Fish and Shellfish Immunology 94 (2019) 497–509

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Transcriptome analysis of hemocytes from the white shrimp *Litopenaeus vannamei* with the injection of dopamine

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ARTICLE INFO

Keywords:
Litopenaeus vannamei
Hemocyte
Dopamine
Neuroendocrine-immune system
Transcriptome

ABSTRACT

As a crucial neuroendocrine-immune factor, dopamine (DA) could regulate the immune system of *Litopenaeus vannamei*. To understand the immune mechanisms and regulatory pathways of DA in *L. vannamei*, the transcriptome analysis of hemocytes of *L. vannamei* with injection of DA (10^{-6} mol/shrimp) at 3 and 12 h were performed in this study. Moreover, quantitative real-time PCR (qPCR) method was applied to validate the accuracy of transcriptome sequencing and analyze the expression pattern of candidate differentially expressed genes (DEGs) at different time points (0, 3, 6, 12, and 24 h) after DA injection. The results showed that a total of 51382 unigenes with a N50 length of 2341 bp were generated. And 1397 and 457 DEGs were obtained by comparative transcriptome at 3 and 12h respectively. Moreover, the results of functional annotation and enriched pathway showed that the DEGs were involved in phagosome (ko04145), lysosome (ko04142), Endocytosis (ko04144), and NOD-like receptor signaling pathway (ko04621). Besides, the Pearson's correlation coefficient (R) between transcriptome sequencing and qPCR was 0.845, which confirmed the reliability of the transcriptome sequencing results and the accuracy of assembly. Furthermore, the expression pattern of 15 candidate DEGs, containing 9 up-regulated and 6 down-regulated DEGs at 3 h, indicated the regulation of DA in physiological functions especially in the immune system. Therefore, these results revealed that DA induced the expressions of membrane receptors or proteins, activated intracellular signaling pathways, regulated cellular and humoral immune systems, controlled antioxidation and apoptosis, and was involved in the regulation of neuroendocrine system. These findings are helpful to promote the understanding on the effects of biogenic amines on physiological functions and regulatory networks of crustacean, and offer a substantial material and foundation for researching the immune response of crustacean.

1. Introduction

The Pacific white shrimp *Litopenaeus vannamei (L. vannamei)* stands as one of the most productive aquatic products around the world with the output of 4.16 million tons and with the commercial value of 24.4 billion dollars in 2016 according to the FAO's (Food and Agriculture Organization of the United Nations, 2018) data. However, the culture of white shrimp has been severely threatened by diseases in recent years. Many studies have reported that environmental stresses such as ammonia nitrogen, temperature, and salinity were the vital factors to shrimp diseases [1–3]. Under environmental stress, the content of secreting neuroendocrine factors such as biogenic amine (BA) would change firstly in white shrimp, and the changes of BA containing dopamine (DA) and 5-hydroxytryptamine could cause stress response, lower immunity and increase the susceptibility to pathogen, which resulted in outbreaks of diseases. More and more attention has been received in view of the important effects of DA on immune function of *L. vannamei*. And previous studies have proven that the injection of DA would lead to the changes of immune parameters such as the decrease in the total hemocyte count, phenoloxidase (PO) activity, superoxide dismutase activity, phagocytic activity and clearance efficiency [4]. The neuroendocrine-immune system of *L. vannamei* currently has become a research hotspot. Nevertheless, the mechanism of DA affecting the immune system is still unclear.

The immune defenses of crustacean wholly depend on the innate system that contains humoral immune and cellular immune [5]. As the donor of the humoral immune, hemocytes of crustacean could also carry out the cellular immune, thus playing important roles in the immune response [6]. For humoral immune, hemocytes released several immunologic active factors, in which the prophenoloxidase (proPO) system containing serine protease (SP) and other associated factors played crucial roles in humoral immune [7]. Recently, several

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https://doi.org/10.1016/j.fsi.2019.09.043
Received 31 May 2019; Received in revised form 10 September 2019; Accepted 16 September 2019
Available online 18 September 2019
1050-4648/

researchers have explored the regulatory mechanism of DA on the immune system in hemocytes of *L. vannamei*. Zhao et al. (2016) have reckoned that dopamine receptor D4 transduced the signals from DA to adenylyl cyclase (AC) and phospholipase C (PLC), which then regulated the concentrations of second messenger cAMP to control phagocytic activity, the PO activity and the expression of C-type lectin in hemocytes [8]. Moreover, it has been reported that phagocytic activity and antibacterial activity of hemocytes were reduced by DA through cyclic adenosine monophosphate (cAMP) and calmodulin (CaM) pathways [9]. Besides, DA could also induce the activation of proPO system in hemocytes through protein kinase C (PKC) signaling pathway [10]. Several studies have reported that DA regulated the immune system of shrimp, but the regulatory network is still not revealed clearly.

In recent years, the availability of transcriptome sequencing has facilitated a new analytical approach for revealing the transcriptional status of genes and the regulation of transcriptional expression at the whole level [11]. This technology has gigantic advantages in understanding the regulatory network in cells and molecular mechanism [12,13], which has been used to research on aquatic animals, including *Gadus morhua* [14], *Oratosquilla oratoria* [15], *Palaemonetes sinensis* [16], *Fenneropenaeus merguiensis* [17], *Gecarcinus lateralis* [18], and *Chlamys farreri* [19]. On the one hand, several researchers investigated the response mechanism of *L. vannamei* under environmental stress via transcriptome sequencing. Under nitrite stress, the genes of hemocytes such as C type lectin 4, serine protease inhibitor 8, and superoxide dismutase were induced to provide defense against nitrite [20,21]. Zhao et al. (2015) have reported that the expressions of many genes related to immune signaling pathways, apoptosis, and energy metabolism, were differentially changed in hemocytes under salinity stress [22]. Moreover, the transcriptome analysis of muscle has also been performed, in which mitogen-activated protein kinases (MAPK) cascade was clustered and might play a significant role in cold adaptation [23]. On the other hand, several researchers have explored the regulatory mechanism of aquatic animals using the method of transcriptome analysis after injection of neuroendocrine factors. Zhou et al. (2016) have reported that Toll-like receptor (TLR) signaling pathway was enriched, the expressions of TLR3 and macrophage inflammatory protein-1β were altered after insulin injection in common carp (*Cyprinus carpio*) [24]. In addition, Zhong et al. (2016) have also noted that the expressions of multiple genes related to immune system process were changed in common carp after growth hormone injection [25]. Several studies have focused on the transcriptome analysis of aquatic animals under environmental stress or the effect of neuroendocrine factor on the immune system, but the understanding is scarce for the molecular expression pattern and pathway regulated by DA in aquatic animals, especially in *L. vannamei*.

Although several researchers have started to investigate the effects of DA on the immune system, none of them focused on the regulatory pathway and response mechanism of DA in hemocytes of *L. vannamei* through transcriptome sequencing. Consequently, to understand the response pathway and the regulatory network of DA in hemocyte of *L. vannamei*, hemocyte transcriptome sequencing and differentially expressed analysis were performed at different hours after DA injection in present study. Furthermore, quantitative real-time PCR (qPCR) were used for validating the accuracy of transcriptome sequencing and analyzing expression pattern of the candidate differential expressed genes (DEGs). The objective of this research is hopeful to promote the understanding on the response pathway and regulatory network of DA in hemocyte of *L. vannamei*, and offer a substantial material and foundation for advanced research.

