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SHORT COMMUNICATION

Immune related genes expression in juveniles of an invasive snail after challenged with Lipopolysaccharide

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Abstract

Invasion by *Pomacea canaliculata* is an increasing problem worldwide. Its rapid invasion and spread depends on the immune mechanism of efficient treatment of unfavorable factors and pathogenic microorganisms in the environment. Transcriptome technology is widely applied in studying the interaction of molluscs and pathogenic microorganisms and the development of disease. In this study, transcriptome technology is used to detect the expression of immune-related genes after the *P. canaliculata* juvenile snails were challenged with Lipopolysaccharide (LPS) for 48 h. A total of 402 immune-related differentially expressed genes(DEGs) were identified in comparison with the control. These DEGs involved in antigen recognition gene, immune stress and detoxification. The results showed that genes associated with detoxification metabolism were significantly up-regulated, such as cytochrome c oxidase, cytochrome b oxidase, NADH dehydrogenase, sterol 17-alpha-hydroxylase and cytochrome P450. These data will provide fundamental information for subsequent studies of the immune defense mechanisms against pathogens in the juvenile snail of *P. canaliculata*.

Key Words: Immune response; apple snail; transcriptome; innate immunity; molluscs

Introduction

Pomacea canaliculata is the only freshwater snail listed among the 100 worst invasive species worldwide (Yang *et al*., 2017). *P. canaliculata* originally distributed in South America, which now has invaded in North America, Asia and Europe (Accorsi *et al*., 2017). Its large size and taste for aquatic plants makes it a threat for causing ecological and economical damage in wetlands and crops fields(Youens and Burks, 2008; Hayes *et al*., 2012). In addition to the impact on crops, *P. canaliculata* also is the intermediate host of the nematode *Angiostrongylus cantonensis* which responsible for potentially lethal encephalitis to human being (Lv *et al*., 2009; Song *et al.,* 2016). At present there are no accepted and efficient strategies for controlling the spread of *P. canaliculata*.

P. canaliculata lack clear evidence of adaptive immunity and yet thrive in freshwater, where is rich in pathogenic bacteria, this means the *P. canaliculata* should have a perfect and effective immune system to disposal pathogens. In the past, research on bacterial challenge mainly focused on adult molluscs (Liu *et al*., 2017). Moreover, pathogens infection can also have serious consequences on mollusc's survival, especially during the juvenile stages (Genard *et al*., 2013). For instance in oysters, severe juvenile mortality was induced when they were treated with a *Vibrio* strain at the concentrations from 10^4 to 10^8 CFU individual (Lacoste *et al*., 2001); while another *Vibrio* strain, named TNEMF6, was also demonstrated to be responsible for the unexplained mortalities of juvenile oyster *C. gigas* living in Bay of Morlaix (Le *et al*., 2002). Although some progresses have been made in the study of the damage caused by pathogens on mollusc juvenile, the mechanism of pathogens that cause great harm to mollusc juvenile remains need further study. In recent years, using transcriptome sequencing can facilitate understanding the immune responsemechanisms of aquatic animals during pathogen stimulation (Dheilly *et al*., 2014), the technology has been used in *Pelodiscus sinensis* (Xu *et al*., 2016), *Biomphalaria glabrata* (Zhang *et al*., 2016)

