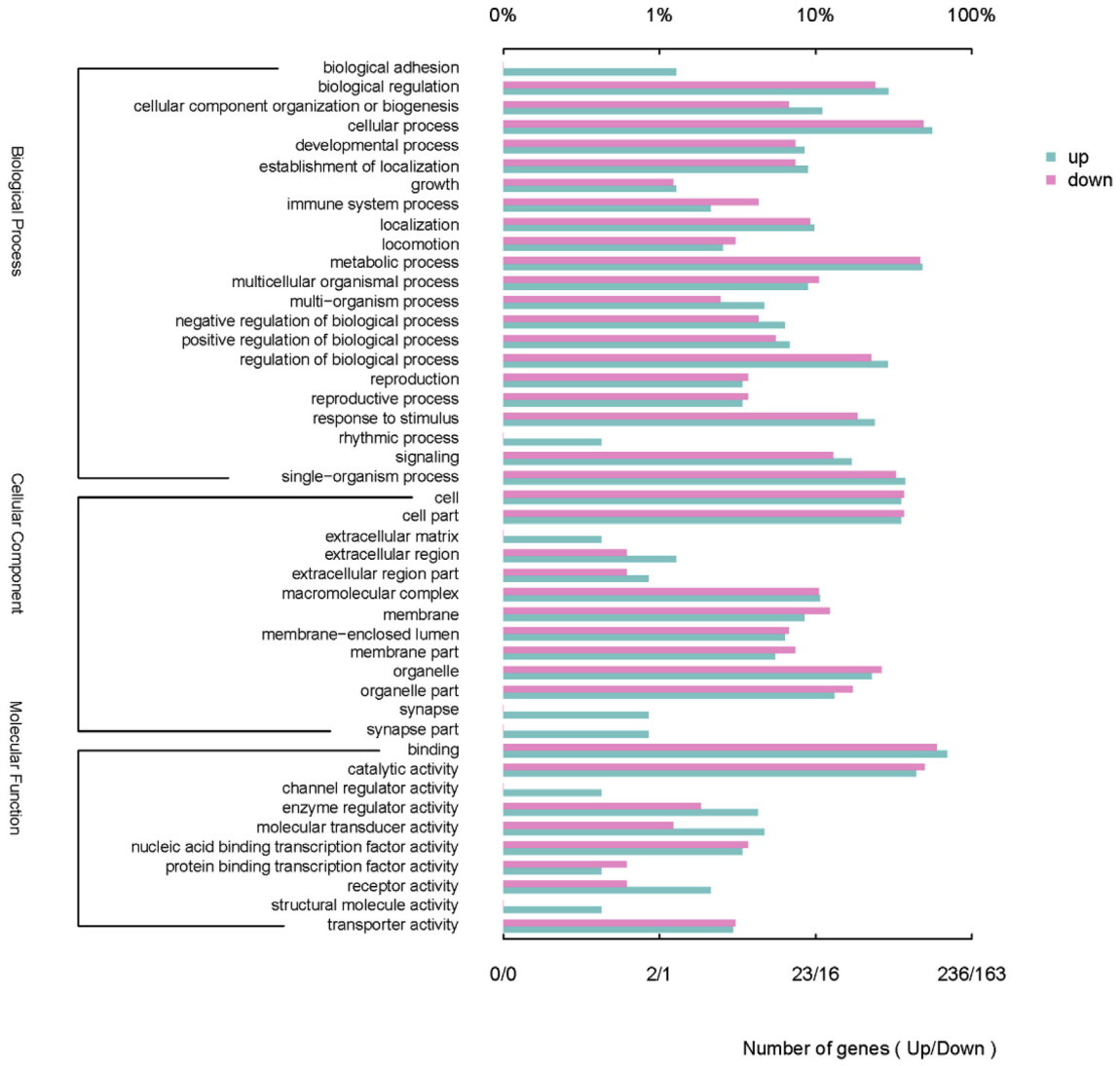

Fig. 5. Up-regulated Gene and down-regulated Gene in W12 vs V12 treatment.

for each type of tissue. Less than 200 μg total RNA was used for cDNA synthesis by ReverTra Ace qPCR RT Master Mix with gDNA Remover Code: FSQ-301 (Toyobo, Japan), The cDNA was kept at −20 °C. The SYBR Green RT-qPCR assay was carried out in Bio-Rad Two Color Real-Time PCR Detection System and the data was calculated according to the $2-\Delta\Delta CT$ comparative CT method by office EXCEL, with the amplification of GAPDH as internal control. Designing and synthesizing of the RT q-PCR primers were entrusted to Generay Shanghai Company, based on the open read frame (ORF). And the primers were shown in Table S1.

2.5. Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). The statistical differences were estimated by one-way analysis of variance (ANOVA) followed by least-significant differences and Duncan's multiple range test. All statistical analyses were carried out using SPSS Statistics version 19. A probability level of 0.01 was used to indicate statistical significance ($P < 0.01$).