2. Materials and methods

2.1. Animals and dopamine injection

Healthy *L. vannamei* (8 ± 0.5 cm) were obtained from a commercial farm in Shazikou, Qingdao, China. The shrimp were maintained in tanks (40 cm × 50 cm × 60 cm) with aerated natural seawater (30 ppt, 22 °C), under the 12:12 h light/dark circle. After one week the health shrimp at intermolt stage were randomly separated into the experimental group and the control group with triplicates, and each replicate contained 8 shrimps (n = 8). The shrimp in the experimental group was injected with 10^{-6} mol/shrimp DA individually, and the shrimp in the control group was injected with sterilizing shrimp normal saline (NaCl 0.40 mol L^{-1}, $CaCl{\cdot}2H_2O$ 0.01 mol L^{-1}, $Na_2HPO_4{\cdot}12H_2O$ 0.0004 mol L^{-1}, KCl 0.009 mol L^{-1}, $MgCl_2{\cdot}6H_2O$ 0.02 mol L^{-1}, Tris 0.035 mol L^{-1}, pH 7.45, osmolality 780 mOsm kg^{-1}). The hemolymph was drawn from the first abdominal segment of the shrimp at 0, 3, 6, 12, and 24 h after injection. The hemolymph samples were centrifuged at 700 × *g* for 10 min at 4 °C.

2.2. cDNA library construction and sequencing

According to the previous experience in our laboratory, total RNA from hemolymph drawn at 0, 3, 6, 12 and 24 h after injection was extracted using TRIzol Reagent (TransGen China) and following the manufacturer's procedure. For the experimental group and the control group, the total RNA from three biological replicates at 0, 3, 6, 12, and 24 h were used for qPCR with three technical replicates respectively, and two biological replicates of total RNA at 0, 3, and 12 h were chosen for RNA-Seq randomly. Therefore, ten sequencing libraries were constructed in this study, four libraries (D3a, D3b, D12a, D12b) from the experimental group and six libraries (C0a, C0b, C3a, C3b, C12a, C12b) from the control group. The total RNA integrity was estimated using Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number > 7.0. Approximately 10 μg of total RNA was subjected to isolate Poly (A) mRNA with poly-T oligo attached magnetic beads for purifying (Invitrogen). The poly(A)- or poly(A)+ RNA fractions were then interrupted into small fragments using divalent cations under elevated temperature. The RNA fragments were reverse-transcribed to create the final cDNA library following the protocol for the mRNA-Seq sample preparation kit (Illumina, San Diego, USA), and the cDNA fragments of 300 bp (± 50 bp) were used for the paired-end libraries. Then the libraries were performed the paired-end sequencing on an Illumina Hiseq 4000 according to the vendor's recommended protocol.

2.3. De novo assembly, unigenes annotation and functional classification

Cutadapt [26] and Perl scripts were used to remove the reads that contained adaptor contamination, low quality bases and undetermined bases. The sequence quality including the Q20, Q30 and GC-content was verified using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The clean data of high quality were used to de novo assembly in Trinity 2.4.0 [27]. Trinity groups transcripts were chosen to generate the 'gene' sequence based on shared sequence content.

All unigenes sequences were aligned against the non-redundant (Nr) protein database (http://www.ncbi.nlm.nih.gov/), Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/), Gene ontology (GO) (http://www.geneontology.org), SwissProt (http://www.expasy.ch/sprot/), and eggNOG (http://eggnogdb.embl.de/) databases using DIAMOND [28] with a threshold of E-value < 10^{-5}.

2.4. Differentially expressed unigenes analysis

Salmon [29] was used to identify the expression levels for unigenes by calculating TPM [30]. The differentially expressed unigenes were chosen with the absolute value of log2 (fold change) > 1 and with statistical significance (p value < 0.05) by R package edgeR [31]. Next, GO and KEGG enrichment analysis were again performed on all differentially expressed unigenes by Perl scripts in house.

Table 1
The sequences of RT-PCR primers.

Annotation	Forward primer (5′-3′)	Reverse primer (5′-3′)
C-type lectin 1(CTL1)	AGATGTGTCAAGCCCAAGGAG	GGAAAAAGAAGTCAGGCGTGTA
scavenger receptor B1 (SR)	CTGCTTCGGACTGGCTTAGG	AACCCGCACCAAAATACCC
HSP90 domain containing protein (HSP90)	TTCAATCCGCCGAAAGTAGA	TTCCCCTCACCCTAACCCTCT
dual specificity MAP kinase phosphatase (MAPKP)	GGCAGAGCATAGAATCGCACA	CCACCCGAAGCCAGACATT
tubulin beta chain isoform X1 (tubulin)	GGAAACAACTGGGCTAAGGG	TGTAAGGCTCAACGACGGTATC
kinesin-like protein KIF2A isoform X1(kinesin)	TCATTTGCCCTTCCGTGC	CACCCAACTCCTTAACCCGA
cathepsin D-like protein (CAT)	GGGGCATTTGACGGCAT	AGAAGGAGAACACGGGCTGG
serine proteinase (SP)	AGACTGCTTTCGCTCCTTGC	ATGCCTCCGTTATCCCCTG
Kazal-type serine proteinase inhibitor 3 (SPI)	CCAAGAGTCCTGCGATTTCC	CAGAACGCAGAGATTCGGATA
nicotinic acetylcholine receptor subunit alpha 12 (NAR)	GCAGTAACCAGCGAAGAATCC	CCAGCAGAACATTTCAATAGCG
copper/zinc superoxide dismutase isoform 2 (SOD)	CTGACCCTGACGCAGAGCAA	CTGGTGGATGTGAAAGCCG
glutathione peroxidase-like isoform X2 (GPx)	ACAACTTTGAGATCCTGGGCTT	CAGCGGGTGTTCATTCTCG
caspase-2 (CAS2)	CACTCGTCACTCACGGCTCA	AAGAACACTCCTGCCATTTACACT
neprilysin-11-like (NEP)	CTGACAACGGCGGTTTAAGA	TGGGGACCAGTACACGGAA
juvenile hormone esterase-like carboxylesterase 1 (CXE)	AGGGCTGAAGGACCAGACAT	TGGCTCGCTTGAACAAACC
β-actin	TGGACTTCGAGCAGGAGATG	GGAATGAGGGCTGGAACAGG
EF-1α	GTATTGGAACAGTGCCCGTG	ACCAGGGACAGCCTCAGTAAG

2.5. Expression pattern analysis and validation using quantitative real-time PCR

To analyze the expression pattern and affirm the transcriptome sequencing results, 15 candidate DEGs were selected for confirmation by qPCR based on the results of the functional annotations and the genes we are interested in, and the gene-specific primers were designed using Oligo 7.37 (Table 1). Housekeeping genes β-actin and EF-1α were the internal reference genes. Total RNA from hemocytes of shrimp in the experimental group and the control group at 0, 3, 6, 12, and 24 h were extracted using Trizol method (TransGen, China) with three replicates, and the RNA sample were reverse-transcribed utilizing SMARTer™ PCR cDNA Synthesis Kit (Clontech, USA). The oligonucleotide primers were tested by qPCR with SYBR® PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China). The RT-PCR reaction mixture (10 μl) was performed by using PikoReal 96-well Real-Time PCR System (Thermo Scientific) in triplicates, which contains 3.6 μl sterile water, 0.2 μl each of forward and reverse primers (10 μM), 5 μl SYBR premix Ex Taq™ (2 ×) and 1ul of cDNA. The relative expression ratio (R) was calculated from the equation: $R = (E_{target})^{\Delta Cp\ target\ (control\text{-}sample)}/(E_{ref})^{\Delta Cp\ ref\ (control\text{-}sample)}$ [32]. PCR efficiency(E) was determined by running standard curves for 10-fold serial dilutions of cDNA templates, and was calculated according to $E = 10^{[-1/slope]}$ [33]. For all standard curves, the primer amplification efficiencies of genes were 96.7–99.6% and $0.983 < R^2 < 0.997$ respectively.

3. Results

3.1. Sequencing and de novo assembly

Ten cDNA libraries from *L. vannamei* were sequenced on Illumina HiSeq 4000 platform. A total of 536244704 raw reads were generated, then 523080972 clean reads were left after removing adapters as well as filtering the low-quality sequences. Thereinto, 209225294 clean reads were generated from 214428512 raw reads at 3 h, and 210427774 clean reads were generated from 215524058 raw reads at 12 h. The whole de novo assembly reads from ten libraries yielded a total length of 55497257 bp with 51382 number of unigenes and a N50 length of 2341 bp. The clean read data were deposited to the US National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra) with the accession No. SRP132193. A detailed summary of the sequencing and assembly results was shown in Table 2.