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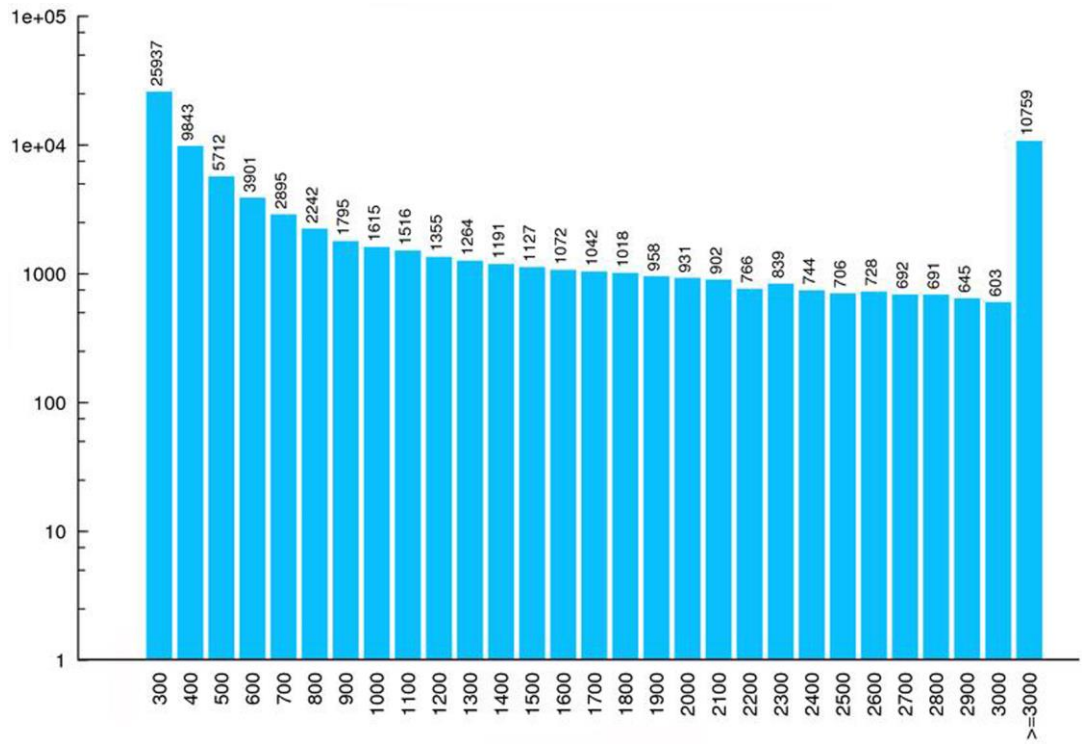

Fig. 1 The length distribution of unigenes. The X axis represents the length of unigene. The Y axis represents the number of unigene.

and *Crassostrea gigas* (Liu *et al*., 2017). The purpose of the study is to reveal the immune-related DEGs expression profiles of *P. canaliculata* juvenile snails between LPS challenged group and control, which can help us understand the immune defense mechanisms of *P. canaliculata* juvenile snails.

Materials and Methods

The fertilized eggs incubation and juvenile snail collection

Eggs were cultured in an incubator at 25 ℃ (Sun *et al.*, 2010). The hatching snails were collected to 100 mm petri dish and washed with sterile water three times. LPS purchased from Sigma (L2630-25MG). Experimental snails were bred in petri dish with 10 mg/ml LPS (LPS dissolve in sterile water), control snails cultured use sterile water. After 48 h cultured, juvenile snails were collected in the frozen tube and placed in liquid nitrogen rapidly. Finally, the samples were stored at -80 ℃ refrigerator for subsequent experiments.

Transcriptome sequencing and de novo assembly

Total RNA was extracted from juvenile snails in each group according to instruction of the manufacturer (TRIzol Reagent, ThermoFisher). Obtained samples of total RNA and use DNase I to digest DNA, after extracting total RNA and treated with DNase I, Oligo(dT) are used to isolate mRNA. The mRNA will fragment and then as templates to synthesize cDNA. The synthesized cDNA was subjected to end-repair and single nucleotide A (adenine) addition. After that, short fragments are connected with adapters. During the quality control steps, Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System are used in quantification and qualification of the sample library. Then the library is sequenced using Illumina HiSeq 4000 platform.The cutadapt used to define raw reads for which containing low-quality, adaptor-polluted and high content of an unknown base reads additionally. Clean reads of each sample were assembled use Trinity software (Haas *et al.,* 2013) and with Tgicl to cluster transcripts to unigenes. The clustered unigenes were aligned to SILVA databases to exclude those matching rRNAs of the score above 60.

Unigenes functional annotation

The unigenes were Blast (Altschul *et al*., 1990) to NT, NR, COG, KEGG and SwissProt to get the annotation function, use Blast2GO (Conesa *et al*., 2005) with NR annotation to get the GO annotation, and use InterProScan5 (Quevillon *et al*., 2005) to get the InterPro annotation. Software parameters settings are based on references.

Identification DEGs and analysis

Unigene expression analysis was clean reads mapped to unigenes using Bowtie2 (Langmead and Salzberg, 2012). Gene expression level was calculated with RSEM according to former study (Li and Dewey, 2011). DEGs detected with PossionDis according as Audic S, *et al. (*Audic and Claverie, 1997).

