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Identification of genes differentially expressed in hemocytes of *Scylla paramamosain* in response to lipopolysaccharide

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ABSTRACT

Although the crab *Scylla paramamosain* has been cultured in China for a long time, little knowledge is available on how crabs respond to infection by bacteria. A forward suppression subtractive hybridization (SSH) cDNA library was constructed from their hemocytes and the up-regulated genes were identified in order to isolate differentially expressed genes in *S. paramamosain* in response to bacterial lipopolysaccharide (LPS). A total of 721 clones on the middle scale in the SSH library were sequenced. Among these genes, 271 potentially functional genes were recognized based on the BLAST searches in NCBI and were categorized into seven groups in association with different biological processes using AmiGO against the Gene Ontology database. Of the 271 genes, 269 translatable DNA sequences were predicted to be proteins, and the putative amino acid sequences were searched for conserved domains and proteins using the CD-Search service and BLASTp. Among 271 genes, 179 (66.1%) were annotated to be involved in different biological processes, while 92 genes (33.9%) were classified as an unknown-function gene group. It was noted that only 18 of the 271 genes (6.6%) had previously been reported in other crustaceans and most of the screened genes showed less similarity to known sequences based on BLASTn results, suggesting that 253 genes were found for the first time in *S. paramamosain*. Furthermore, two up-regulated genes screened from the SSH library were selected for full-length cDNA sequence cloning and in vivo expression study, including *Sp*-superoxide dismutase (*Sp*-Cu-ZnSOD) gene and *Sp*-serpin gene. The differential expression pattern of the two genes during the time course of LPS challenge was analyzed using real-time PCR. We found that both genes were significantly expressed in LPS-challenged crabs in comparison with control. Taken together, the study primarily provides the data of the up-regulated genes associated with different biological processes in *S. paramamosain* in response to LPS, by which the interesting genes or proteins potentially involved in the innate immune defense of *S. paramamosain* will be investigated in future.

1. Introduction

The crab *Scylla paramamosain*, as a commercial species, has been cultured in China for many years and the animals are usually raised in ponds at high densities which has led to disease epidemics. It is generally accepted that crustaceans including crabs lack the adaptive immune response found in vertebrates and are principally dependent on a series of non-specific responses against foreign invaders. Therefore, elucidation of potential immune-related components involved in innate immunity is very important in crabs, by which we could understand how this animal resists pathogens and then bring the knowledge into crab farming to maintain the animals health. Some immune-related proteins or genes have been reported in crabs including antimicrobial peptides like scygonadin from *Scylla serrata* [1,2], prophenoloxidase (proPO) [3], α_2-macroglobulin [4], anti-lipopolysaccharide factor (ALF) [5–7] and carcinin [8] from various crab species. Besides, many immune factors have been identified from horseshoe crab and LPS challenge could induce hemocyte exocytotic degranulation which results in the secretion of different defense molecules such as coagulation factors, antimicrobial peptides and lectins [9]. However, these studies provide relatively limited knowledge and in this study, the genes differentially expressed in *S. paramamosain* in response to bacterial LPS were screened using suppression subtractive hybridization (SSH). Two potential immune-related genes were further selected for full-length cDNA sequence cloning and their gene expressions were also investigated in hemocytes and hepatopancreas during the LPS challenge.

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2. Materials and methods

2.1. Experimental animals

Live healthy female *S. paramamosain* (300 ± 50 g in weight) purchased from a local commercial crab farm were acclimated at 25 ± 2 °C for one week before the experiments were carried out.

2.2. LPS challenge and hemocyte preparation

LPS from *Escherichia coli* (L2880, Sigma, USA) was dissolved with a modified crab saline solution (NaCl, 496 mM; KCl, 9.52 mM; $MgSO_4$, 12.8 mM; $CaCl_2$, 16.2 mM; $MgCl_2$, 0.84 mM; $NaHCO_3$, 5.95 mM; HEPES, 20 mM; pH 7.4) [10] to be 5 mg mL^{-1} for animal challenge.