3. Results

3.1. Transcriptome analysis

In the present study, a transcriptome profile was used to study the immune response to WSSV or *V. alginolyticus* in shrimp hemocytes using Illumina sequencing technologies. Shrimp *M. japonicus* is a non-model organism and has no reference genome sequence. The transcriptome from *M. japonicus* comprised 79,525,942 total clean reads and a GC percentage of 44.33%. We obtained 40,231 isogenes (ranging from 351 to 18945 bp, mean length = 1557 bp) from *M. japonicus* hemocytes after de novo assembly (Table 1). Most of the isogenes (23.53%) were 401–600 bp in length, followed by 601–800 bp (11.57%), and 1–400 bp in length (10.39%) (Fig. 1). A comparison with GenBank protein and nucleotide sequences identified 28,746 genes in *M. japonicus* hemocytes. These genes were found to be related to the innate immune system in other species, such as pattern recognition receptors, antimicrobial peptides, proteases and protease inhibitors, signal transduction proteins, apoptosis-related proteins, and antioxidant proteins. There were more than 98% high-quality bases (above Q20, Table 1), indicating an excellent quality (Q20 indicates 1 error per 100 sequenced bases). The ratio statistics of mapping from healthy shrimps was over 90% in all six samples.

Table 3
Candidate genes involved in the immune response of *M. japonicus*.

Gene ID	WSSV FC	*V. anguillarium* FC	Homologous function	Species
comp16935_c0	2.97	23.41	crustin-like protein	*Litopenaeus vannamei*
comp115129_c0	3.49	0.19	unknown	
comp11890_c0	5.76	0	unknown	
comp16340_c0	2.68	0.22	unknown	
comp17608_c0	13.09	0.96	unknown	
comp19544_c0	2.09	18.5	unknown	
comp135892_c0	1.15	0	prepro-calcitonin-like diuretic hormone	*Homarus americanus*
comp144955_c0	5.52	0.06	unknown	
comp6853_c0	2.5	0	conserved hypothetical protein	*Tribolium castaneum*
comp150797_c0	6.55	0.36	HR3 isoform B2	*Blattella germanica*
comp2737_c0	6.34	0.38	WD repeat-containing protein 85	*Ceratotherium simum simum*
comp87977_c0	1.57	0	putative nuclease HARBI1-like	*Amphimedon queenslandica*
comp163681_c0	0	4.12	hypothetical protein DAPPUDRAFT_303826	*Daphnia pulex*
comp19254_c0	13.78	1.39	unknown	
comp208166_c0	5.59	0.34	similar to HR3	*Tribolium castaneum*
comp308819_c0	0.23	4.36	unknown	
comp114591_c0	0.17	17.97	unknown	
comp15328_c0	31.11	2.88	unknown	
comp247791_c0	10.65	0.09	unknown	
comp295508_c0	0.18	19.51	unknown	
comp298067_c0	6.51	0.11	hypothetical protein M91_09415, partial	*Bos grunniens mutus*
comp10817_c0	17.6	2.08	unknown	
comp9461_c0	3.46	0.19	unknown	
comp16658_c0	5.96	0.31	interferon alpha-inducible protein 27-like protein 2-like	*Cricetulus griseus*
comp1883_c0	0.11	2.79	putative H23L24.5	*Danaus plexippus*
comp304295_c0	2.36	0	unknown	

3.2. Gene ontology assignments and COG analysis

A total of 19,872 putative proteins were classified functionally into 25 molecular families in the COG database (Fig. 2). Among these 25 functional categories, a cluster for signal transduction mechanisms represents the largest group with 1034 unique sequences, followed by general function prediction only (1,012), transcription, posttranslational modification, and others (Fig. 2).

The GO classification of the hemocyte transcriptome in healthy *M. japonicus* was analyzed. By alignment to GO terms, these genes were mainly divided into three categories with 59 functional groups: biological processes (23 functional groups), cellular components (18 functional groups), and molecular functions (18 functional groups). Among the biological processes, cellular processes represent the largest group, followed by metabolic processes, single-organism processes and biological regulation (Fig. 3). Among the cellular components, the cell group represented the largest group, followed by cell part and organelle (Fig. 3). Among molecular function, binding represented the largest group, followed by catalytic activity and transporter activity (Fig. 3). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification was performed to obtain important pathways. The KEGG pathway analysis indicated that metabolic pathways (1358 genes) were the most important, followed by biosynthesis of secondary metabolites, Huntington's disease, and RNA transport (Fig. 4).