Table 1 Summary of functional annotation results of unigene

Database	Numbers	Percentage
Annotated in NR	10788	30.86%
Annotated in NT	11729	33.55%
Annotated in Swissprot	11830	33.84%
Annotated in KEGG	23977	68.58%
Annotated in COG	12251	35.04%
Annotated in Interpro	12544	35.88%
Annotated in GO	6925	19.81%
Annotated in all databases	2561	7.32%
Total sequences number	34963	100%

Results and Discussion

RNA-seq de novo assembly

Next-generation sequencing technology was used in the study to detect immune-related genes expression pattern of LPS challenge, and generated about 17.87 Gb bases in total after Illumina Hiseq4000 sequencing (SRA accession number SRX3029507). Then assemble all samples together. The average length is 1,080 bp and N50 is 2,231 bp. These results demonstrated that the sequencing quality was quite fine and suitable for subsequent transcriptome analysis. Length distribution of all unigenes is shown in Figure 1. Finally, RNA-Seq data had been submitted to the sequence read archive (SRA) database of NCBI with the accession number for SRX3029507.

Unigene functional annotation

The unigenes were aligned with sequences recorded in the major functional databases (NR, NT, Swissprot, COG, KEGG, GO and Interpro). A total of 34,963 unigenes were been annotated and 2,561 (7.3 %) unigenes were annotated in all databases (Table 1).

In this study, the species distribution was statistically analyzed for unigenes which annotated in NR database (Fig. 2), the highest proportion of the best Blastx hit was found for *Aplysia californica* (33.32 %), *Lottia gigantean* (22.69 %) and *Crassostrea gigas* (12.27 %). The result means more than 68.28 % of the unigenes best matched to molluscan species, despite the limited number of molluscan sequences in the public databases.

Using GO and KEGG database to analyze the functional classification of annotated sequences. Unigenes were assigned to GO database to determine their functional classifications, a total of 6,925 (19.81 %) unigenes were classified into three categories of molecular function, cellular component and biological process, comprised of 19, 19 and 25 subcategories (Fig. 3). According to the results of the GO analysis, immune-related genes involved in the response to the LPS challenge belong to the GO terms of “immune system process” and “response to stimulus” as well, and the result revealed 133 and 952 unigenes were belonged to the subcategory “immune system process” and “response to stimulus” respectively.

