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RESEARCH REPORT

Transcriptome analysis of hemocytes and hepatopancreas in red swamp crayfish, *Procambarus clarkii*, challenged with white spot syndrome virus

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Abstract

Red swamp crayfish *Procambarus clarkii* is used for the innate immune defense of crustaceans due to its convenience for laboratory culture and study. To know more about the transcriptome of the crayfish, we constructed and sequenced a cDNA library from a mixture of hemocytes and hepatopancreas from white spot syndrome virus (WSSV)-infected crayfish. By random sequencing, we obtained 9115 high-quality expressed sequence tags with a mean length of 370 bp, representing 3033 unigenes. Most of the unigenes are first reports for the red swamp crayfish. Besides the metabolic genes, many genes that may be involved in the innate immune system of the crayfish are also obtained from the library, such as antimicrobial peptides, pattern recognition receptors, proteases and protease inhibitors, signal transduction proteins, apoptosis-, antioxidant-, and RNA interference-related proteins. We chose ten immune-related genes to analyze their expression pattern by quantitative real time polymerase chain reaction (qRT-PCR) from the hemocytes of normal and WSSV-challenged crayfish. Seven of them, including anti-lipopolysaccharide factor, astacidin, crustin 1, H3 histone family 3A, serine/threonine protein kinase, TGF beta-inducible nuclear protein, and tar RNA binding protein, were upregulated after WSSV injection, but the mRNA expression levels of crustin 2, a lectin, and a digestive cysteine protease decreased after WSSV infection. Our results showed that the transcriptome analysis provides a useful resource for identification of immune related genes and understanding the immune responses of the crayfish.

Key Words: hemocytes; hepatopancreas; expression sequence tags; white spot syndrome virus; red swamp crayfish; *Procambarus clarkii*

Introduction

The crustacean farming industry has been suffering serious problems and enormous economic losses from an outbreak of white spot syndrome virus (WSSV) since 1993 (Yan *et al.,* 2007). WSSV is a serious pathogen and can infect numerous crustaceans including shrimp, crab, and lobster (Shi *et al.,* 2005; Zeng *et al.,* 2009). The cultivation of red swamp crayfish (*Procambarus clarkii*) has become an important economic activity in China as crayfish is a delicious food. *P. clarkii* can be infected by white spot syndrome virus (WSSV) and is easy to be used as a laboratory model for immunity analysis. The investigation of the immune defense mechanisms of red swamp crayfish could be helpful in the control of the WSSV disease of cultivated crustaceans by enhancing the defense activity.

Crustaceans lack an adaptive immune system and rely totally on the innate defense system to resist pathogen invasion. The innate immune reaction comprises cellular reactions such as phagocytosis, nodule formation and encapsulation performed by hemocytes, and humoral reactions mediated by antimicrobial peptides. Crayfish hemocytes play important roles in the initiation of several immune responses and production of antimicrobial peptides (Jiravanichpaisal *et al.,* 2007). The hemocytes and hepatopancreas are crucial for the immune system in crustaceans as it is the main production site for immune recognition molecules, initiates the humoral reaction, and takes part in the cellular reaction by some specialized cells and phagocytes (Gross *et al.,* 2001). Many immune-related genes have been found in hemocytes of WSSV-challenged crayfish (Jiravanichpaisal *et al.,* 2007; Zeng *et al.,* 2009).

Expressed sequence tag analysis is widely used to identify novel and differentially expressed genes

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(Leu *et al.,* 2007; Jiang *et al.,* 2008). Liu (Liu *et al.,* 2006) firstly used suppression subtractive technique on crayfish to study transcripts after WSSV infection. By suppression subtractive hybridization analysis, Zeng and Lu (Zeng *et al.,* 2009) found thirty-three differently expressed genes in WSSV-infected hemocytes of crayfish. The transcriptome information for the hepatopancreas is still limited, so we constructed and sequenced a cDNA library from WSSV-challenged hemocytes and hepatopancreas in red swamp crayfish. The aim of this study is to identify and annotate more immune-related genes in the two immune organs and also provide some information to uncover the mechanisms of WSSV pathogenesis in crustaceans.

In this study, we obtained 3033 unigenes; most of them are novel for red swamp crayfish. Many genes in the library 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, antioxidant proteins, RNA silencing-related proteins, molecular chaperones, cuticle proteins, and calcium-binding proteins.

Materials and Methods

WSSV challenge of crayfish and collection of hepatopancreas and hemocytes

Red swamp crayfish (*Procambarus clarkii*) (about 10-20 g each) were obtained from a seafood market in Jinan, Shandong Province, China. They were cultured in 500 L tanks in fresh water at room temperature (~20 °C) in the laboratory. WSSV was extracted from the gills of infected crayfish, and diluted in PBS (Phosphate Buffered Saline, containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). Using a 100 μl syringe, 3.2×10^7 virus particles were injected into the abdominal segment of each crayfish. The preparation and quantification of viral inocula followed a previously described method (Wang *et al.,* 2009b). Ninety-six hours after injection, the hemolymph was collected from the ventral sinus of ten crayfish and mixed with 1⁄10 volume of anticoagulant buffer (10 % sodium citrate, pH 7) containing 200 mM phenylthiourea as a melanization inhibitor. The hemolymph was then centrifuged at 800×*g* for 5 min (4 °C) to collect the hemocytes. The hepatopancreas was also collected from the crayfish for RNA extraction 96 h after WSSV injection.