To discover the differentially expressed genes in *M. japonicus* after WSSV or *V. alginolyticus* infection, two infection experiments (WSSV, W12, and *V. alginolyticus,* V12) were carried out. In a comparison of the expression of genes after W12 and V12 treatment, 26 genes were up-regulated or down-regulated (Table 2). The metabolic pathways (mainly energy production and conversion) were the most important pathway for the genes that were up-regulated or down-regulated in W12 *vs.* V12 treatment, followed by signal transduction and transcription (Data not shown). The important immune pathways included: NF-κB signaling pathway, p53 signaling pathway, lysosome, phagosome, apoptosis, Wnt signaling pathway, Toll-like receptor signaling pathway, JAK-STAT signaling pathway, and Fc gamma R-mediated phagocytosis (Data not shown). The important immune functions, such as apoptosis, phagocytosis, and lysosome, were anticipated in the response to WSSV and *V. alginolyticus* infection (Data not shown). We determined the genes up-regulated and down-regulated in W12 vs V12 treatment, cellular processes, metabolic processes, and single organism processes were the most important (Fig. 5). We found that cells, cell parts, and organelles were important cellular components, and binding and catalytic activity were important molecular functions (Fig. 5).

3.3. Identification of differentially expressed genes

Overall, 26 transcripts were upregulated or downregulated significantly in both WSSV and *V. alginolyticus* infection, but most of the genes were up-regulated or down-regulated conversely in the two transcriptomes (Table 3). Five candidate genes, *endonuclease-reverse transcriptase*, *putative nuclease HARBI1-like*, *diphthamide biosynthesis protein 7*, *hormone receptor 3* and *crustin-like*, were characterized by real-time PCR assay (Fig. 6). The expression of *putative nuclease HARBI1-like* showed a similar trend to the transcriptome results after the WSSV challenge but was down-regulated significantly after the challenge with *V. alginolyticus* (Fig. 6A and B). The expression of *hormone receptor 3* was up-regulated significantly at 12 h after the challenge with WSSV and *V. alginolyticus* but was down-regulated at 24 h after challenged with *V. alginolyticus* (Fig. 6A and B). The expression of *diphthamide biosynthesis protein 7* showed a different trend to the transcriptome results, it was up-regulated significantly at 12 h and 24 h after the challenge with *V. alginolyticus* but was not changed significantly by the WSSV challenge (Fig. 6A and B). The expression of *endonuclease-reverse transcriptase* was up-regulated significantly at 12 h and 24 h after the challenge with WSSV (Fig. 6A and B). The expression of *crustin-like* was down-regulated significantly at 12 h and 24 h after the challenge with *V. alginolyticus* (Fig. 6A and B).

4. Discussion

Most known immune genes of crustaceans support antibacterial immunity but not antiviral immunity. On the contrary, many known immune genes of crustaceans will be exploited by powerful viruses like WSSV to facilitate the replication of viruses. In addition, WSSV could adjust the expression of immune genes at different time intervals