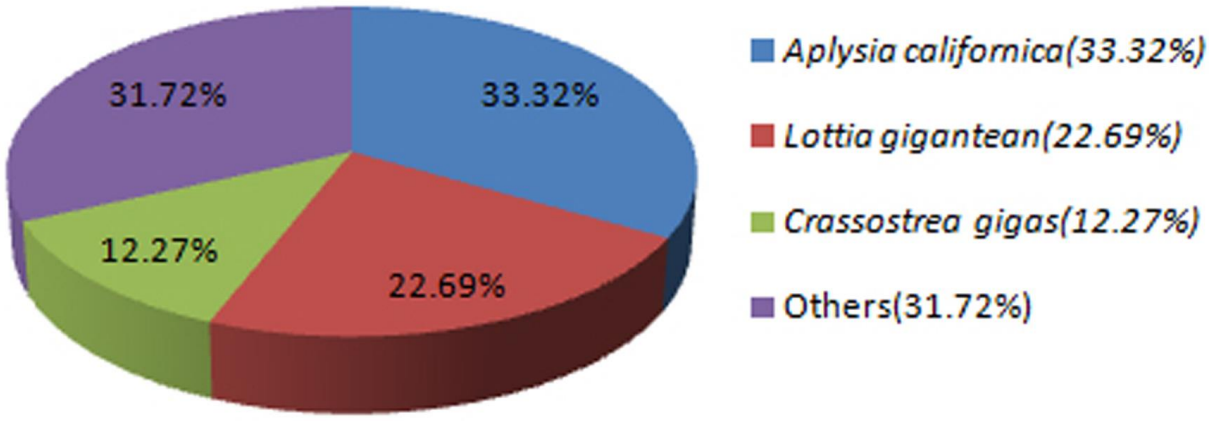

Fig. 2 Species distribution of best matched in NR database

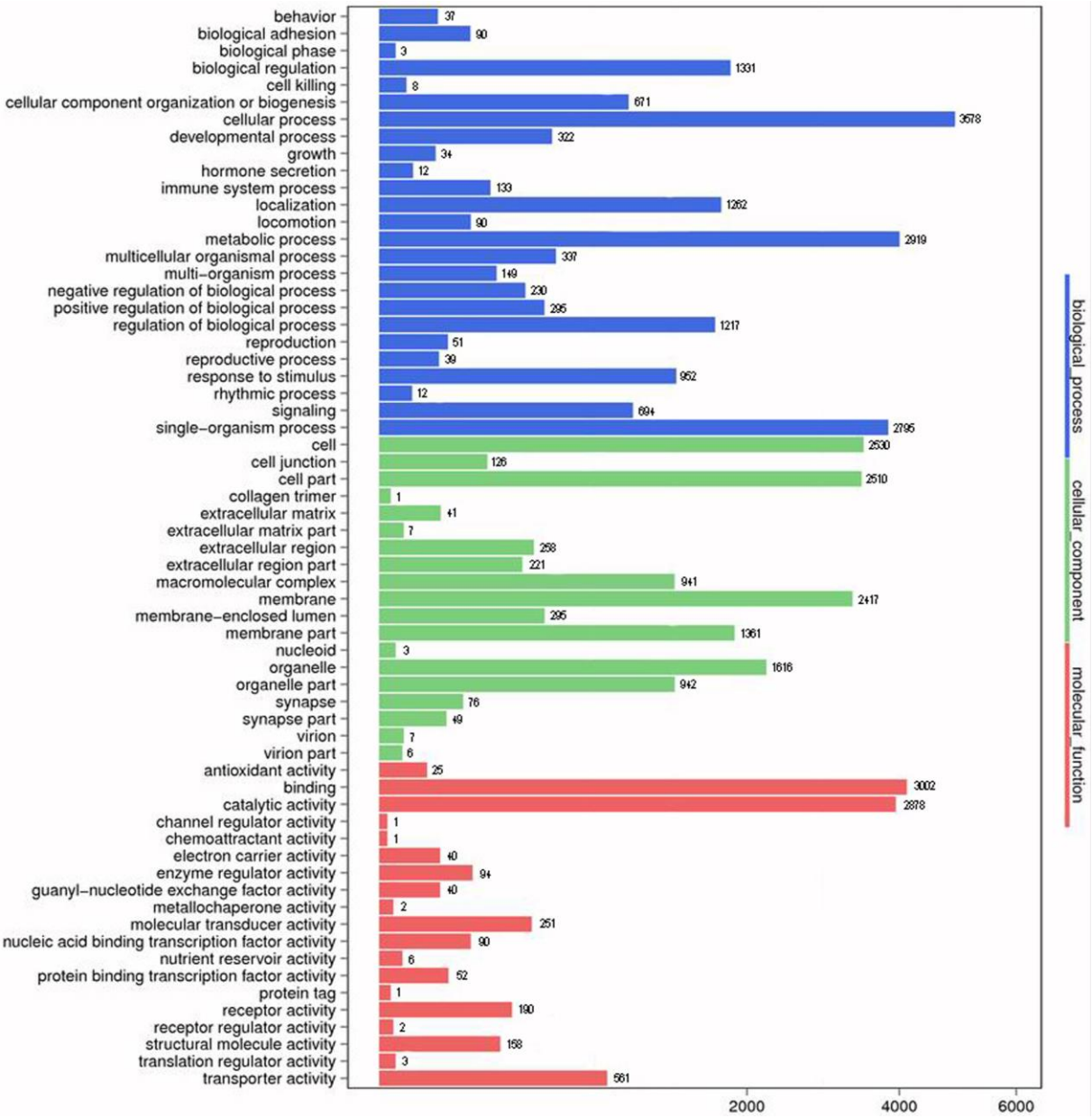

Fig. 3 Functional distribution of GO annotation. X axis represents the number of unigenes. Y axis represents the GO functional category.