Table 3
Summary of function annotation of *Litopenaeus vannamei*.

Database	Number of annotated unigenes	Ratio (%) of annotated unigenes
All	51382	100.00
GO	11617	22.61
KEGG	7363	14.33
Pfam	11205	21.81
swissprot	10751	20.92
eggNOG	13191	25.67
NR	13374	26.03

3.2. Functional annotation and classification of unigenes

All the 51382 unigenes were searched against the Nr protein, Pfam, Swissprot protein, KEGG, GO and eggNOG database with E-value threshold of 1E-5. Among them, 100% unigenes had at least one significant match to an existing gene model, and 13191 and 13374 unigenes had hits by the eggNOG and Nr databases, the percentage of annotated unigenes were 25.67% and 26.03%. The detailed results were summarized in Table 3. Moreover, 28.03% of the unigenes sequences were homologous to genes of *Hyalella azteca*, and 4.73% of the unigenes sequences were homologous to genes of *Zootermopsis nevadensis* (Supplementary Fig. S1).

Gene ontology (GO) analysis is a comprehensive system of gene function classification that has three main ontologies to describe molecular functions, cellular components, and biological process. Among the 51382 unigenes, 11617 unigenes were annotated and classified into three main functional categories according to GO analysis: biological process (52.54%), cellular component (22.47%), and molecular function (24.99%) (Fig. 1A). Within biological process, regulation of transcription, DNA-dependent (GO:0006355), biological_process

Table 2
Summary of assembly results of *Litopenaeus vannamei*.

Index	All	GC%	Min Length	Median Length	Max Length	Total Assembled Bases	N50
Transcript	89428	42.88	201	518.00	32812	105742085	2480
Gene	51382	42.74	201	457.00	32812	55497257	2341

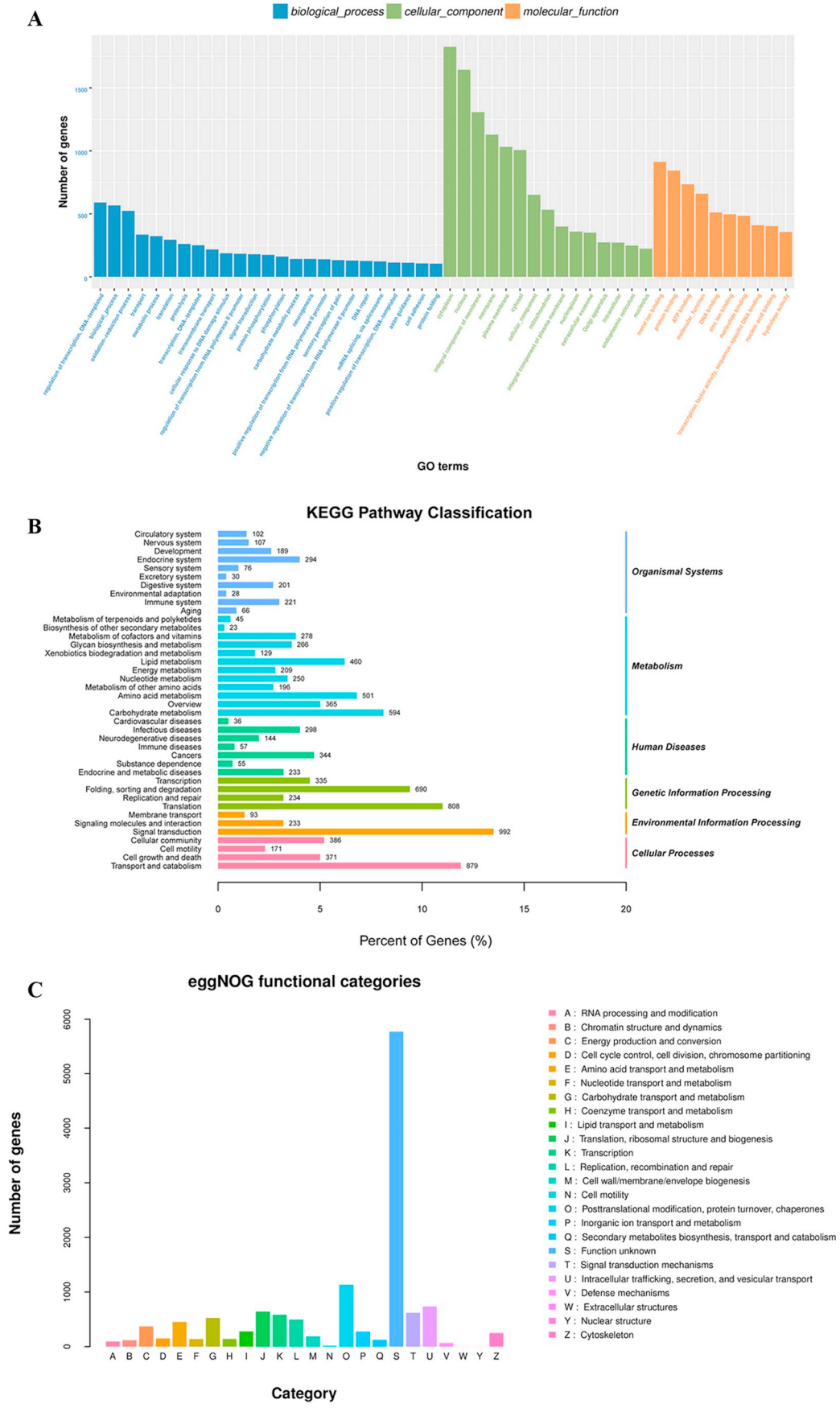

Fig. 1. Functional enrichment of unigenes. (A) Gene ontology (GO) classifications of unigenes in Litopenaeus vannamei. Three major GO categories were enriched: biological process, cellular component, and molecular function. (B) KEGG pathway classifications of unigenes in Litopenaeus vannamei. Unigenes were assigned to 6 special KEGG pathways, including Organismal Systems, Metabolism, Human Diseases, Genetic Information Processing, Environmental Information Processing, and Cellular Processes. (C) eggNOG function classifications of unigenes in Litopenaeus vannamei. Each bar represents the number of unigenes classified into each of the 26 KOG functional categories.

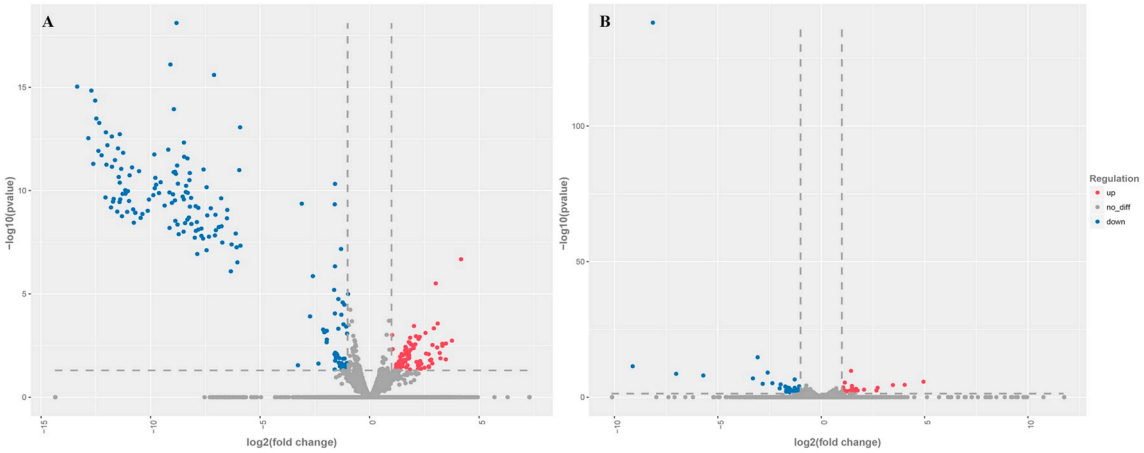

Fig. 2. Volcano diagram of differentially expressed genes in *Litopenaeus vannamei*. The x-axis indicates the fold change and the y-axis indicates the statistical significance of the differences. (A) Volcano diagram of differentially expressed unigenes in group X3-1 vs. X3-0. (B) Volcano diagram of differentially expressed unigenes in group X12-1 vs. X12-0.