RNA extraction and cDNA library construction

The total RNA was extracted from hemocytes and hepatopancreas of 96 h infected crayfish using Unizol reagent following the manufacturer's instructions (Biostar, Shanghai, China). Messenger RNA (mRNA) was extracted with the PolyATract mRNA isolation system (Promega, USA). The mRNAs from hemocytes and hepatopancreas were mixed together and used to construct a cDNA library. The Creator SMART cDNA Library Construction Kit (Clontech, USA) was used for the cDNA library construction following the manufacturer's instructions. The double strand cDNA was digested and ligated with the pDNR-LIB vector, and then transformed into competent DH5α cells. Individual colonies were randomly selected, and plasmids were extracted for sequencing from the 5'-ends.

Analysis and classification of the expression sequence tags (ESTs)

High quality ESTs (more than 100 bp after removal of the vector sequence) were assembled by CAP3 (http://seq.cs.iastate.edu./) in order to obtain contig sequences. Unigenes (including contigs and singlets) were analyzed by BLASTx against the National Center for Biotechnology Information (NCBI) protein database, BLASTn against the NCBI nucleotide database, and BLASTx against the SWISSPROT protein database. The E-value of significant matches by the BLASTx and BLASTn is less than 0.001, and the alignment score is more than 30.

The concrete functional information and metabolic profiles of Unigenes were obtained using the Kyoto Encyclopedia for Genes and Genomes (KEGG) (Kanehisa *et al.,* 2004). Unigenes were assembled into different functional classes with Clusters of Orthologous Groups of proteins (COGs) (http://www.ncbi.nlm.nih.gov/COG/).

Quantitative real time PCR (qRT-PCR)

Total RNAs were extracted from hemocytes of ten normal and 96 h WSSV-challenged crayfish using Unizol reagent separately. Then RNAs were reverse transcribed to the first strand cDNAs. The cDNAs were diluted 100 fold and used as the template for qRT-PCR. Ten genes including anti-lipopolysaccharide factor, astacidin, crustin 1, crustin 2, H3 histone family 3A, serine/threonine protein kinase (SRK), TGF beta-inducible nuclear protein, tar RNA binding protein (TRBP), lectin and digestive cysteine protease from this cDNA library were chosen for qRT-PCR analysis. These genes were chosen because they belonged to different groups and were firstly found in crayfish. We wanted to know whether they participate the antivirus innate immune reaction of crayfish. 18S rRNA was used as the control. Primer sequences used in this study are listed in Table 1. qRT-PCR was performed following the manufacturer's instruction with SYBR Premix Ex Taq (Takara, Japan) using a real-time thermal cycler (Bio-Rad, USA). The qRT-PCR was performed in 10 μl reactions containing 5 μl of 2×Premix ExTaq, 1 μl of the 1:100 diluted cDNA, 2 μl each of the forward and reverse primer (1 μM). The amplification conditions were initial denaturation at 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 60 °C for 50 s, followed by a melting curve from 60 to 95 °C. PCR products were detected by agarose gel analysis after each PCR reaction to confirm the specific gene amplification. Data were analyzed using a comparative method ($2^{-\Delta\Delta Ct}$). qRT-PCR analysis was repeated three times for each sample.

Results and Discussion

General of the cDNA library

To get more information on the virus immunity-related proteins from crayfish, total RNA was extracted from important immune tissues including hemocytes and hepatopancreas at 96 h

Table 1 Primers used in the study

Gene	Primer	Sequence(5′-3′)
Astacidin	Astacidin-F	ATGCGTCTTCTCCATCTCC
	Astacidin-R	TTACTTGCCTGGACGGTA
Anti lipopolysaccharide factor	ALF-F	CCGCCTCCTTCACCCCACA
	ALF-R	TCCACCTCACCGTTCCGCC
Crustin1	Crustin1-F	TATTCCTCGCTGCACAAACA
	Crustin1-R	CACATAGCACCTCCCTCTCA
Crustin2	Crustin2-F	GGGAAGAAAAGCACAATGGT
	Crustin2-R	GGTATGGAGGTCGAGACAGG
Digestivecysteine protease	Dcysp-F	AAGTATGTTGACGCAGAGGAGG
	Dcysp-R	AAATTGGTTCATTGCCAGGTTG
H3 histone family 3A	H3-F	GTCACCATCATGCCCAAGGATA
	H3-R	GCCAGCACTGCGAAGTCAATTC
Lectin	Lectin-F	GTTATTGACGACTCCACCTT
	Lectin-R	GTCTTCCCATTGACCCACTT
Serine/threonine protein kinase	SPK-F	TGCTATGTGAAGCTCGGCTCT
	SPK-R:	GCGATCTGATGCTCCTCCTCT
TGF beta-inducible nuclear protein	TGFINP-F	GCCTGGGTGCTGGTATCTTGG
	TGFINP-R	GTTCGTTCTGTGGCATTGTGT
Tar RNA binding protein	TRBP-F	AAAATGTATCGTCAACCACCAC
	TRBP-R	CACCCTCTATCTGCAACAAGTC
18SRNA	18SRNA-F	ACCGATTGAATGATTTAGTGAG
	18SRNA-R	TACGGAAACCTTGTTACGAC

after WSSV challenge, and then a cDNA library was constructed and randomly sequenced. As shown in Table 2, a total of 10145 clones were sequenced, and among these, 9115 ESTs were equal to or longer than a cutoff length (100 bp) after removal of vector sequences. They are high quality ESTs with an average length of 370 bp. In total, 3033 unigenes were acquired including 859 contigs which have more than one EST and 2174 singlets which have only one EST; the singlet rate is 71.68 %. The unigene hemocyanin has 213 ESTs, which is the largest unigene size (Table 3). The novelty of the library is 33.27 %. Among the unigenes, only a small fraction is previously reported genes and most of them are novel genes in red swamp crayfish. We discovered many new genes from this library, and it also provides some information about the transcriptome of crayfish hemocytes and hepatopancreas.