For SSH library construction, haemolymph of one healthy crab (about 300 g in weight) was taken from the base of right chelate leg after 7 days acclimation, and kept in a seawater tank until next sampling. Two millilitres of haemolymph were collected into an equal volume of anti-coagulant solution (NaCl 510 mM; glucose 100 mM; citric acid 200 mM; Na-citrate 30 mM; EDTA-Na_2 10 mM; pH 7.3) [11] followed by centrifugation at 800 × g at 4 °C for 20 min. The resulting hemocyte pellet was used for total RNA isolation (Sample-A). The crab was then challenged with a dose of 0.5 mg kg^{-1} LPS solution at the base of the right fourth leg after one week of first sampling. Haemolymph was sampled and processed as described above 20 h after the LPS challenge (Sample-B). The cDNA was prepared as driver (Sample-A) and tester (Sample-B), respectively, for the following SSH process.

For the gene expression study, 15 crabs were injected with a dose of 0.5 mg kg^{-1} LPS and the other 15 individuals were injected with an equal volume of sterile saline solution as control treatments, respectively. The crabs for each group (3 crabs/group) were separately reared in individual tanks under the same conditions. Meanwhile, three normal crabs were reared in an individual tank as a normal control group. Sampling was performed at different time intervals (0, 3, 6, 12, 24 and 48 h) after LPS challenge. Haemolymph was collected from the third pereiopod, diluted in an equal volume of anti-coagulant solution and centrifuged for 20 min at 800 × g at 4 °C. Hemocyte pellets were preserved in Trizol reagent (Invitrogen) immediately for RNA extraction. Samples from the hepatopancreas were also separately collected from each individual animal and were frozen immediately in liquid nitrogen, and stored at −80 °C for later use.

2.3. RNA isolation and cDNA synthesis

Total RNAs were extracted from samples using Trizol reagent following the manufacturer's instructions. For SSH library construction, SMART PCR cDNAs were synthesized, amplified and digested with Ras I from 1 μg of total RNA for each group using the Super SMART™ PCR cDNA Synthesis Kit (Clontech) according to the manufacturer's protocol.

2.4. Construction of an SSH library

SSH was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech) following the manufacturer's instructions. Briefly, the cDNA synthesized from the crab challenged with LPS (Sample-B) was used as the "tester" cDNA and that from the same crab before LPS challenge (Sample-A) as the "driver" cDNA for the forward subtraction. The SSH was performed as described by the manufacture's instructions. The forward SSH library was then plated on LB agar (supplemented with 100 μg/mL ampicillin, 20 μg/cm^2 X-gal and 12.1 μg/cm^2 IPTG and incubated overnight at 37 °C) for the library screening.

2.5. Identification of positive clones and DNA sequencing

White clones were randomly picked and transferred into 5 mL LB-ampicillin (100 μg/mL) agar plate overnight at 37 °C. The inserted fragment size of the picked clones was identified using PCR and 1% agarose gel electrophoresis. The PCR reaction was performed using 1 μL bacterial culture, primers M13−47 and M13-48, and rTaq DNA polymerase (TaKaRa). The PCR amplification was carried out as: 3 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C; and 3 min at 72 °C for the final extension. Selected clones were sequenced using ABI 3730 automated sequencers (Applied Biosystems, USA) at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China).

2.6. Sequence analysis

The sequences obtained were analyzed using DNAstar 7.0 and GeneTool 1.0 Lite. Homology searches were performed using BLASTn, BLASTx and BLASTp programs, with default parameters against the non-redundant database, by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). The CD-Search service [12] was used to identify the conserved domains (CD) present in predicted protein sequences against NCBI's Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The best annotated hit from the similarity search was retained. Gene ontology (GO) annotation [13] based on BLAST analysis was performed using AmiGO against the GO database (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi). Novel sequences were submitted to GenBank at the NCBI and the accession numbers were assigned.