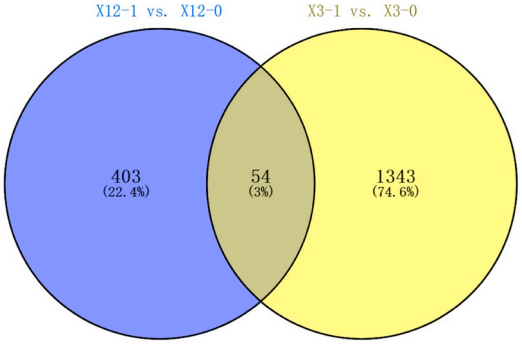

Fig. 3. Venn diagram of differentially expressed unigenes between two compare groups.

(GO:0008150), and oxidation-reduction process (GO:0055114) were the dominant subcategories. Meanwhile, cytoplasm (GO:0005737), nucleus (GO:0005634), and integral component of membrane (GO:0016021) were significantly enriched in the cellular component categories. Among the molecular function terms, unigenes were mainly assigned to metal ion binding (GO:0046872), protein binding (GO:0005515), and ATP binding (GO:0005524).

Using KEGG, a total of 7363 unigenes were mapped to six specific pathways, including organism system, metabolism, human diseases, cellular processes, genetic information processing and environmental information processing (Fig. 1B). Of these unigenes, the largest categories were signal transduction (992 unigenes), followed by Transport and Catabolism (879 unigenes), translation (808 unigenes), and Folding, Sorting and Degradation (690 unigenes). In addition, 294 unigenes were related to endocrine system, and 221 unigenes were related to immune system.

For eggNOG annotation, a total of 13191 unigenes were assigned to 25 eggNOG categories (Fig. 1C). Excluding Function unknow (5780 unigenes), the largest groups were Posttranslational modification, protein turnover, chaperones (1139 unigenes), followed by Intracellular trafficking, secretion, and vesicular transport (733 unigenes), Translation, ribosomal structure and biogenesis (645 unigenes), and Signal transduction mechanisms (636 unigenes).

3.3. The analysis and functional annotation of the differentially expressed genes

In this study, the different analysis methods were as follows: the experimental group X3-1 was compared with the control group X3-0 at 3 h (X3-1 vs. X3-0), and the experimental group X12-1 was compared with the control group X12-0 at 12 h (X12-1 vs. X12-0). In total, 1397 significantly DEGs were found in group X3-1 vs. X3-0, containing 453 up-regulated genes and 944 down-regulated genes (Fig. 2A). For GO analysis, membrane (GO:0016020), transport (GO:0006810), and protein binding (GO:0005515) were the dominant terms in cellular component, biological process, and molecular function respectively (Supplementary Fig. S2). Carbon metabolism (ko01200), Biosynthesis of amino acids (ko01230), and ABC transporters (ko02010) were main clusters in KEGG analysis (Supplementary Fig. S3). While a total 457 significantly DEGs were identified in group X12-1 vs. X12-0, of which 139 up-regulated genes and 318 down-regulated genes (Fig. 2B). Through GO analysis, molecular_function (GO:0003674), nucleus (GO:0005634), and proteolysis (GO:0006508) were the main terms in molecular function, cellular component and biological process respectively (Supplementary Fig. S4). And for KEGG analysis, Glycolysis/Gluconeogenesis (ko00010), Neuroactive ligand-receptor interaction (ko04080), and Influenza A (ko05164) were principal categories (Supplementary Fig. S5). Besides, the immune-regulated pathways, such as MAPK signaling pathway (ko04010), PI3K-Akt signaling pathway (ko04151), and Phosphatidylinositol signaling system (ko04070), were enriched in both two compared groups, while NOD-like receptor signaling pathway (ko04621) and Wnt signaling pathway (ko04310) were just enriched in group X12-1 vs. X12-0.

Moreover, DEGs were identified via Venn diagram (Fig. 3). 1343 unigenes were only expressed at 3 h, consisting of 426 unigenes up-regulated genes and 917 down-regulated genes. Among them, metabolic process (GO:0008152), membrane (GO:0016020), and protein binding (GO:0005515) were the dominant subcategories in biological process, cellular component and molecular function respectively with GO analysis (Fig. 4A). Carbon metabolism (ko01200), Two-component system (ko02020), and Biosynthesis of amino acids (ko01230) were the largest class in KEGG pathway (Fig. 5A). Besides, 403 unigenes were only expressed at 12 h, of which had 127 up-regulated genes and 276 down-regulated genes. Among the unigenes, negative regulation of transcription from RNA polymerase II promoter (GO:0000122), nucleus (GO:0005634), and protein binding (GO:0005515) were the primary subcategories in biological process, cellular component and molecular function respectively according GO analysis (Fig. 4B). Glycolysis/Gluconeogenesis (ko00010), Neuroactive ligand-receptor interaction (ko04080), and Lysine degradation (ko00310) were the largest categories in KEGG pathway (Fig. 5B). As well, 54 unigenes were expressed both at 3 and 12 h, including 23 unigenes that up-regulate at 3 h but down-regulate at 12 h, while 13 unigenes down-regulated both at 3 and 12 h. Thereinto, proteolysis (GO:0006508), extracellular region