All the unigenes were aligned to the NCBI nr database using BLASTx, and to the NCBI nt database using BLASTn as a complementary analysis. Moreover, the unigenes were repeatedly aligned against the BLASTx SWISSPROT database. From the annotation of the alignments, we found that most of the ESTs shared high similarities with nucleotide sequences mainly from

Table 2 Summary statistical analysis of the cDNA library from hemocytes and hepatopancreas

Description	Number
Total ESTs	10145
Low Quality ESTs	1030
High Quality ESTs	9115
Unigenes	3033
Contigs	859
Singlets	2174
Novelty (%)	33.27
Redundancy (%)	66.73

Total ESTs: Low and high quality ESTs; Low Quality ESTs: After remove Empty ESTs and Vector ESTs, the length of EST less than a cutoff length (100bp); High Quality ESTs: After remove Empty ESTs and Vector ESTs, the length of EST more than a cutoff length (100bp); Unigenes: Contigs and Singlets; Contigs: the number of ESTs is equal to or bigger than 2; Singlets: the number of ESTs is 1; Novelty: Unigenes/Assembled ESTs; Redundancy: 1-Novelty

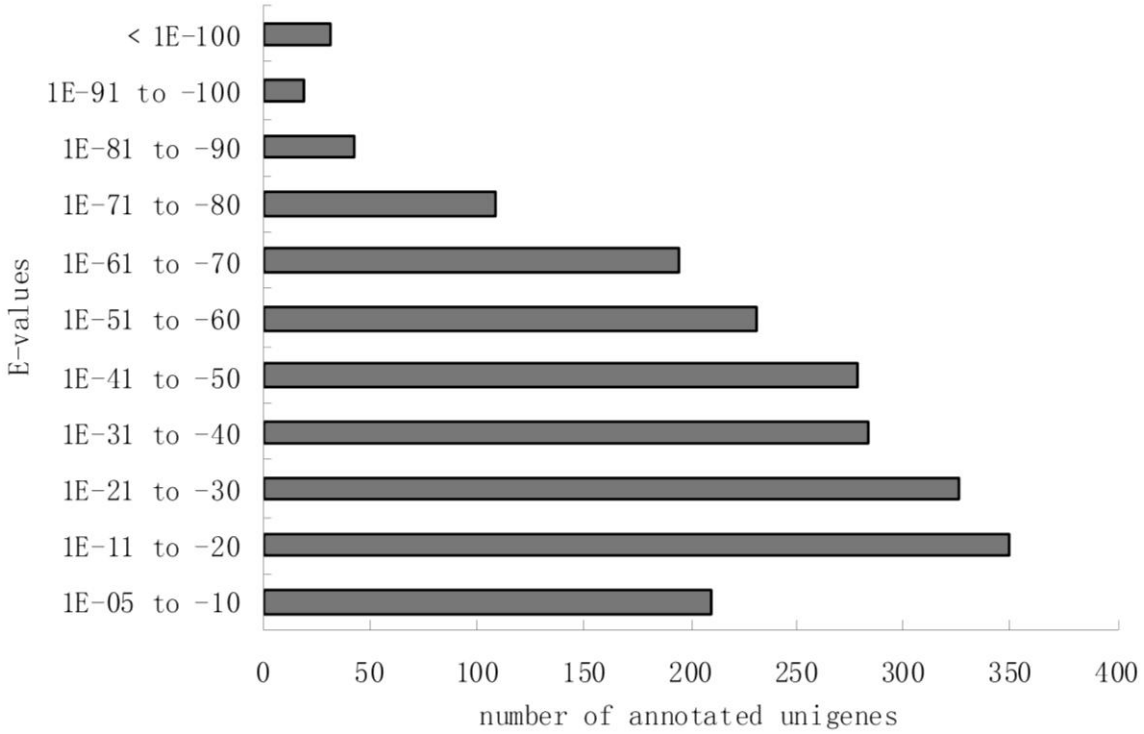

Fig. 1 The number of annotated unigenes with different E-values.

crayfish (*Pacifastacus leniusculus*), narrow-fingered crayfish (*Pontastacus leptodactylus*), broad-fingered crayfish (*Astacus fluviatilis*), and California spiny lobster (*Panulirus interruptus*). About 68 % of the unigenes have best hits and annotations against available databases, and the other 961 unigenes that have no hits may be undiscovered or unknown function genes in the database. A number of genes had identity scores of a given E value (1.00E−10). From the E-value distribution profile of all the annotated unigenes shown in Fig. 1, we could see that about 90 % of the annotated unigenes had an E-value <1.00E−10, so the annotation results of the blast searches in three databases are believable.

The most highly expressed gene is hemocyanin 2, and its contig contains 213 ESTs. In arthropods, hemocyanin is a multifunctional protein which can transport oxygen in the hemolymph, shows phenoloxidase activity in its proteolytically cleaved N-terminal, and the C-terminal part sequence serves as antimicrobial peptides (Lee *et al.,* 2004). The hemocyanin of horseshoe crab could be directly activated by microbial protease and was enhanced by pathogen associated molecular patterns. The activated hemocyanin can generated highly reactive oxygen intermediates that kills microbes (Jiang *et al*., 2007). Therefore, hemocyanin plays important roles in the immune system of arthropods. Here we obtained 213 ESTs of hemocyanin in our cDNA library, and nine kinds of hemocyanin can be divided by alignment and phylogenetic analysis. This suggested that the hemocyanin might have function in antiviral immune response. Megalin is also a highly expressed gene which contains 152 ESTs. Megalin is a low-density lipoprotein receptor-related protein that is a cell surface endocytic receptor and could bind extracellular ligands for degradation (Li *et al.,* 2001). Several proteases such as cathepsin L, trypsin, and zinc protease are all highly abundant expressed genes and have more than 100 ESTs.