2.7. Identification of the potential up-regulated genes in LPS-challenged crabs

To confirm the up-regulation of gene expression after LPS challenge from the forward SSH library, two immune-related genes including *Sp*-Cu-ZnSOD and *Sp*-serpin, induced by LPS challenge were selected for in vivo expression study. The candidate genes after LPS challenge were both evaluated in hemocytes and hepatopancreas by quantitative real-time PCR. Total RNA was extracted as described above and quantified with an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences, Sweden). Five micrograms of total RNA for each group was separately reverse transcribed in a final volume of 100 μL using a PrimeScript™ RT reagent kit (Perfect Real Time) (TaKaRa) following the manufacturer's instructions. Real-time PCR was performed in a reaction mixture of 20 μL containing 0.5 ng of total transcribed cDNA, 5 pmol of each gene-specific primer and 10 μL of Power SYBR Green PCR Master Mix (Applied Biosystems, UK). The forward and reverse primers are shown in Table 2. The standard cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Data of raw relative quantification were calculated using 7500 system SDS software version 1.3.1.21 and the actin gene was employed as the internal standard. The healthy group was used as the calibrator. Anova comparison tests were used for statistical analysis by SPSS software (version 11.5). Values were considered to be significant at $P < 0.05$.

2.8. Determination of the full-length cDNA of candidate genes

To isolate the full-length cDNAs of two candidate genes, *Sp*-Cu-ZnSOD and *Sp*-serpin, of *S. paramamosain*, 5′-RACE and 3′-RACE were carried out. Specific primers for each candidate gene cDNA were designed according to the obtained partial cDNA sequence as shown in Table 2. The RACE cDNA were performed with an SMART

Table 1
The genes were categorized in different groups according to GO annotation.