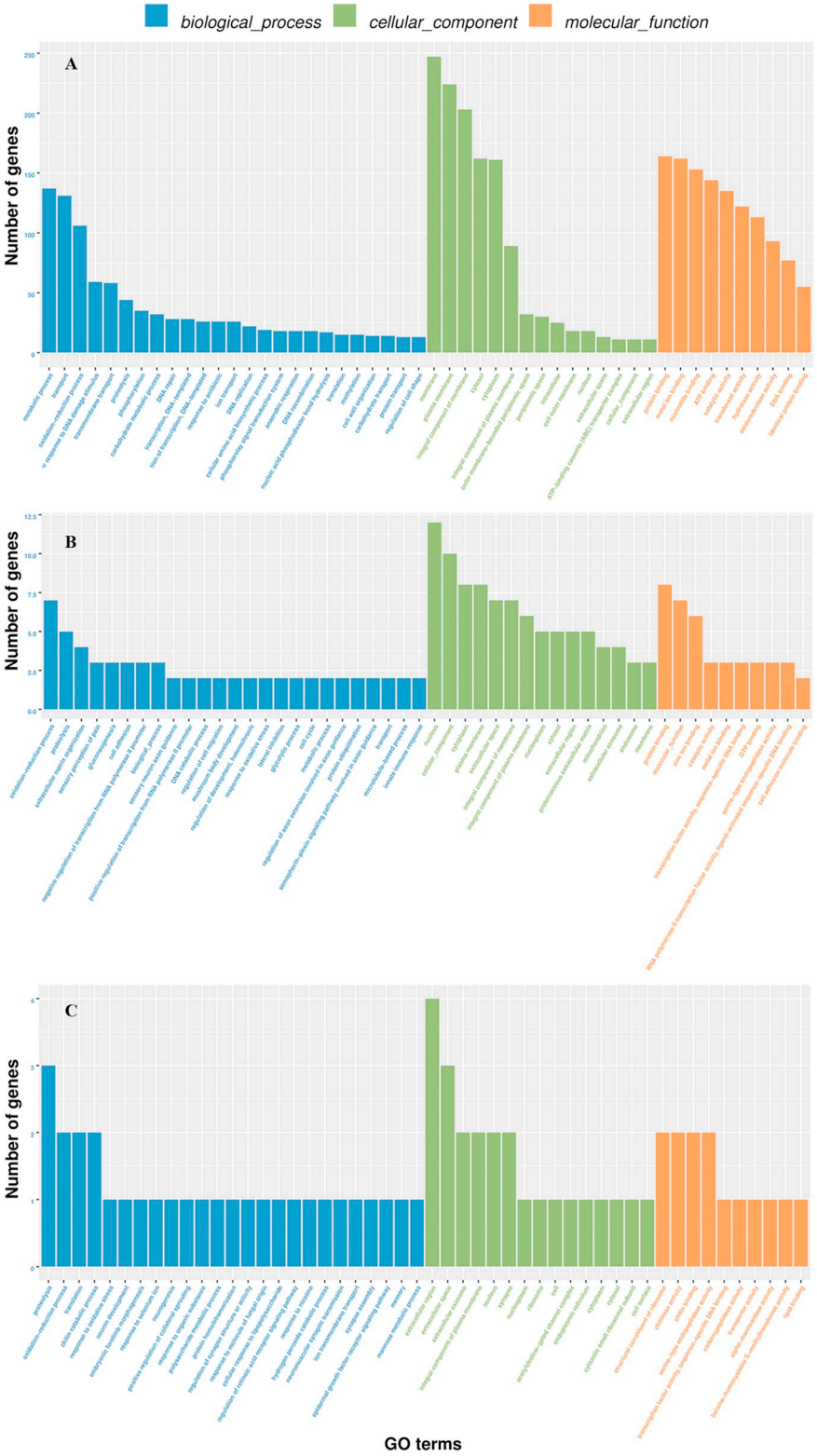

Fig. 4. GO enrichment analysis of differentially expressed genes. Three major GO categories were enriched: biological process, cellular component, and molecular function. The x-axis indicates the GO terms and the y-axis indicates enrichment score. (A) GO enrichment analysis of differentially expressed genes that were only expressed at 3 h. (B) GO enrichment analysis of differentially expressed genes that were only expressed at 12 h. (C) GO enrichment analysis of the unigenes that were expressed in both 3 h and 12 h.

(GO:0005576), and structural constituent of ribosome (GO:0003735) were the dominant categories in biological process, cellular component and molecular function respectively based on GO analysis (Fig. 4C). Meanwhile, neuroactive ligand-receptor interaction (ko04080), amino sugar and nucleotide sugar metabolism (ko00520), and lysosome (ko04142) were significantly enriched in KEGG pathway (Fig. 5C).

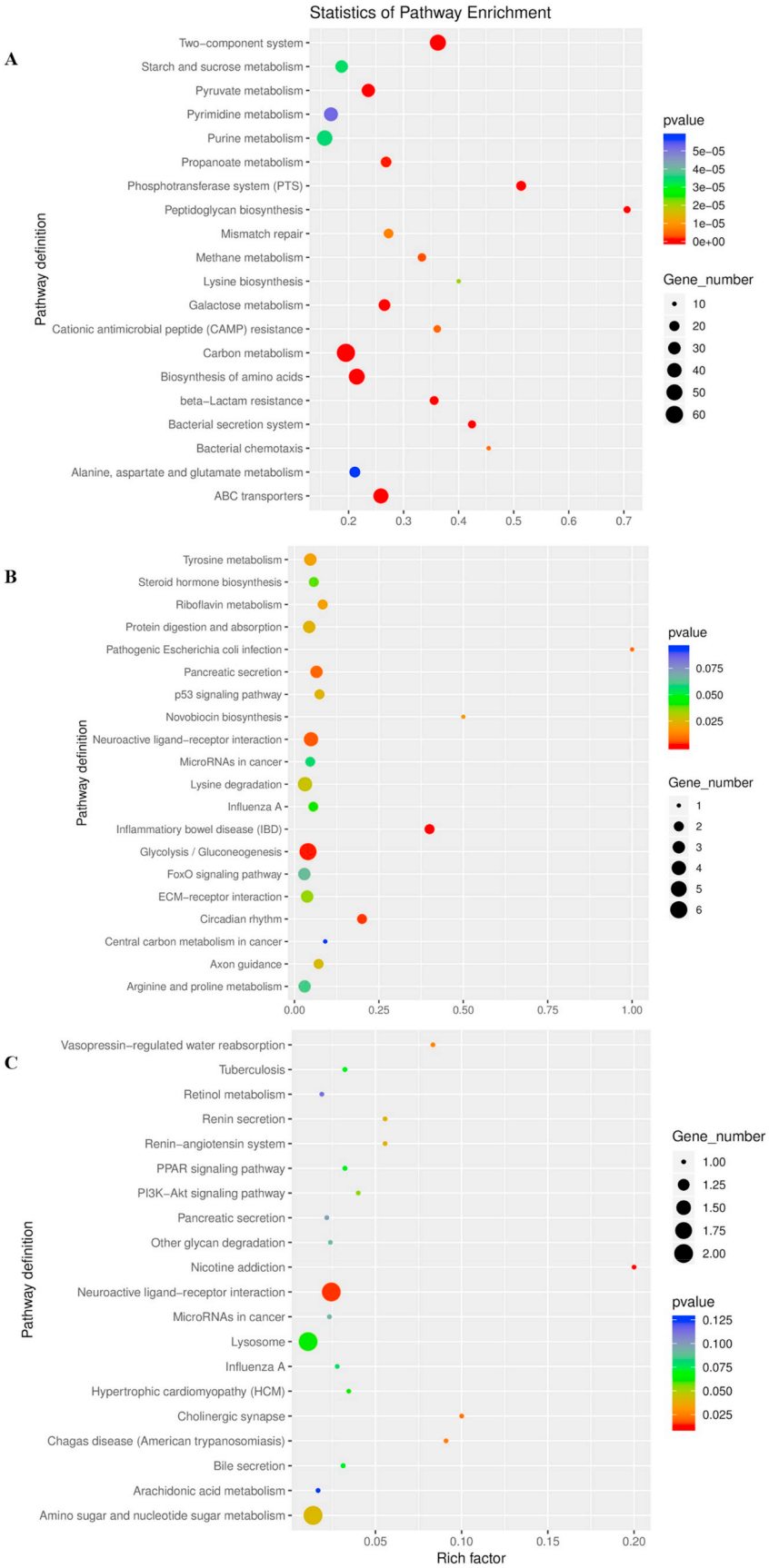

Fig. 5. KEGG enrichment pathway of differentially expressed genes. The x-axis represents the corresponding enrichment factor and the y-axis represents the pathway name. (A) KEGG enrichment pathway of differentially expressed genes that were only expressed at 3 h. (B) KEGG enrichment pathway of differentially expressed genes that were only expressed at 12 h. (C) KEGG enrichment pathway of the unigenes that were expressed in both 3 h and 12 h.

Table 4
The differential expressed genes about immune function, antioxidation, apoptosis, and neuroendocrine system.

function/gene	3h	12h
	up/down	up/down
immune function		
C-type lectin 1	up	–
C-type lectin	up	
C-type lectin 2	up	–
C-type lectin 5	up	–
mutant C-type lectin	up	–
scavenger receptor B1	up	–
tetraspanin-like protein CD9	up	–
C-type mannose receptor 2-like	up	–
leucine rich repeat only protein	up	–
HSP90 domain containing protein	down	–
dual specificity MAP kinase phosphatase	up	–
kinesin-like protein KIF2A isoform X1	down	–
tubulin beta chain isoform X1	down	–
alpha-2-tubulin	down	–
cathepsin D-like protein	up	–
cathepsin l	up	–
lysosomal alpha-mannosidase-like	up	down
legumain	up	down
beta-galactosidase-1-like protein 2	up	–
Beta-hexosaminidase subunit beta, partial	down	–
N-sulphoglucosamine sulphohydrolase-like	down	–
serine proteinase	down	down
serine proteinase homologue, partial	down	–
clip domain serine proteinase 2	down	–
Kazal-type serine proteinase inhibitor 3	up	–
peroxisomal sarcosine oxidase-like	down	–
nicotinic acetylcholine receptor subunit alpha 12	up	down
fibrinogen-like protein	up	down
antioxidation		
copper/zinc superoxide dismutase isoform 2	down	–
glutathione peroxidase-like isoform X2	down	–
putative oxidoreductase YgcW	down	–
apoptosis		
caspase-2	up	–
neuroendocrine systom		
neprilysin-11-like	up	–
juvenile hormone esterase-like protein 1	up	–
juvenile hormone esterase-like carboxylesterase 1	up	–