Table 3 Analysis of unigenes including contigs and singlets

Unigene Size	Number of Unigenes	Percent of Unigenes(%)
1	2174	71.68
2	358	11.8
3	157	5.18
4–5	116	3.82
6–10	103	3.4
11–20	60	1.98
21–50	40	1.32
51–100	17	0.56
>100	8	0.26

Max Unigene Size：213

*Unigene Size: The Number of ESTs in a Unigene

Genes potentially involved in the defense reactions

Hemocytes and hepatopancreas are important immune-related cells and organs in arthropods, and 33 differentially expressed genes have been discovered in hemocytes of WSSV-challenged crayfish by suppression subtractive hybridization (SSH) and cDNA microarrays (Zeng *et al.,* 2009). Many upregulated genes in the SSH library such as serine protease inhibitor, tubulin, zinc finger protein, synaptosome-associated protein, fatty acid binding protein, superoxide dismutase precursor, arginine kinase, and heat shock protein were also found in our cDNA library from hemocytes and hepatopancreas of WSSV-challenged red swamp crayfish.

Among the newly discovered genes of our cDNA library, there are many genes potentially involved in the defense reaction of crayfish (Table 4). They could be assembled into seven groups such as antimicrobial peptides, pattern recognition receptors, proteases and protease inhibitors, signal transduction proteins, apoptosis-related proteins, antioxidant proteins, and others which could not be classified into groups.

Antimicrobial peptides

In this cDNA library, five kinds of antimicrobial peptides have been found, including anti-lipopolysaccharide factor (ALF), astacidin, lysozymes, single WAP domain-containing protein, and crustins. ALFs are potential antimicrobial peptides which could bind to the lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria and inhibit the LPS-mediated coagulation cascade in arthropod (Rosa *et al.,* 2008). ALF could interfere with WSSV replication in crayfish *Pacifastacus leniusculus* and protect shrimp from WSSV infection (Liu *et al.,* 2006). ALF was upregulated in hemocytes 96 h after WSSV challenge in qRT-PCR analysis (Fig. 2A), and it may be related to the anti-WSSV reaction in crayfish *P. clarkii*. Astacidin 2 is a small proline/arginine-rich antibacterial peptide that shows strong antimicrobial activity against Gram-positive and Gram-negative bacteria, and has been purified from the hemolymph of freshwater crayfish *P. leniusculus* (Jiravanichpaisal *et al.,* 2007). It was upregulated in hemocytes 96 h after WSSV challenge (Fig. 2B). Lysozymes are well known antimicrobial proteins that show lytic activity to a range of Gram-positive and Gram-negative bacteria (de-la-Re-Vega *et al.,* 2006). Both the single WAP domain-containing protein (SWD) and two crustins belong to the WAP-containing proteins were also found in the crayfish. SWD was upregulated in the WSSV-challenged shrimp (Amparyup *et al*., 2008; Du *et al*., 2010).

Crustins are a large antimicrobial peptide family. They can be divided into three groups according to structural characters (Smith *et al.,* 2008). From an antimicrobial experiment *in vitro*, we could see that crustins mainly showed strong bactericidal activity against Gram-positive bacteria and Gram-negative bacteria so far (Amparyup *et al.,* 2008; Smith *et al.,* 2008). Also in vivo experiment showed that after crustin was depleted, the mortality increased significantly after shrimp were infected by *V. penaeicida* (Shockey *et al*., 2009). Crustin 2 is a kind of carcinin like protein. Crustin 1 and Crustin 2 belong to different groups. Crustin 1 was upregulated and crustin 2 was downregulated in hemocytes 96 h after challenge of WSSV (Figs 2C, D).

Pattern recognition receptors (PRRs)

In invertebrates, pattern recognition receptors can recognize and bind molecules present on the surface of pathogens such as β-1,3-glucans, lipopolysaccharides, lipoteichoic acid, and peptidoglycans, which are called pathogen-associated molecular patterns (Janeway, 1989). We discovered several kinds of pattern recognition receptors including C-type lectins, β-1,3-glucan binding protein, lipopolysaccharide and β-1,3-glucan binding protein, and LysM and putative peptidoglycan binding domain-containing protein. C-type lectins have been reported to participate in the innate defense reaction of nonself recognition by recognizing and binding to the molecules on the surfaces of cell walls and then inducing pathogen phagocytosis and agglutination (Sun *et al.,* 2007; Ma *et al.,* 2008). Reports on Chinese shrimp showed that C-type lectin served not only as a pattern recognition receptor but also an effector (Sun *et al.,* 2008). C-type lectin from the shrimp *Litopenaeus vannamei* also has activity against WSSV (Zhao *et al.,* 2009). A C-type lectin was downregulated 96 h after WSSV injection, which was the same pattern as a C-type lectin, PmAV, from *Penaeus monodon* (Leu *et al.,* 2007) (Fig. 2E).

In crustaceans, β-1,3-glucan binding protein (BGBP) and lipopolysaccharide and β-1,3-glucan binding protein (LGBP) serve as pattern recognition receptors to recognize cell wall components of bacteria and fungi, such as β-1,3-glucans and lipopolysaccharide. BGBP binding to β-1,3-glucan induces hemocyte degranulation and subsequently activates the prophenoloxidase (proPO) system (Lin *et al.,* 2008). LGBP from the crayfish *P. leniusculus* have been reported to participate in the activation of the proPO system (Lee *et al.,* 2000).