To classify the biological pathways in the juvenile snail of *P. canaliculata*, the unigenes were mapped to the KEGG database, which is a bioinformatics database for systematic analysis (Kanehisa and Goto, 2000). A total of 23,977 (68.58 %) unigenes were annotated in KEGG and grouped into 32 level 2 terms (Fig. 4). Among the 32 predicted pathways, immune-related unigenes were grouped into the immune system cluster and divided into 11 immune-related pathways, including platelet activation, leukocyte transendothelial migration, hematopoietic cell lineage, chemokine signaling pathway, complement and coagulation cascades, NOD-like receptor signaling pathway, Toll-like receptor signaling pathway, cytosolic DNA-sensing pathway, RIG-I-like receptor signaling pathway, TNF signaling pathway and NF-kappa-B signaling pathway.

Immune-related DEGs analysis

In order to obtain further details on the immune response of *P. canaliculata* challenged with LPS, immune-related DEGs were analyzed according to GO and KEGG enrichment result. In the study, a total of 402 immune-related DEGs were obtained between the experimental group and the control group, including 189 genes up-regulated and 213 genes down. 54 immune-related DEGs were enriched in different functional classes of GO, such as the immune system

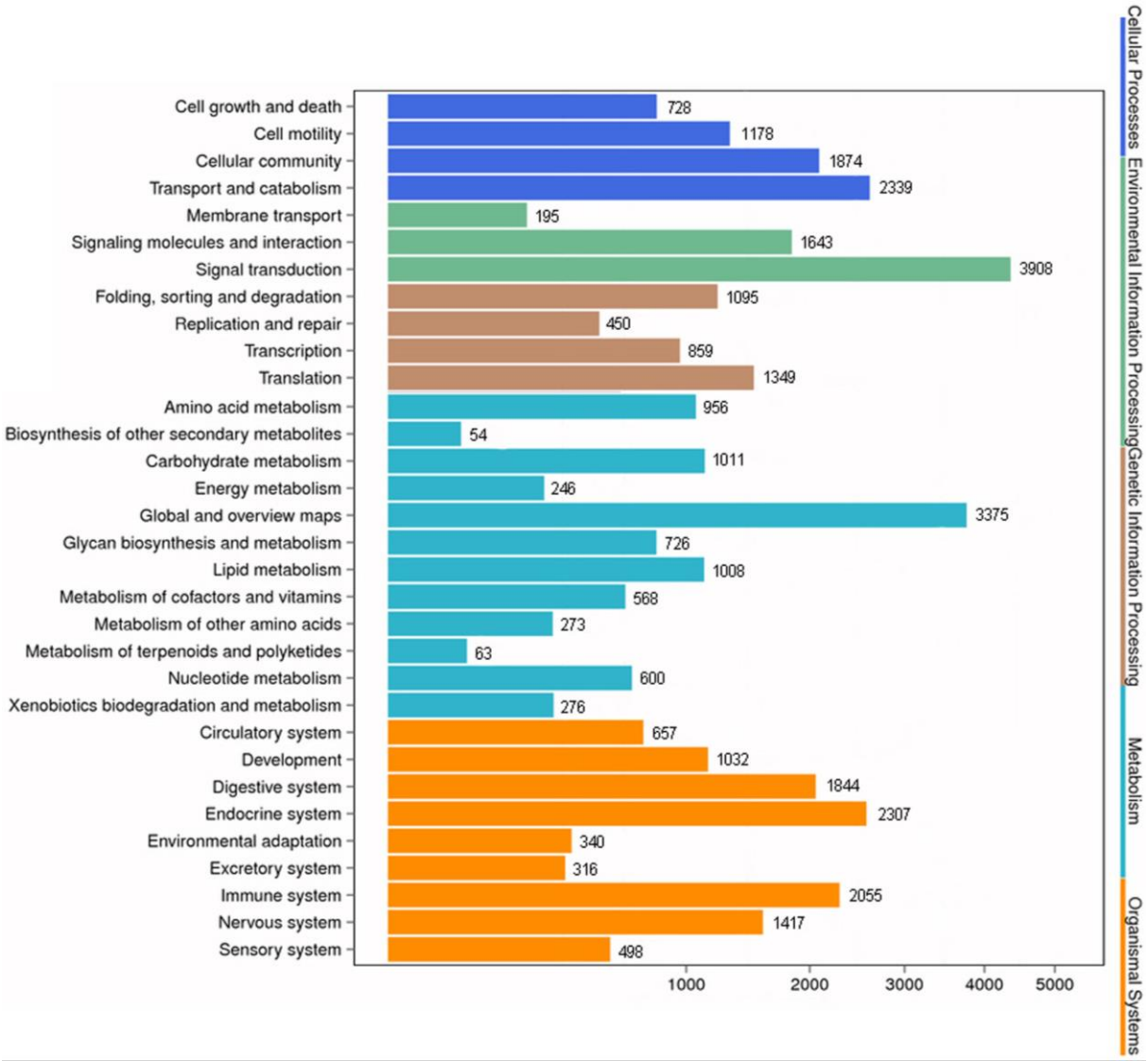

Fig. 4 Functional distribution of KEGG annotation. X axis represents the number of unigenes. Y axis represents the KEGG functional category.

Table 2 List of immune-related DEGs enrichment in KEGG pathways

Item	Pathway ID	Up-regulated	Down-regulated
Hematopoietic cell lineage	ko04640	48	67
Complement and coagulation cascades	ko04610	27	33
Platelet activation	ko04611	111	73
Leukocyte transendothelial migration	ko04670	65	103
NF-kappa B signaling pathway	ko04064	12	46
Toll-like receptor signaling pathway	ko04620	31	6
NOD-like receptor signaling pathway	ko04621	12	20
TNF signaling pathway	ko04668	42	14
RIG-I-like receptor signaling pathway	ko04622	3	14