3.4. Identification of DEGs related to immune function, antioxidation, apoptosis, and neuroendocrine system

In the present study, KEGG enriched analysis showed that 5 immune pathways were significantly enriched at 3 h, which included Phagosome (ko04145), Lysosome (ko04142), Endocytosis (ko04144), Hematopoietic cell lineage (ko04640), and Complement and coagulation cascades (ko04610). And 2 immune pathways were significantly enriched at 12 h, which included Lysosome (ko04142) and NOD-like receptor signaling pathway (ko04621). After 3 h of injection, C-type lectin 1 (CTL1), scavenger receptor B1, leucine rich repeat only protein, very low-density lipoprotein receptor-like, the C-type mannose receptor 2-like, tetraspanin-like protein CD9, cathepsin D-like protein (CAT), and cathepsin L were significantly up-regulated. While tubulin beta chain isoform X1, kinesin-like protein KIF2A isoform X1, serine proteinase homologue, N-sulphoglucosamine sulphohydrolase-like, serine proteinase (SP), Kazal-type serine proteinase inhibitor 3 (SPI), clip domain serine proteinase 2, and Beta-hexosaminidase subunit beta were significantly down-regulated. However, after 12 h of injection, most of immune related genes were returned to the control level, while the C-type mannose receptor 2-like, SP, clip domain serine proteinase 1, tubulin beta chain isoform X1, and putative serine proteinase inhibitor still kept down-regulated level. Moreover, nicotinic acetylcholine receptor subunit alpha 12 (NAR) were significantly up-regulated at 3 h then down-regulated at 12 h. NAR played a key role in the regulation of proinflammatory cytokine production. In addition, copper/zinc superoxide dismutase isoform 2 (SOD) and glutathione peroxidase-like isoform X2 (GPx) were significantly enriched in antioxidant pathway, while SOD was down-regulated at 3 h then up-regulated at 12 h, and GPx was down-regulated at 3 h but not significantly expressed at 12 h. Both SOD and GPx were important antioxidase, playing crucial roles in free radical scavenging, and *L. vannamei* could deal with environment stress by means of controlling the expression of antioxidase. Besides, apoptosis related gene caspase-2 (CAS2) was significantly up-regulated at 3 h while not significantly expressed at 12 h, which may induce the apoptosis of hemolymph. Furthermore, the DEGs related to neuroendocrine system, including neprilysin-11-like (NEP) and juvenile hormone esterase-like carboxylesterase 1 (CXE), were significantly up-regulated expressed at 3 h, and recovered to the control level at 12 h. Neprilysin and CXE could degrade enkephalin and methyl farnesol respectively, which could further control the body color, hemolymph glucose levels, molting and gonad development. Table 4 listed the primary DEGs related to immunity function, antioxidation, apoptosis, and neuroendocrine system.

3.5. Expression pattern analysis and validation using quantitative real-time PCR

In order to validate the transcriptome results of DEGs, 15 candidate genes were chosen to perform qPCR. Correlation between candidate DEGs and qPCR was assessed using Pearson's coefficient, which revealed that the log2(fold change) of candidate DEGs were highly correlated with the log2(fold change) of qPCR (regression $P < 0.001$, Pearson's correlation coefficient $r = 0.845$, Fig. 6). These results suggested that the transcriptome sequencing results were reliable and the assembly was accurate. The expression pattern analysis of the genes indicated that the mRNA expression levels of CTL1, SR, dual specificity MAP kinase phosphatase (MAPKP), CAS2 and CXE were significantly stimulated at 3 and 6 h. The mRNA expressions of CAT, SPI, and NEP were significantly stimulated at 3 h. The mRNA expressions of tubulin, kinesin, HSP90 domain containing protein, SOD, and GPx were significantly inhibited at 3 and 6 h. The mRNA expression of SP was significantly stimulated at 3, 6, and 12 h. The NAR mRNA expression was significantly stimulated at 3 h then inhibited at 12 and 24 h (Fig. 7**).**

4. Discussion

Environmental stress stands as a vital threat on the shrimp aquaculture, and DA is the critical response factor for environmental stress in *L. vannamei*, which received much attention. However, the regulatory mechanism of DA in *L. vannamei* is still unclear. To fill the gap on how *L. vannamei* responds to DA at the molecular level, the transcriptome of hemocytes was used to analyze the systemic gene expression and regulatory pathways after DA injection at different time points in *L. vannamei*. In this study, 1397 DEGs were generated in X3-1 vs. X3-0, while 457 DEGs were generated in X12-1 vs. X12-0, it means that there were more DEGs at 3 h than 12 h, which is probable due to the expression of DEGs returned to the control group level at 12 h. Besides, GO enrichment and KEGG analysis were used to explore the pathway of DEGs, which manifested that the DEGs were involved in the immune-related pathway such as phagosome (ko04145), lysosome (ko04142), and NOD-like receptor signaling pathway (ko04621), and the other biological functions including antioxidation, signal transduction and neuroendocrine system. Furthermore, the expression pattern and validation of candidate DEGs were performed by qPCR. The results of qPCR were consistent with the transcriptome results, which indicated that the results of transcriptome sequencing were reliable. Therefore, the transcriptome data in this study obtained many key genes and crucial pathways, which uncovered that DA induced the expressions of membrane receptors or proteins, activated intracellular signaling pathways, regulated cellular and humoral immune systems, controlled

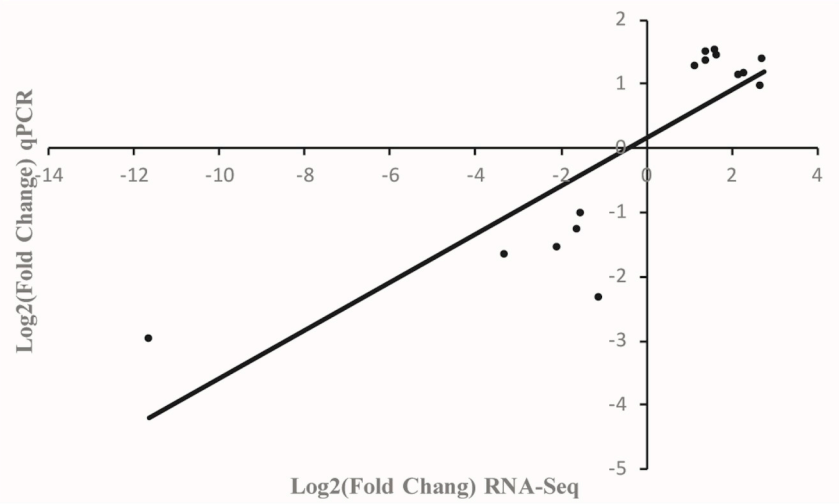

Fig. 6. Comparison of relative fold change obtained from DEGs and qPCR. **15** differentially expressed transcripts (DETs) were measured by qPCR. The regression indicates a close correlation between methods (linear regression of log2fold change: $P < 0.001$; $r = 0.845$).

antioxidation and apoptosis, and was also involved in the regulation of neuroendocrine system.