Proteases and protease inhibitors

The hepatopancreas was reportedly the site of synthesis of digestive enzymes in crustaceans. Several protease and protease inhibitors were found in the crayfish. The proteases include astacin, zinc protease, zinc metalloproteinase, cathepsin, cysteine protease, carboxypeptidase, and trypsin. The protease inhibitors include three kinds of serine protease inhibitors such as hemocyte-specific Kazal-type protease inhibitor, hepatopancreas Kazal-type protease inhibitor, and the putative serine protease inhibitor serpin.

Astacin is a small zinc protease that can digest fibrillar collagen and other proteins (Reyda *et al.,* 1999). It was significantly upregulated by bacterial or LPS challenge in oyster (Roberts *et al.,* 2009). Zinc metalloproteinase is a protease family involved in growth factor activation, polypeptide degradation and extracellular protein processing (Bond *et al.,* 1995).

Trypsin is one of the most important proteases and contributes about 6 % of soluble protein in the

Table 4 Genes potentially included in innate immune response systems

Gene name	Species most similar to	E-value	No. of ESTs
Antibacterial peptide			
Pl-crustin 1	*Pacifastacus leniusculus*	2E-37	3
Pl-crustin 2	*Pacifastacus leniusculus*	3E-13	2
anti-lipopolysaccharide factor	*Pacifastacus leniusculus*	1E-19	3
astacidin 2	*Pacifastacus leniusculus*	4E-20	1
Lysozyme precursor	*Crassostrea gigas*	2E-06	2
single WAP domain-containing protein	*Marsupenaeus japonicus*	1E-09	1
Pattern recognition receptor			
C-type lectins	*Fenneropenaeus chinensis*	2E-06	72
β-1,3-glucan binding protein	*Penaeus monodon*	1E-11	5
Lipopolysaccharide and β-1,3-glucan binding protein	*Pacifastacus leniusculus*	7E-56	1
Proteases/protease inhibitors			
Astacin	*Astacus fluviatilis*	5E-96	1
zinc proteases	*Astacus astacus*	4E-25	123
Trypsin	*Pacifastacus leniusculus*	1E-12	124
cysteine protease	*Homarus americanus*	4E-11	60
cathepsin C	*Marsupenaeus japonicus*	7E-44	1
cathepsin D	*Apriona germari*	8E-34	1
cathepsin L	*Nephrops norvegicus*	2E-16	141
carboxypeptidase A1	*Scophthalmus maximus*	4E-06	1
carboxypeptidase B	*Astacus fluviatilis*	7E-20	7
carboxypeptidase A5 precursor	*Mus musculus*	1E-06	8
Serine carboxypeptidase precursor	*Mus musculus*	3E-10	3
semigranular hemocyte specific Kazal-type protease inhibitor	*Pacifastacus leniusculus*	3E-13	1
hepatopancreas Kazal-type protease inhibitor	*Penaeus monodon*	1E-07	17
zinc metalloproteinase	*Caenorhabditis elegans*	2E-07	1
putative serine protease inhibitor serpin	*Pacifastacus leniusculus*	4E-21	1
Signal transduction proteins			
Ras-related protein Rab-1A	*Lymnaea stagnalis*	2E-40	3
Ras-related protein Rab-18	*Gallus gallus*	2E-63	1
Ras-related protein Rab-5B	*Pongo pygmaeus*	2E-49	1
Ras-related protein Rab-7a	*Rattus norvegicus*	3E-34	1
Ras-related GTP-binding protein C	*Mus musculus*	1E-10	1
Ras-like GTP-binding protein Rho1 isoform 1	*Apis mellifera*	2E-25	2

Rho family small GTP binding protein cdc42	*Nasonia vitripennis*	6E-69	1
vacuolar ATPase G subunit-like protein	*Maconellicoccus hirsutu*	2E-19	1
Nucleolar GTP-binding protein 1	*Homo sapiens*	2E-56	1
GTP-binding nuclear protein Ran (GTPase Ran)	*Xenopus tropicalis*	2E-45	3
Ran-binding protein 16	*Mus musculus*	7E-51	1
protein kinase N2	*Tribolium castaneum*	2E-38	1
receptor for activated protein kinase C-like protein	*Lepeophtheirus salmonis*	9E-73	1
Toll-like receptor	*Anopheles gambiae*	1E-06	2
Relish	*Litopenaeus vannamei*	2E-25	1
Insulin like growth factor binding protein	*Mus musculus*	2E-10	1
Casein kinase I isoform alpha	*Xenopus laevis*	2E-06	1
mitogen-activated protein-binding protein-interacting protein	*Nasonia vitripennis*	5E-29	1
Serine/threonine-protein phosphatase PP2A	*Drosophila melanogaster*	1E-25	1
Arginine kinase	*Homarus gammarus*	3E-46	6
Serine/threonine-protein kinase N2	*Homo sapiens*	2E-38	1
Apoptosis			
senescence-associated protein	*Pisum sativum*	1E-19	2
zinc finger protein	*Xenopus laevis*	2E-11	3
Antioxidation			
thioredoxin-like protein	*Maconellicoccus hirsutus*	4E-33	1
thioredoxin domain containing 11 isoform 1	*Apis mellifera*	1E-31	1
thioredoxin reductase 1	*Rattus norvegicus*	9E-08	1
superoxide dismutase	*Pontastacus leptodactylus*	6E-18	1
glutathione peroxidase 5	*Mus musculus*	1E-09	23
glutathione peroxidase 1	*Canis familiaris*	4E-21	14
ferritin	*Pacifastacus leniusculus*	1E-09	11
glutathione S-transferase Mu 2	*Mus musculus*	1E-32	6
glutathione-S-transferase-like protein	*Galleria mellonella*	4E-40	1
glutathione S-transferase 1-1 (GST class-theta)	*Tribolium castaneum*	1E-36	1
glutamine synthetase	*Panulirus argus*	1E-42	1
Microsomal glutathione S-transferase 3	*Mus musculus*	1E-24	1
Putative thiosulfate sulfurtransferase FMP31	*Saccharomyces cerevisiae*	2E-13	1
omega class glutathione S-transferase	*Crassostrea gigas*	1E-27	1
farnesoic acid O-methyltransferase	*Marsupenaeus japonicus*	4E-08	6
O-methyltransferase	*Fenneropenaeus chinensis*	5E-25	1
Acyl-Coenzyme A oxidase 3	*Strongylocentrotus purpuratus*	9E-07	2
		1E-12	