Cytosolic DNA-sensing pathway	ko04623	10	5
Chemokine signaling pathway	ko04062	19	20

Table 3 Representative of significantly up-regulated immune related DEGs after challenge with LPS

Contig	Description	Fold change	P-value
CL4201.Contig1_All	Cytochrome c oxidase subunit II	14.24	2.54E-204
Unigene8090_All	Cytochrome b	12.23	1.36E-58
Unigene48594_All	NADH dehydrogenase subunit 4	10.90	6.46E-10
CL5717.Contig1_All	Steroid 17-alpha-hydroxylase	10.45	1.29E-84
CL5310.Contig6_All	Cytochrome P450	5.52	6.52E-28
Unigene36519_All	Fibrinogen-related molecule	7.61	5.11E-05
CL3301.Contig3_All	TNF receptor-associated factor 4	7.61	1.24E-20
Unigene23546_All	TNF receptor-associated factor 3	8.18	2.90E-17
CL4150.Contig2_All	TNF-inducible gene 6 protein	7.20	4.44E-08
Unigene25880_All	Latrophilin-2	4.57	1.18E-06
Unigene24146_All	Peptidoglycan recognition protein S1L	4.39	0
CL2173.Contig3_All	Cell division cycle protein 16-like protein	7.38	2.19E-08
Unigene15602_All	C-type lectin domain family 4	7.38	1.07E-284
Unigene25286_All	C-type lectin domain family 4 member E	9.29	1.42E-26
CL705.Contig1_All	Complement C1q-like protein 2	5.30	8.52E-21
CL4389.Contig2_All	C1q domain protein	3.21	5.71E-54
Unigene14048_All	C1q domain profile	4.65	0
CL364.Contig12_All	NF-kappa-B inhibitor-like protein 1	6.75	7.45E-07
CL2261.Contig3_All	NF-kappa-B inhibitor cactus	3.15	0
Unigene3864_All	Neurotrypsin	6.20	0
Unigene14909_All	HSP70	6.05	4.28E-19
Unigene23401_All	Apoptosis 2 inhibitor	5.73	5.20E-156
CL4290.Contig2_All	Inhibitor of apoptosis protein	2.26	3.52E-45
Unigene757_All	Baculoviral IAP repeat-containing protein 7	4.63	7.83E-43
Unigene21173_All	Baculoviral IAP repeat-containing protein 2	4.22	2.30E-109
Unigene14450_All	Dermatopontin 2	5.33	4.76E-11
Unigene5788_All	Dermatopontin 3	3.58	2.63E-24
Unigene9600_All	Toll-like receptor 2	3.45	1.30E-10
Unigene5098_All	Toll-like receptor 4	9.76	2.41E-13
CL7289.Contig3_All	Collagen alpha-1	3.89	1.05E-14
CL6048.Contig2_All	Protein AMBP	2.95	9.84E-51
CL2116.Contig6_All	Kunitz-like protease inhibitor	2.56	0