Gene name	GenBank accession	Species with homology to	E-value (Blastp)	Number of genes
1. Metabolism and biogenesis				80
16S rRNA	FJ774905	*Scylla paramamosain*	0.00E+00	1
18S rRNA	FJ774906	*Charybdis variegate*	0.00E+00	1
Acetyltransferase	FJ774764	*Acyrthosiphon pisum*	1.00E-34	1
Aconitate hydratase	FJ774744	*Laccaria bicolor S238N-H82*	3.00E-99	1
Acyl carrier protein	FJ774745	*Drosophila melanogaster*	1.00E-33	1
Adenosylhomocysteinase	FJ774746	*Brugia malayi*	4.00E-51	1
Alcohol dehydrogenase	FJ774658	*Nasonia vitripennis*	2.00E-68	1
Arginine kinase	FJ774747	*Callinectes sapidus*	3.00E-48	1
Arginine N-methyltransferase	FJ774748	*Culex quinquefasciatus*	2.00E-42	1
Aspartyl-tRNA synthetase	FJ774749	*Culex quinquefasciatus*	1.00E-54	1
ATP synthase related gene	FJ774698	*Tribolium castaneum*	4.00E-99	3*
ATP/ADP translocase	FJ774701	*Marsupenaeus japonicus*	6.00E-34	1
Chloride intracellular channel	FJ774704	*Bombyx mori*	2.00E-51	1
Cysteine desulfurase related gene	FJ774913	*Pediculus humanus corporis*	1.00E-54	2*
Cytochrome *c* oxidase related gene	FJ774692	*Aedes aegypti*	2.00E-06	2*
Glucosyl/glucuronosyl transferase	FJ774755	*Culex quinquefasciatus*	5.00E-04	1
Glyceraldehyde-3-phosphate dehydrogenase	FJ774697	*Procambarus clarkii*	3.00E-92	1
Glycosyl-phosphatidylinositol-linked carbonic anhydrase	FJ774680	*Callinectes sapidus*	7.00E-36	1
Inorganic pyrophosphatase	FJ774756	*Nasonia vitripennis*	2.00E-57	1
Lysyl-tRNA synthetase related gene	FJ774911	*Tribolium castaneum*	5.00E-60	3*
L-asparaginase	FJ774757	*Tribolium castaneum*	7.00E-07	1
L-lactate dehydrogenase	FJ774758	*Culex quinquefasciatus*	5.00E-77	1
Lysosomal acid lipase	FJ774759	*Nasonia vitripennis*	1.00E-17	1
Malate dehydrogenase	FJ774760	*Sphyraena idiastes*	1.00E-61	1
Na+/K+-ATPase related gene	FJ774673	*Carcinus maenas*	1.00E-68	2*
NADH:ubiquinone dehydrogenase	FJ774761	*Culex quinquefasciatus*	1.00E-14	1
Phosphatase 2C	FJ774675	*Tribolium castaneum*	4.00E-77	1
Proteasome	FJ774765	*Drosophila melanogaster*	1.00E-48	4*
Pyruvate dehydrogenase kinase	FJ774678	*Apis mellifera*	3.00E-25	1
Ribosomal protein related gene	FJ774826	*Drosophila melanogaster*	3.00E-40	36*
Triose phosphate isomerase	FJ774782	*Metapenaeus ensis*	7.00E-12	1
Ubiquinol–cytochrome *c* reductase	FJ774783	*Artemia franciscana*	2.00E-16	1
Ubiquitin associated protein	FJ774785	*Ornithorhynchus anatinus*	3.00E-06	1
Ufm1-conjugating enzyme related gene	FJ774784	*Culex quinquefasciatus*	2.00E-23	2*
2. Cytoskeletal component organization				15
Adaptin beta	FJ774657	*Acyrthosiphon pisum*	4.00E-145	1
Adaptin delta	FJ774656	*Mus musculus*	7.00E-91	1
Calreticulin	FJ774917	*Paralichthys olivaceus*	8.00E-15	1
Collagen alpha-2(IV) chain precursor	FJ774706	*Ascaris sum*	1.00E-18	1
Gelsolin	FJ774722	*Homarus americanus*	1.00E-32	1
Myosin II	FJ774723	*Tribolium castaneum*	3.00E-64	1
Myosin regulatory light chain 2	FJ774718	*Maconellicoccus hirsutus*	6.00E-85	1
Profilin	FJ774724	*Bombyx mori*	3.00E-23	1
Rab11A	FJ774654	*Macaca mulatta*	3.00E-46	1
Rab35	FJ774725	*Tribolium castaneum*	5.00E-87	1
Septin-2	FJ774772	*Apis mellifera*	2.00E-122	1
Suppressor of profilin	FJ825624	*Culex quinquefasciatus*	3.00E-117	1
Thymosin beta	FJ774741	*Triatoma infestans*	3.00E-37	1
Tubulin related gene	FJ774729	*Homarus americanus*	2.00E-156	2*
3. Biological regulation				50
28 kDa heat- and acid-stable phosphoprotein	FJ774664	*Tribolium castaneum*	6.00E-17	1
ADP ribosylation factor	FJ774666	*Drosophila melanogaster*	1.00E-101	1
Cathepsin A	FJ774750	*Strongylocentrotus purpuratus*	4.00E-29	1
Chaperonin 10	FJ774665	*Danio rerio*	3.00E-26	1
Chromatin modifying protein 1A	FJ774705	*Danio rerio*	1.00E-03	1
Coat-associated protein related gene	FJ774708	*Tribolium castaneum*	5.00E-34	2*
Cyclin t	FJ774662	*Nasonia vitripennis*	2.00E-81	1
Cyclophilin A	FJ774707	*Penaeus monodon*	8.00E-34	1
DEAD box polypeptide	FJ774714	*Bos taurus*	4.00E-83	1
Dendritic cell protein	FJ774655	*Aedes aegypti*	2.00E-18	1
Elongation factor	FJ774751	*Procambarus clarkii*	5.00E-52	4*
Eukaryotic translation initiation factor related gene	FJ825623	*Penaeus monodon*	1.00E-76	3*
Failed axon connections protein	FJ774922	*Aedes aegypti*	8.00E-13	1
FK506-binding protein 1A	FJ774667	*Drosophila melanogaster*	1.00E-42	1
GDP dissociation inhibitor	FJ774710	*Drosophila melanogaster*	1.00E-34	1
Histone H1	FJ774715	*Mytilus galloprovincialis*	4.00E-07	1
Importin 2	FJ774909	*Ictalurus punctatus*	2.00E-16	1
Importin 3	FJ774683	*Tribolium castaneum*	1.00E-20	1
LUC7-like	FJ774717	*Canis lupus familiaris*	5.00E-30	1
Mesoderm development candidate	FJ774693	*Culex quinquefasciatus*	4.00E-10	1
Nop56	FJ774823	*Tribolium castaneum*	1.00E-19	1