Others			
LysM and putative peptidoglycan binding domain containing protein	*Xenopus tropicalis*	4E-09	1
megalin	*Danio rerio*	1E-16	152
hemocyanin 2	*Pacifastacus leniusculus*	2E-15	213
ATP-dependent RNA helicase	*Mus musculus*	1E-23	6
PAZ and PIWI domain protein/ Piwi-like protein	*Paramecium tetraurelia*	5E-19	1
Cuticle protein 6	*Blaberus craniifer*	2E-11	1
Crustacean calcium-binding protein 23	*Orconectes limosus*	2E-46	1
heat shock 70 kDa protein	*Blastocladiella emersonii*	4E-37	3
heat shock protein 27	*Drosophila melanogaster*	2E-06	1
TGF beta-inducible nuclear protein	*Brugia malayi*	8E-07	1
H3 histone family 3A	*Salmo salar*	3E-29	1
tar RNA binding protein	*Fenneropenaeus chinensis*	1E-09	1
chitinase	*Fenneropenaeus chinensis*	9E-14	43

digestive gland. Trypsins as important digestive proteases mainly take part in food digestion, hydrolysis, and activation of zymogens (Muhlia-Almazan *et al.,* 2008).

Three kinds of cathepsins have been discovered in the crayfish, including cathepsin C, cathepsin D, and cathepsin L. Cathepsin C is a multifunctional protease and is essential for the activation of other enzymes (Turk *et al.,* 2001). Cathepsin D is an aspartic protease involved in several physiological functions such as protein degradation, apoptosis and autophagy (Zaidi *et al.,* 2008). Cathepsin L could digest food in the gastrointestinal juice of crustaceans (Hu *et al.,* 2007). A digestive cysteine protease was downregulated 96 h after WSSV injection (Fig. 2F).

Carboxypeptidases are hydrolases that cleave in the C-terminus of proteins. Carboxypeptidases A and B are digestive carboxypeptidases and serve in the degradation of proteins in the digestive tract.

Serine protease inhibitors of arthropods include the Kazal, Kunitz, α-macroglobulin, and serpin families. They play important roles in prophenoloxidase and cytokine activation, blood coagulation, and pathogen digestion. Three kinds of serine protease inhibitors including hemocyte-specific Kazal-type protease inhibitor, hepatopancreas Kazal-type protease inhibitor, and the putative serine protease inhibitor serpin have been isolated from this library. Kazal-type protease inhibitors have been found in the hemocytes of shrimp, and were suggested to be involved in the host defense against WSSV challenge (Jarasrassamee *et al.,* 2005). Serpin from hemocytes cDNA library of Chinese shrimp has been shown to fluctuate after WSSV and bacteria challenges (Liu *et al.,* 2009).

Proteins in signal transduction pathway

Ras-related protein Rab, Ran-binding protein, and Ras-like GTP-binding protein Rho were found in the cDNA library; they all belong to the Ras-related protein superfamily. The Ras family of small GTPases plays roles in a series of cellular processes such as phagocytosis, vesicle transportation, and development. Rab GTPase from Japanese shrimp participates in the defense response to virus and might function as an intracellular virus recognition protein in virus-infected shrimp (Wu *et al.,* 2008). Moreover Ran from Japanese shrimp is involved in antiviral defense immunity and could regulate hemocytic phagocytosis by interacting with myosin (Liu *et al.,* 2009). The studies of Rho GTPase have mainly focused on phagocytosis and actin dynamics control (Pan *et al.,* 2005).

Toll is a receptor of the Toll signal pathway responsible for antifungal and anti-Gram-positive bacterial response in *Drosophila*. Toll like receptor from invertebrates could not bind pathogens directly, but it was activated by binding to Spaetzle (Hoffmann, 2003). It was also found in the crayfish cDNA library. Relish is a downstream NF-κB transcription factor in the Imd signal pathway. The translocation of cleaved Relish to the nucleus could activate the signal pathway (Hoffmann, 2003). This means that the Toll and Imd pathways might existed in the crayfish, and the pathways might have functions in antiviral responses.

Insulin-like growth factor binding protein (IGFBP) was also found in the crayfish. It is a member of the

insulin-like growth factor pathway. The insulin-like growth factor pathway takes part in growth and development, metabolic homeostasis, fecundity and stress resistance, and also lifespan in multicellular organisms (Broughton *et al.,* 2009).

Serine/threonine-protein kinases and phosphatases play roles in signal transduction of anoxia in the crayfish *Orconectes virilis* (Cowan *et al.,* 2001). Serine/threonine-protein kinase N2 and serine/threonine-protein phosphatase PP2A were isolated from our cDNA library. Casein kinase1 is a member of the serine/threonine protein kinase family, and it has seven isoforms in mammals and vertebrates. Casein kinase1α could enhance receptor interacting protein-mediated NF-κB activation (Wang *et al.,* 2008). The mRNA expression level of serine/threonine-protein kinase N2 from this cDNA library increased 96 h after WSSV injection (Fig. 2G), and it may be involved in the antiviral innate immune reaction of crayfish.