process(37), immune system development(3), response to stress(19), innate immune response(8), response to lipopolysaccharide(2). In addition, 402 immune-related DEGs were enriched into 11 KEGG database metabolic pathway subcategories, including platelet activation(184), leukocyte transendothelial migration(168), hematopoietic cell lineage(115), chemokine signaling pathway(39), complement and coagulation cascades(60), NOD-like receptor signaling pathway(32), Toll-like receptor signaling pathway(37), cytosolic DNA-sensing pathway(15), RIG-I-like receptor signaling pathway(17), TNF signaling pathway(56) and NF-kappa-B signaling pathway(58) (Table 2). Notably, many immune-related DEGs were involved in more than one function owing to their multiple roles in regulation of immune response. So the sum of each term DEGs is larger than total immune-related DEGs.

When *P. canaliculata* juvenile snails were challenged by LPS, some genes related to the

immune defense system were significant up-regulated (>2 fold change), these genes including Toll-like receptor, C-type lectin, C1q domain containing proteins, peptidoglycan-recognition proteins, fibrinogen-related proteins (FREPs), cytochrome c oxidase and TNF receptor-associated factor (Table 3). In invertebrates, the innate immune system mainly relies on pattern-recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMP) (Akira *et al*., 2006). Toll-like receptors (TLRs) are very important PRRs, which play a critical role to recognize PAMPs in innate immune (Lu *et al*., 2016). In this study, the results showed that TLR2 and TLR4 genes were up-regulated after challenged by LPS. LPS is a component of the cell wall of Gram negative bacteria, is a potent activator of innate immunity in mammals, which can recognise by TLR4 and leads to subsequent cell signaling pathway responses. Ren *et al*. (2014) reported that TLR2 is involved in regulation of antimicrobial peptides (AMP) gene expression of *Hyriopsis cumingii*, but the mechanism of how TLR2 regulates AMP expression is unclear. Some Lectin-like proteins also have been detected up-regulated in this study. Lectin-like proteins play an important role in the defense of gastropods against pathogen infection (Bulat *et al*., 2016). Lectin proteins often recognize a specific carbohydrate moieties of the pathogen's surface (Vasta *et al*., 2015). These proteins are including Galectin-4, Calnexin, Calreticulin, C-type lectins, I-type lectins and C1q domain containing proteins. Inhibitors of nuclear factor kappa B (IκBs) are major control components of the Rel/NF-κB signaling pathway, a key regulator in the modulation of the expression of immune-related genes in vertebrates and invertebrates (Gao *et al*., 2016; Oyanedel *et al*., 2016). In Zhikong scallop *Chlamys farreri*, the mRNA expression of *CfIκB* and *CfNF-κB* genes increase expression after LPS stimulation (Wang *et al.*, 2011).

The present study revealed that cytochrome c oxidase was the most highly up-regulated DEGs after challenged with LPS. Indeed, increase expression of a number of genes associated with degradation or detoxification was observed, including cytochrome c oxidase subunit II, cytochrome b, NADH dehydrogenase subunit 4, steroid 17-alpha-hydroxylase and cytochrome P450. The immune system of molluscs consists of blood cells (hemocytes), which are the main cellular components of the hemolymph that covers all soft tissue. Hemocytes depends on a multi-enzyme (referred to NADPH and cytochrome oxidase) production of cytotoxic molecules (like nitric oxide and hydrogen peroxide) for killing and elimination pathogen (Humphries and Yoshino, 2008). The production of nitric oxide (NO) by hemocytes of bivalve molluscs has been reported in *M. edulis* (Ottaviani *et al*., 1993), *V. ater* (Franchini *et al.*, 1995), and the carpet shell clam *Ruditapes decussatus* (Tafalla *et al*., 2003). The hemocytes of these species are able to produce NO in response to various stimuli, such as LPS, zymosan, and live bacterial challenge by *Vibrio tapetis* (Tafalla *et al.,* 2003).

In conclusion, the study first using high-throughput transcriptome technology analyzed the expression of immune-related genes after the *P. canaliculata* juvenile snails were challenged with LPS. The work will provide a molecular resource for further functional studies of *P. canaliculata.*

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