(*continued on next page*)

Table 1 (*continued*)

Gene name	GenBank accession	Species with homology to	E-value (Blastp)	Number of genes
Protein FAM60A	FJ774734	*Apis mellifera*	2.00E-41	1
Putative RNA polymerase II transcriptional coactivator	FJ774736	*Tetrahymena thermophila SB210*	2.00E-10	1
Replication factor	FJ774767	*Macaca mulatta*	2.00E-129	1
RNA binding motif protein	FJ774699	*Gallus gallus*	1.00E-09	1
RuvB-like 2	FJ774676	*Apis mellifera*	3.00E-101	1
Sex-determining protein fem-1	FJ774737	*Nasonia vitripennis*	1.00E-59	1
Small nuclear ribonucleoprotein polypeptide A	FJ774874	*Xenopus tropicalis*	2.00E-47	1
Small nuclear ribonucleoprotein SM D3	FJ774875	*Tribolium castaneum*	3.00E-25	1
Sodium-dependent phosphate transporter	FJ774738	*Culex quinquefasciatus*	8.00E-28	1
Splicing factor related gene	FJ774775	*Culex quinquefasciatus*	8.00E-79	2*
T-complex protein 1 related gene	FJ774781	*Aedes aegypti*	2.00E-124	5*
TIA-1 homolog	FJ774742	*Bombyx mori*	2.00E-84	1
Trafficking protein related gene	FJ774727	*Mus musculus*	1.00E-43	2*
Translocon-associated protein, delta subunit	FJ774743	*Tribolium castaneum*	6.00E-10	1
Vacuolar protein sorting 4B	FJ774733	*Danio rerio*	2.00E-35	1
Zinc finger related gene	FJ774686	*Macaca mulatta*	3.00E-34	2*
4. Signal transduction				11
Arfaptin-2	FJ774709	*Canis lupus familiaris*	1.00E-72	1
G protein related gene	FJ774713	*Homarus americanus*	2.00E-153	2*
Innexin inx2	FJ774668	*Homarus gammarus*	6.00E-09	1
MAP kinase-interacting serine/threonine	FJ774689	*Tribolium castaneum*	2.00E-119	1
Ezrin/radixin/moesin homolog	FJ825625	*Drosophila melanogaster*	1.00E-55	1
Rab1A	FJ774681	*Lymnaea stagnalis*	4.00E-57	1
Rab5	FJ774735	*Aedes aegypti*	2.00E-35	1
Rap1A	FJ774682	*Nasonia vitripennis*	2.00E-35	1
Rheb	FJ774684	*Nasonia vitripennis*	1.00E-75	1
Serine/threonine kinase 32B	FJ774685	*Apis mellifera*	7.00E-16	1
5. Response to stimuli				9
Catalase	FJ774660	*Fenneropenaeus chinensis*	0.00E+00	1
HSP16.2	FJ774659	*Caenorhabditis elegans*	4.00E-10	1
HSP70	ACE79213	*Scylla paramamosain*	1.00E-145	1
HSP90	FJ774653	*Callinectes sapidus*	0.00E+00	1
Matrix metalloproteinase	FJ774679	*Bombyx mori*	0.00E+00	1
Metallothionein	FJ774671	*Scylla serrata*	2.00E-13 (Blast X)	1
Oncoprotein nm23	FJ774730	*Litopenaeus vannamei*	5.00E-70	1
Prohibitin	FJ774731	*Litopenaeus vannamei*	3.00E-123	1
Solute carrier family	FJ774695	*Canis lupus familiaris*	3.00E-35	1
6. Immune system process				14
CD53 antigen	FJ774702	*Bos Taurus*	1.00E-08	1
Cu–ZnSOD	FJ774661	*Macrobrachium rosenbergii*	6.00E-40	1
Ferritin	FJ774652	*Fenneropenaeus chinensis*	6.00E-79	1
Flotillin-1	FJ774690	*Culex quinquefasciatus*	2.00E-65	1
Histone H2A	FJ774663	*Nasonia vitripennis*	8.00E-65	1
Integrin	FJ774672	*Pacifastacus leniusculus*	3.00E-11	1
Integrin kinase	FJ774674	*Nebalia hessleri*	3.00E-58	1
Kazal-type serine protease inhibitor	FJ774716	*Phytophthora infestans*	3.00E-07	1
Leucine-rich repeat (LRR) protein	FJ774669	*Penaeus monodon*	2.00E-35	1
Lysosomal-associated membrane protein	FJ774691	*Pan troglodytes*	3.00E-06	1
Serine protease homology	FJ774773	*Drosophila melanogaster*	4.00E-21	1
Serpin	FJ774918	*Cyanothece* sp. *CCY 0110*	4.00E-49	1
Thioredoxin-like protein	FJ774740	*Nasonia vitripennis*	2.00E-16	1
Translationally controlled tumor protein	FJ774739	*Marsupenaeus japonicus*	1.00E-66	1
7. Unknown				92
Hypothetical protein related gene	FJ774799	*Branchiostoma floridae*	9.00E-18	2*
Unknown proteins	FJ790492			90