The transforming growth factor β (TGF-β) signal pathway plays important roles in diverse biological processes. In *Caenorhabditis elegans*, the TGF-β pathway participates in immune responses (Schulenburg *et al.,* 2004). TGF beta-inducible nuclear protein could be induced by stimulation of TGF-β, so it may be involved in the defense reaction. TGF beta-inducible nuclear protein was upregulated in hemocytes of crayfish after WSSV challenge, so it may participate in the anti-virus immune response (Fig. 2H).

Apoptosis-related proteins

A senescence-associated protein was also found from this cDNA library. The senescence-associated protein takes part in the early embryonic development of silkworm *Bombyx mori* (Hong *et al.,* 2006).

Several zinc finger proteins were also found in the library, just as in the SSH library of hemocytes from WSSV-challenged crayfish. One zinc finger protein could bind to viral mRNA and prevent its accumulation in the cytoplasm (Garcia *et al.,* 2007). Thus the zinc finger proteins of our cDNA library may be involved in the anti-virus immune defense reaction of crayfish.

Antioxidant proteins

Reactive oxygen species, the products of normal aerobic metabolism, can cause oxidative damage to organisms. Antioxidant proteins are needed to eliminate the reactive oxygen species and regulate the redox homeostasis. To protect against damage and regulate redox homeostasis, molecules in the glutaredoxin and thioredoxin systems are employed. Glutaredoxins and thioredoxins are conserved proteins involved in many cellular processes including repair of oxidatively damaged proteins and protein refolding and regulation (Grant, 2001). The thioredoxin system contains thioredoxin and thioredoxin reductase, and thioredoxin is catalyzed directly by thioredoxin reductase and electrons from nicotinamide adenine dinucleotide (NADH) (Aispuro-Hernandez *et al.,* 2008). By the random sequencing of the cDNA library, thioredoxin-like proteins and thioredoxin reductase were isolated from the WSSV-challenged crayfish. Thioredoxin was characterized in Pacific white shrimp (*L. vannamei*) and the antioxidant activity of the recombinant protein was tested by reducing insulin disulfides using the Trolox Equivalent Antioxidant Capacity assay. The results showed that thioredoxin is an important antioxidant (Aispuro-Hernandez *et al.,* 2008). Glutathione peroxidase is a component of the glutaredoxin system. Glutathione S-transferases belong to a multigene family that plays important roles in detoxification of xenobiotic compounds (Rosa de Lima *et al.,* 2002). A selenium-dependent glutathione peroxidase and two glutathione S-transferases have been cloned from Chinese shrimp and were involved in detoxification defense reactions (Ren *et al.,* 2009). A glutamine synthetase was also found in this library. Superoxide dismutase is a kind of antioxidant enzyme. It was also found in the SSH library of WSSV-challenged crayfish and upregulated after virus injection (Zeng *et al.,* 2009).

Ferritin is an iron storage protein that participates in iron metabolism and detoxification. Ferritins from shrimp have been reported to be involved in the anti-virus defense reaction (Zhang *et al.,* 2006).

Others

LysM and putative peptidoglycan binding domain-containing protein (PBP) has not been found in crustaceans so far, so this PBP may be the first one. It may also be involved in the innate immune reaction in crayfish.

We found PAZ and PIWI domain protein and ATP-dependent RNA helicase, which are involved in RNA-mediated silencing and the defense response against viruses (Cerutti *et al.,* 2006). In humans, the trans-activation response (Tar) RNA-binding protein plays a role in passing small interfering RNA to the RNA-induced silencing complex and functions in RNA interference(Wang *et al.,* 2009a). A tar RNA binding protein was found in the cDNA library; it was upregulated after WSSV challenge in the hemocytes of crayfish (Fig. 2I), so it may take part in the antiviral immune reaction.

Chitinases are members of the glycoside hydrolase family. It has been reported that in the mollusc *Crassostrea gigas*, two chitinases are stimulated in response to LPS challenge, which suggested that they are involved in the immune defense reaction of oyster (Badariotti *et al.,* 2007). Chitinase was upregulated in hepatopancreas of WSSV-resistant shrimp (Pan *et al.,* 2005).

Cuticle protein and crustacean calcium-binding protein were also found by an EST approach in the WSSV-infected shrimp *P. mondon*. Calcium-binding proteins act as cytosolic Ca^{2+} buffers and were significantly downregulated in WSSV-injected shrimp (Leu *et al.,* 2007). A calcium-binding peptide from the exoskeleton of crayfish functions as a regulator of exoskeleton calcification (Inoue *et al.,* 2004). Cuticle proteins were upregulated as a result of WSSV infection, as the WSSV mainly infects the cuticular epidermis in shrimp (Leu *et al.,* 2007).