The immune system of crustaceans depends on innate immunity basically, which triggers cellular immune or humoral immunity via membrane receptors or proteins. The pathogen-recognition receptor could trigger various immune activities through intracellular signal transduction pathways. C-type lectin receptors, which belong to C-type lectin superfamily, and scavenger receptors are identified as pattern recognition receptors that could recognize pathogen-associated molecular [34]. In this study, the transcript encoding CTL1 and scavenger receptor B1 were up-regulated at 3 h, and the qPCR revealed that the expressions of CTL1 and scavenger receptor B1 were significantly stimulated at 3 and 6 h. For C-type lectin, it has been revealed that exocytosis PO activity was provoked by recombinant C-type lectin protein via cGMP-PKA pathway to involve in phagocytic activity and hemagglutinating activity in *L. vannamei* hemocytes [35]. Besides, some C-type lectin receptors can induce signaling pathways that directly activate nuclear factor kappa-B (NF-κB), while other C-type lectin receptors affect signaling by Toll-like receptors [36]. In the black tiger shrimp (*Penaeus monodon*), a novel C-type lectin was identified as a pattern recognition receptor by binding and causing bacterial agglutination [37]. Moreover, C-type lectin also could be induced under nitrite or salinity stress except pathogens [21,22]. For scavenger receptors, previous studies have proven that scavenger receptor B promotes bacterial clearance by enhancing phagocytosis, diminishes white spot syndrome virus (WSSV) proliferation, and regulates the expressions of antimicrobial peptides in shrimp [38,39]. Furthermore, the other membrane receptor or protein were also identified in this study. Leucine rich repeat only protein, tetraspanin-like protein CD9, very low-density lipoprotein receptor-like, the C-type mannose receptor 2-like, and NAR were also identified at 3 h. Leucine rich repeat only protein plays an important role in protein-protein interactions and the recognition process [40,41], as well as regulates the expression of antimicrobial peptide and participates in bacterial clearance [42]. Tetraspanin-like protein CD9 might play a crucial role in WSSV penetration and invasion [43]. Very low-density lipoprotein receptor-like is a member of low-density lipoprotein receptor family, which could participate in immune response against bacterial infection [44,45]. The C-type mannose receptor 2-like might be involved in recognizing pathogens and the phagocytosis of bacteria [46]. And it has been discovered that NAR plays a critical role in the regulation of proinflammatory cytokine production and leads to a modulation of antibody production in mammals [47]. Above results suggest that DA is a vital neuroendocrine-immune factor and functions at immunity through regulating membrane receptor or protein.

After binding the ligand, the membrane receptors regulate biological functions through intracellular signaling pathways. In this study, signal transduction pathways including NOD-like receptor signaling pathway (ko04621), MAPK signaling pathway (ko04010), phosphatidylinositol 3 kinase (PI3K)-Akt signaling pathway (ko04151), Wnt signaling pathway (ko04310), and Phosphatidylinositol signaling system (ko04070) were enriched at 3 or 12 h. The qPCR results showed that HSP90 domain containing protein, enriched in NOD-like receptor signaling pathway (ko04621), was down-regulated by DA at 3 and 6h. It has been reported that the SGT1-HSP90 pair as a chaperone maintained the correct folding status and a recognition-competent state of NOD protein [48], which contained nucleotide binding domain and leucine-rich repeats, NOD protein therefore played a crucial role in microbial recognition and innate immune response [49]. However, the leucine rich repeat only protein, probably as a part of NOD, was induced by DA at 3 h. The difference of the expression between the HSP90 domain containing protein and the leucine rich repeat only protein is probably due to NOD was not only regulated by HSP90 domain containing protein. In mammals, it has been reported that MAPK signaling pathway (ko04010) regulated cytoskeleton, especially tubulin proteins, and participated in lipopolysaccharide (LPS)-induced inflammatory responses [50]. In addition, dopamine D1 receptor improved apoptosis of osteosarcoma cells via changes of MAPK signaling pathway (ko04010) [51]. Besides, NOD1 and NOD2 could activate MAPK signaling pathway (ko04010) and transcription factors NF-κB to improve the production of proinflammatory cytokines and antimicrobial peptides, and ultimately lead to immune response and cell apoptosis [52,53], which was similarly with the expression of leucine rich repeat only protein. Transcriptome data revealed that the genes especially MAPKP in MAPK signaling pathway (ko04010) were significantly induced at 3 h, which was consistent with the qPCR results that the expression of MAPKP was actuated at 3 and 6 h, suggesting MAPKP was enhanced to participate in the immune regulation of DA. In addition, PI3K-Akt signaling pathway (ko04151) [54], Wnt signaling pathway (ko04310), and Phosphatidylinositol signaling system (ko04070) [55,56] could change the content of diacylglycerol (DAG) and inositol triphosphate (IP3), as well as improve the production of proinflammatory cytokines and macrophage motility. Thus, it could be speculated that DA probably regulates immune defense and induces apoptosis of hemocytes via several signal transduction pathways.

The crustacean immune system consists of cellular immune and humoral immune response. In this study, Phagosome (ko04145), Lysosome (ko04142), Endocytosis (ko04144), Hematopoietic cell

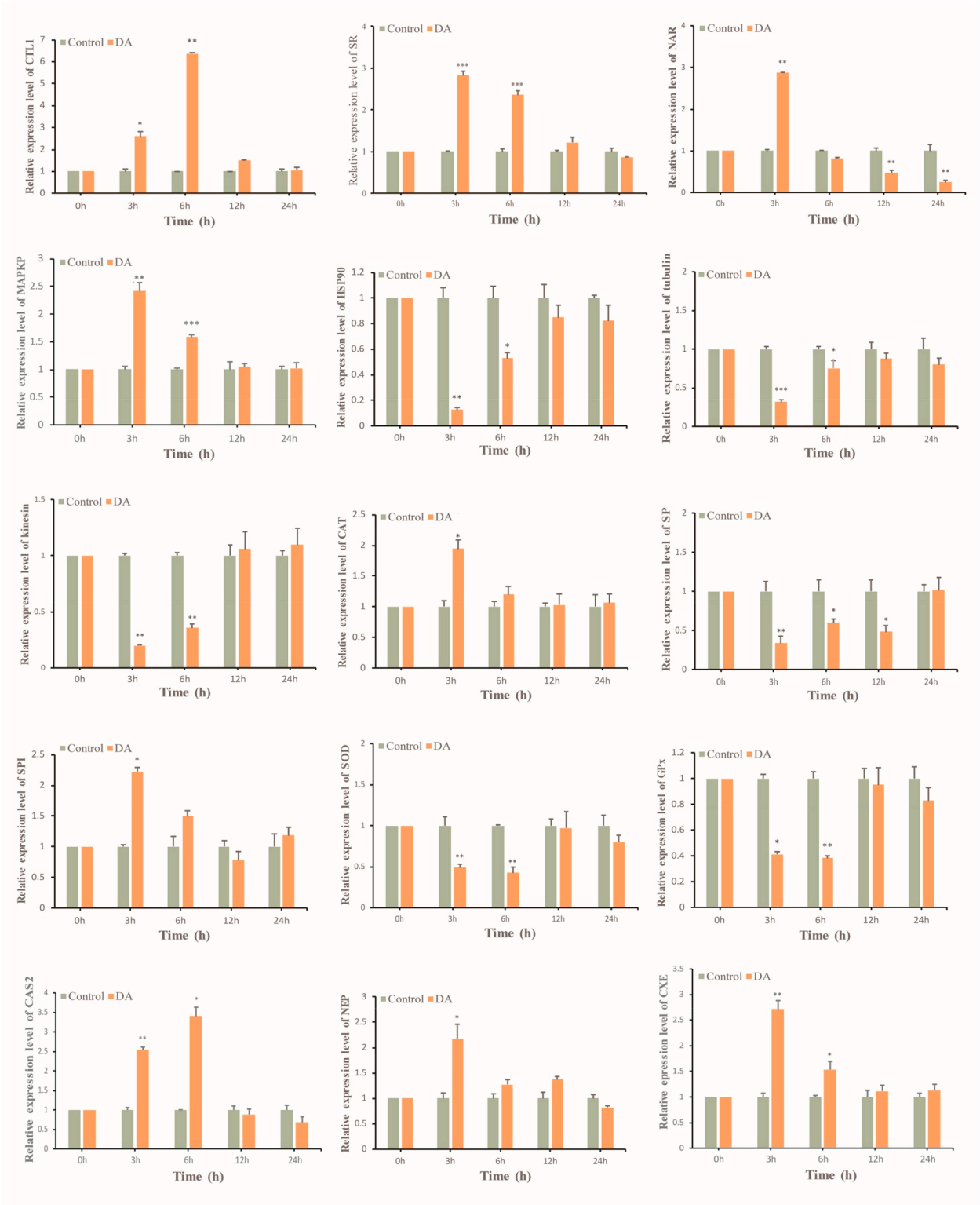

Fig. 7. Expression changes of 15 DETs after DA injection at different timepoints (0, 3, 6, 12, and 24 h). These data are expressed as the mean ± SD relative to the control. *p < 0.05 vs. control; **p < 0.01 vs. control; ***p < 0.001 vs. control.