Among 271 genes involved in different biological processes, 80 genes (29.5%) were categorized as a group in association with metabolism and biogenesis; 15 were associated with cytoskeletal component organizations (5.5%); 50 were involved in a biological regulations group (18.5%); 11 were listed as a signal transductions group (4.1%); 9 were response to stimuli (3.3%); 14 were taken part in immune system processes (5.2%); and 92 genes (33.9%) were classified as an unknown-function gene group. *Only one GenBank identity accession number and the highest Blastp E-value were given here because of the table capacity limitation.

RACE cDNA Amplification kit (Clontech, USA) according to the manufacturer's protocol. PCR conditions were as follows: 94 °C for 1 min, 35 cycles consisting of 94 °C for 40 s, 60 °C for 30 s and 72 °C for 2 min. The final extension was carried out at 72 °C for 7 min. The expected DNA fragment was eluted from agarose gel and ligated to pMD18-T vector (Takara). The ligation product was transformed into *E. coli*. The recombinant clones were identified by bacterial colony PCR as described above. Briefly, white clones were randomly selected and cultured overnight at 37 °C. The recombinant clones were identified by PCR. The PCR reaction was performed using 1 μL bacterial culture, primers M13–47 and M13–48, and rTaq DNA polymerase (TaKaRa). The amplification conditions were performed as: 94 °C 3 min for denaturation; followed by 30 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 90 s; and with a final extension at 72 °C for 3 min. The positive recombinant clones were sequenced at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China). The resulting sequences were verified and subjected to cluster analysis.