Heat shock proteins are members of the chaperone family and take part in protein folding. In shrimp, heat shock protein 70 expression was influenced by WSSV infection, and the association

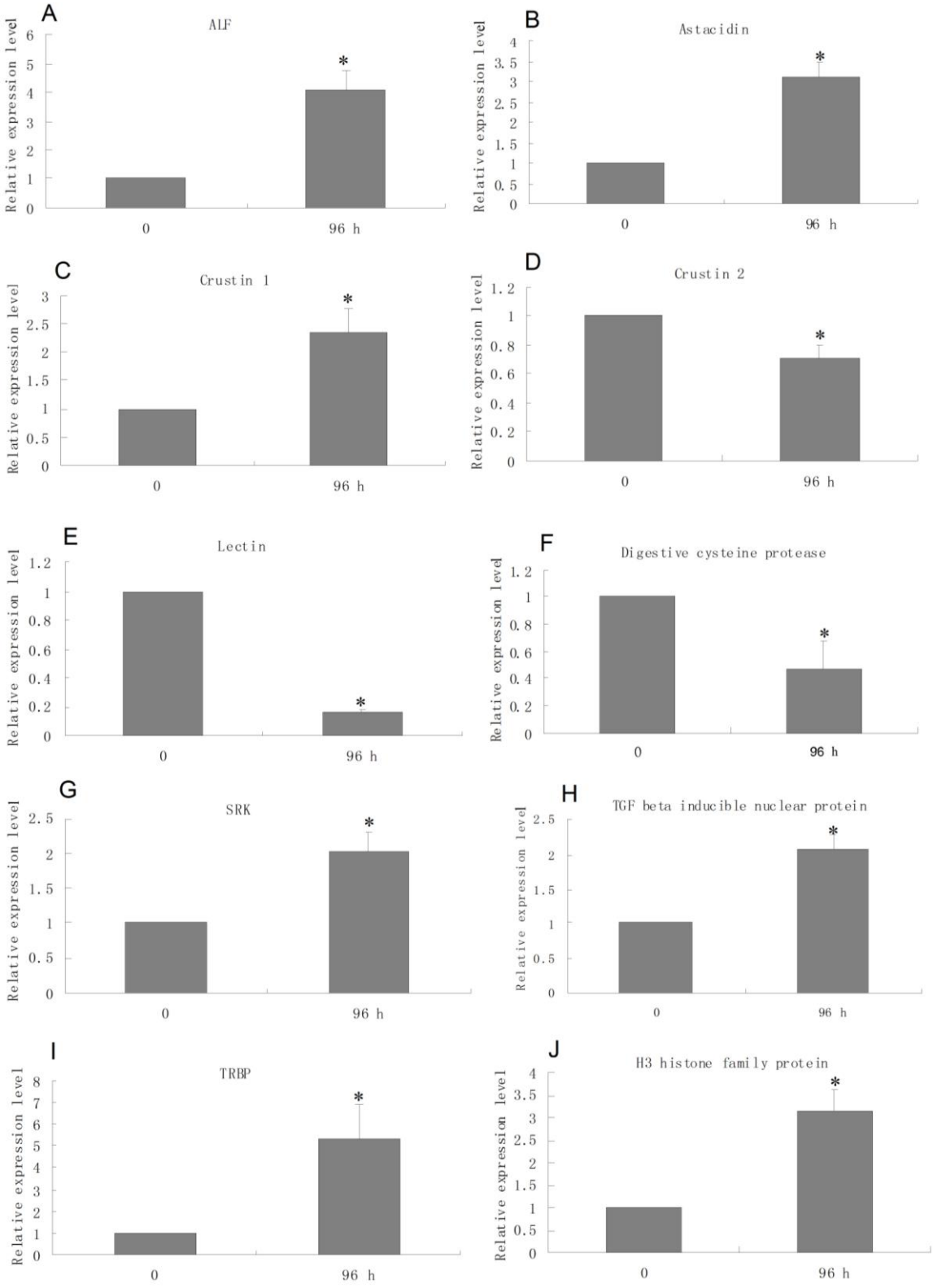

Fig. 2 qRT-PCR analysis of ten genes in total RNA extracted from red swamp crayfish hemocytes 96 h after WSSV challenge. The qRT-PCR data of the expression level of these genes in response to bacterial and viral challenge were calculated by $2^{-\Delta\Delta Ct}$. Bars stand for the mean±SD. of three independent PCR amplifications and quantifications. The data obtained were subjected to the statistical analysis followed by an unpaired sample *t*-test. Asterisks indicate significant differences (*P<0.05) when comparing to that in the healthy hemocytes (0 h). Astacidin (GenBank accession no. GQ301199); crustin 1 (GenBank accession no. GQ301201); crustin 2 (GenBank accession no. GQ301202); Anti-lipopolysaccharide factor (ALF) HM005306; Digestive cysteine proteinase HM005305; H3 histone family protein HM005304;Serine/threonine protein kinase(SER) HM005303; TGF beta-inducible nuclear protein HM005302; Tar RNA binding protein (TRBP)HM005301; Lectin HM005300.

between the major envelope protein VP28 of WSSV and heat shock protein 70 is direct and ATP-dependent (Xu *et al.,* 2009). Heat shock protein 70 was upregulated after WSSV infection and involved in the anti-virus response in crayfish (Zeng *et al.,* 2009). Another heat shock protein of this cDNA library showed similarity to heat shock protein 27 from *Drosophila melanogaster*, and it had anti-apoptotic activity by inhibition of cytochrome C and TNF-mediated cell death (Arya *et al.,* 2007).

Histones are basic protein components of chromatin. As early as 1942, some reports showed that histone possessed antibacterial properties (Miller *et al.*, 1942). Recent work demonstrate that the histones and histone-derived fragments have antimicrobial activities in diverse range of organisms from shrimp to human (Kawasaki and Iwamuro, 2008). The H3 family contains two replacement histone genes, H3 histone family 3A and 3B. Here we found a H3 histone family 3A. It was upregulated after WSSV infection (Fig. 2J). It was reported that histone H2B could mediate anti-virus immune defense reactions, so the upregulation of H3 histone 3A may also be involved in the antiviral defense reaction of crayfish (Kobiyama *et al.,* 2010).

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