lineage (ko04640), and Complement and coagulation cascades (ko04610) were enriched and involved in cellular immune and humoral immune response. Cellular immune, including phagocytosis, encapsulation, nodule formation and aggregation, is an important part of crustacean immune system [57,58]. Shrimp granule cells could kill the invaded pathogens via phagocytotic [59]. Microbial pathogen is engulfed to form phagosome when the pattern recognition receptors are ligated, and then phagosome fuses selectively with primary lysosomes or the product of the endoplasmic reticulum (ER) and Golgi complex to form a secondary phagolysosome [60]. It has been reported that abnormal re-organization of actin and tubulin might interfere with phagosome closure and inhibit phagocytosis [61], kinesin is also necessary

for efficient phagocytosis [62]. As same as the results of qPCR, after 3 h of DA injection, the transcript encoding tubulin beta chain isoform X1 and kinesin-like protein KIF2A isoform X1 were down-regulated, which might suggest that DA could inhibit the phagocytosis. Moreover, for the lysosome (ko04142) pathway, DA stimulated the expressions of protases containing cathepsin D-like protein, cathepsin L, and legumain. The protases including legumain, cathepsin D and L were not only involved in the degradation of protein after uptake of the exogenous particles [63], but also played crucial roles in innate immune system against bacterial or virus challenge [64–66]. After 12 h of injection, most of immune related genes were recovered to the control level. In conclusion, these phenomena might suggest that DA could regulate the phagocytosis through tubulin and kinesin.

The humoral immune genes were found beside the cellular immune system in this study. Humoral immunity includes the production of antimicrobial peptides, lectins, complement-like factors and prophenoloxidase cascade that regulates clotting or melanization of hemolymph [57,58]. Previous studies have delineated that serine proteinase plays a key role in the activation of proPO system [67,68]. In addition, the serine proteinase inhibitor (PmSERPIN3) from *P. monodon* could inhibit the activation of proPO system [69]. In the present study, serine proteinase, serine proteinase homologue, and clip domain serine proteinase 2 were significantly down-regulated while Kazal-type serine proteinase inhibitor 3 was up-regulated due to the injection of DA. Similarly, the results of qPCR revealed that SP was inhibited at 3, 6, and 12 h while SPI was induced at 3 h after DA injection. Similar conclusions were also reported by Pan et al. (2011) [70], in which the SP activity reached the minimum at 3 h after the DA injection. Therefore, these results might imply that DA is involved in humoral immune via regulating the expressions of the genes in proPO system such as serine proteinase and serine proteinase inhibitor.

Oxidative burst is one of the crucial defense strategies of crustacean against microbial invasion, but the imbalance between oxidants and antioxidants would probably lead to cellular damage. This study found that in addition to immune-related genes and pathways, antioxidant and apoptotic genes were also screened out at 3 h after DA injection. The antioxidant enzymes such as SOD and GPx were down-regulated, which were consistent with the results of qPCR that SOD and GPx were inhibited at 3 and 6 h after treatment with DA. Oxidative burst produced a large number of reactive oxygen species (ROS) against pathogen infection in crustaceans [71]. However, the massive accumulation of ROS would cause DNA damage and cell apoptosis, and for detoxifying harmful ROS, the antioxidant enzymes were produced [72]. DA inhibited the expressions of antioxidant enzymes, which might cause higher levels of ROS, and then increased the rates of hemocytes apoptosis [73]. In addition, the qPCR results showed that the expression of CAS2 was stimulated by DA at 3 and 6 h in this study, caspases are proteases that trigger programmed cell death, so CAS2 could induce apoptosis as a direct effector of the mitochondrial apoptotic pathway [74,75]. Therefore, reducing the expressions of antioxidant genes and inducing the expressions of apoptotic genes might be the one key reason for the total hemocyte count reduction, which have been also noted by Guo et al. (2013) [76]. Therefore, these results suggest that the expressions of antioxidant and apoptotic genes were controlled by DA to affect antioxidation and apoptosis.

Many researchers believe that multidirectional interactions consist in the immune, endocrine, and nervous systems. The neuroendocrine system is regulated by input from immune system and vice versa. It was noteworthy that neuroendocrine genes, NEP and CXE, were significantly up-regulated at 3 h after DA injection, which was similarly with the qPCR results that NEP was stimulated at 3 h and CXE was stimulated at 3 and 6 h. The neprilysin rapidly inactivates extracellular enkephalins to regulate the enkephalin concentration [77]. It has been reported that methionine-enkephalin produces hyperglycemia in the Estuarine Crab (*Scylla serrata)* by triggering release of crustacean hyperglycemic hormone (CHH) from the eyestalks [78], which did not contract with the conclusion that DA mediated the release of CHH into hemolymph [79], indicating that DA and enkephalin might be competitive. Besides, it has been revealed that [Met]enkephalin could stimulate the migration of immunocompetent hemocytes and reduce the adherence [80], thus it may be involved in immune mechanisms [81]. The induction of NEP protein was also similarly with the present results, thus proving immunocytes had the ability to release and control neuropeptides [80]. It is also important to note that the expression of CXE was stimulated by DA at 3 h, which was consistent with the previous study that the increase of DA levels caused an increase in JH degradation in sexually mature *Drosophila virilis* [82]. In addition, CXE could regulate physiological process such as molting, reproduction, and metamorphosis in crustacean by the degradation of methyl farnesoate (MF) [83,84]. Thus, these results prove that DA could play a role not only in immune system via controling NEP, but also in other physiological functions via regulating the expression of CXE.

5. Conclusion

The present study obtained the transcriptome data of hemocytes in *L. vannamei* after injected with dopamine. A total of 51382 unigenes were obtained, while 1397 and 457 DEGs were identified in 3 h and 12 h respectively. Moreover, based on the transcriptome data, the changes in the expression pattern of C-type lectin, scavenger receptor B1, HSP90 domain containing protein, tubulin, serine proteinase, serine proteinase inhibitor, SOD, GPx, caspase-2, neprilysin-11-like, and juvenile hormone esterase-like carboxylesterase 1 were clearly detected in hemocytes during 24 h after DA injection. Enriched pathways such as Phagosome (ko04145), Lysosome (ko04142), Endocytosis (ko04144), Hematopoietic cell lineage (ko04640), Complement and coagulation cascades (ko04610), NOD-like receptor signaling pathway (ko04621), MAPK signaling pathway (ko04010), and PI3k-Akt signaling pathway (ko04151) were also identified. In conclusion, DA inhibited the cellular immunity by inhibiting the expressions of phagocytosis-related proteins such as tubulin beta chain isoform X1 and kinesin-like protein KIF2A isoform X. Meanwhile, DA inhibited the humoral immunity by reducing the expression of serine proteinase as well as inducing the expression of serine proteinase inhibitor. It proved that DA could inhibit the immune system by inhibiting the phagocytosis-related proteins and the proPO system. Besides, DA also controlled antioxidation and apoptosis, and was involved in the regulation of neuroendocrine system. These results promoted the apprehension on the effects of biogenic amines on physiological function and regulatory network, and provided a substantial material and foundation for researching the immune response of crustacean. To enrich the immune response of DA on crustacean, the immune regulatory function of the differentially expressed genes will be explored and characterized in more details in future research, which could improve the understanding on immune regulatory network of crustacean.

Disclosure summary

The authors have nothing to disclose.

Acknowledgements

The work was supported by State Oceanic Administration Specific Public Project of China (201305005). The authors are thankful to the staff at the Laboratory of Environmental Physiology of Aquatic Animal for their help with sampling and taking care of the shrimps.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.043.

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