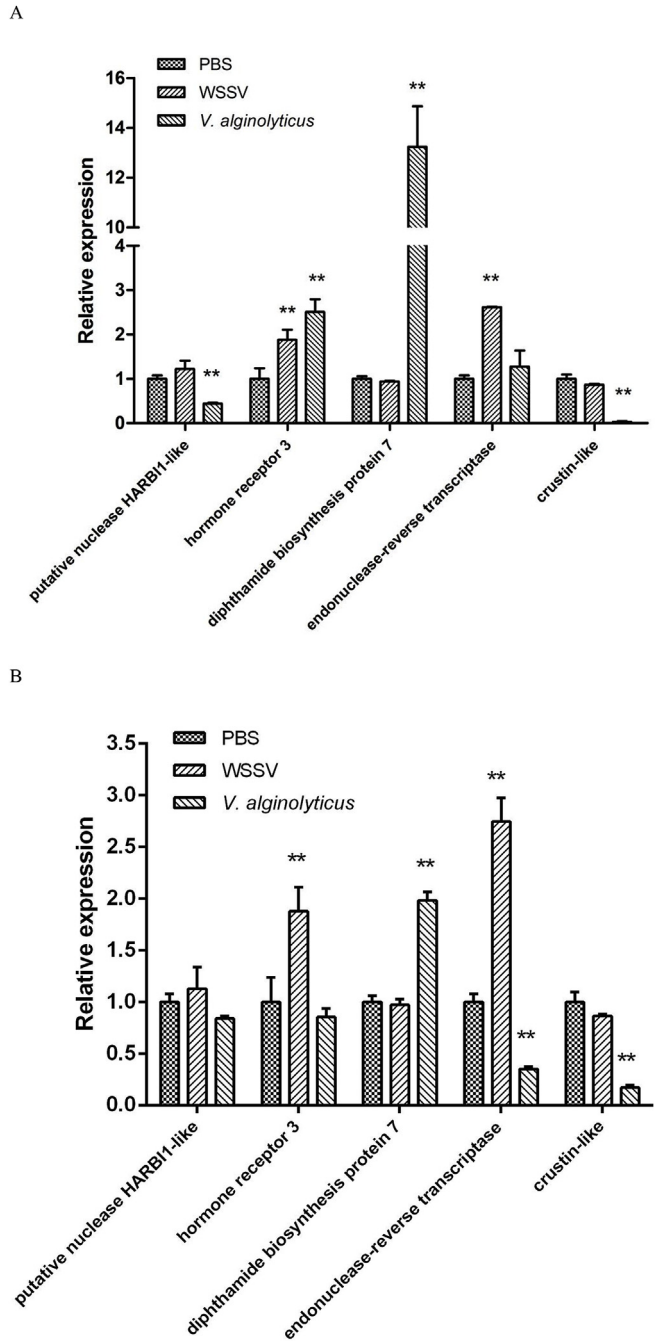

Fig. 6. The expression of the selected genes in *S. paramamosain* hemocytes in response to WSSV or *V. alginolyticus*. At 12 h (A) and at 24 h (B) post-challenge. Data are shown as the means ± standard deviation (SD) of three separate individuals. Double asterisks indicate a significant difference ($P < 0.01$) between two samples.

according to the needs of its different life cycles. Therefore, the expression of the same gene in two transcripts shows different changes in gene expression, which is a normal phenomenon.

In eukaryotic genomes, an endonuclease-reverse transcriptase is a form of protein containing an RT_nLTR_like domain which has the function of reverse transcriptase, a multifunctional enzyme with RNA-directed DNA polymerase, DNA-directed DNA polymerase, and ribonuclease hybrid (RNase H) activities [20]. We found that *endonuclease-reverse transcriptase* expression was up-regulated significantly after challenges with WSSV in this study. The endonuclease-reverse transcriptase may promote the immune response against *V. alginolyticus* by regulating phagocytosis, SOD activity and PO activity in shrimp [21]. HARBI1 proteins may act as DNA nucleases involved in crucial cellular functions, including possible DNA rearrangements of the genome [22]. Therefore, HARBI1 functions are expected to be quite general and universally important. HARBI1 mRNAs are expressed in a wide variety of adult and embryonic tissues. Such a diverse set of tissues expressing HARBI1 at different developmental stages implies that this gene is of paramount biological importance. HARBI1-like was found to play an important role in the progression of WSSV and *V. alginolyticus* infection in shrimp [23]. In this study, the expression of *putative nuclease HARBI1-like* was up-regulated significantly after WSSV challenge but was down-regulated after the *V. alginolyticus* challenge. Diphthamide, the target of diphtheria toxin (DT) and Pseudomonas exotoxin A (ETA), is a posttranslational derivative of histidine that exists in eEF-2 [24]. Diphthamide biosynthesis protein 7, also referred to as WDR85, contains WD40 domains which are known to mediate protein-protein interactions, and is a scaffold protein involved in the third step which converts methylated diphthine to diphthine so that diphthamide biosynthesis protein 6 can create diphthamide [25]. The diphthamide biosynthesis protein 7 may promote the *anti*-WSSV immune response of shrimp by regulating apoptosis, SOD and PO activity, and can influence the progression of WSSV infection in shrimp [26]. In the present study, the expression of *diphthamide biosynthesis protein 7* was found to be up-regulated significantly after *V. alginolyticus* challenge but not changed significantly by the WSSV challenge. The nuclear hormone receptor (NHR) is a ligand-regulated sequence-specific transcription factor that may activate or repress gene expression [27,28]. Generally, the mammalian NHR functions to regulate a variety of cellular processes including the mevalonate pathway, apoptosis, T-cell response, and development-induced autoimmune disease, secondary development of lymphoid organs, and the immunity response in some non-immune tissues [29]. *Vibrio alginolyticus* could cause significant up-regulation of *hormone receptor*, which functions in an anti-apoptosis and anti-inflammation manner to prevent shrimp death caused by an over-load of immunity responses [30]. The expression of *hormone receptor 3* was up-regulated significantly by the WSSV challenge but up-regulated significantly only at 12 h after the *V. alginolyticus* challenge. Therefore, we confirmed that the metabolic pathway was the most important pathway in the process of either WSSV or *V. alginolyticus* infection. Crustin-like, an antimicrobial peptide, has various biological activities, including activities that are antimicrobial, protease inhibitory, and immunoregulatory during recovery from wounding or physiological stress [31,32]. Crustin was found to be involved in the antimicrobial and antiviral innate immunity in shrimp and crab through regulating the innate immunity [33–35]. The expression of *crustin-like* was found to be down-regulated significantly after the *V. alginolyticus* challenge in this study. The evidence above suggested that these genes might be related to immune function and the differential expression of these genes after WSSV or *V. alginolyticus* stimulation reinforced this view.

Acknowledgments

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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.2019.06.034.

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