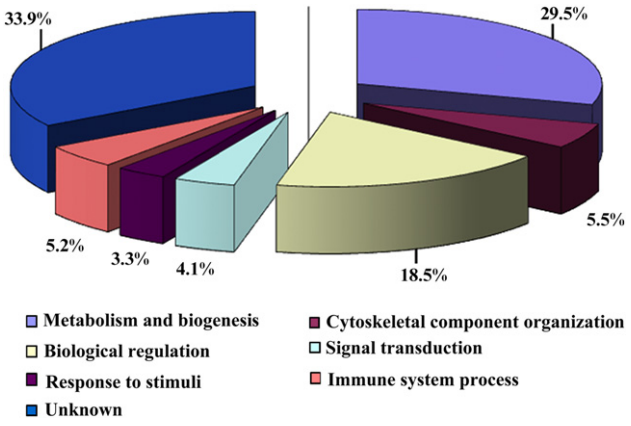

Fig. 1. Distribution of up-regulated genes screened from SSH library from *S. paramamosain* challenged with LPS. The genes were categorized in different groups according to GO annotation. Among 271 genes involved in different biological processes, 80 genes (29.5%) were categorized as a group in association with metabolism and biogenesis; 15 were associated with cytoskeletal component organizations (5.5%); 50 were involved in a biological regulations group (18.5%); 11 were listed as a signal transductions group (4.1%); 9 were response to stimuli (3.3%); 14 were taken part in immune system processes (5.2%); and 92 genes (33.9%) were classified as an unknown-function gene group.

3. Results

3.1. Sequencing and analysis of clones from the SSH library

A forward subtracted cDNA library was constructed from the hemocytes of the *S. paramamosain* challenged with LPS using SSH. Due to lack of information on immune-related genes in mud crabs, we randomly selected and sequenced 721 clones on the middle scale in this library. Among these genes, 269 potentially functional genes and two rRNA genes (including 18S and 16S rRNA) were submitted to GenBank after all sequences were trimmed to exclude redundancy, assembled and edited. Of 271 genes, 269 translatable DNA sequences were predicted to be proteins, and the putative amino acid sequences were searched for conserved domains and proteins using the CD-Search service and BLASTp with default parameters against NCBI's Conserved Domain Database and the non-redundant database. In these genes, 179 (66.1%) were identified using CD with creditable expectation values (E-value $\leq 10^{-3}$). Among 271 genes, 92 (33.9%) showed no significant similarity in sequences to homologous proteins searched by BLASTx and BLASTp. Gene functions were predicted by AmiGO (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) in search of the Gene Ontology database (Table 1). All 271 genes were categorized into seven groups including metabolism and biogenesis, cytoskeletal component organization, biological regulation, signal transduction, response to stimuli, immune system process and unknown (Fig. 1). Among these genes, 179 (66.1%) were annotated to be involved in different biological processes, while 92 genes (33.9%) were classified as an unknown-function gene group. It was noted that only 18 of the 271 genes (6.6%) had previously been reported in other crustaceans and most of the screened genes in the present study showed less similarity to known sequences based on BLASTn results (Table 1), suggesting that 253 genes (93.4%) were found for the first time in *S. paramamosain*.

3.2. Analysis of expression pattern of differential expressed genes in hemocytes and hepatopancreas using quantitative real-time PCR

To confirm the increased gene expression of the SSH library clones, two up-regulated cDNAs screened from the SSH library encoding *Sp*-Cu-ZnSOD gene and *Sp*-serpin were selected for in vivo expression study. Because of the important roles of hemocytes and hepatopancreas for generation of immune defense molecules, the differential expression pattern of each gene in hemocytes and hepatopancreas during the time course of LPS challenge was analyzed using real-time PCR. The primers used were described in Table 2. After LPS challenge, *Sp*-Cu-ZnSOD gene was significantly up-regulated in hemocytes (5.3-fold, Fig. 2A) and hepatopancreas (4.7-fold, Fig. 2B) at 3 h ($P < 0.01$) and maintained high expression to 6 h (3.8-fold and 4.3-fold for hemocytes and hepatopancreas, respectively). However, for *Sp*-serpin transcripts, the increased expression in hemocytes was found only at 3 h (24.6-fold) and its expression decreased from 6 h after LPS challenge (Fig. 2C). In hepatopancreas, the *Sp*-serpin gene was also observed for a significantly increased expression at 3 h (10.7-fold, $P < 0.05$) and remained highly expressed till 6 h (13.9-fold) after LPS challenge (Fig. 2D). Both genes decreased after 12 h and no obvious up-regulation was observed at 48 h after challenge even though a slight increase occurred at 24 h for *Sp*-Cu-ZnSOD gene in the hemocytes, whereas for the *Sp*-serpin gene a relative increased expression was observed at 48 h again in the hepatopancreas (2.7-fold, Fig. 2). These results suggested that both genes had tissue dependent expression patterns in the crab after LPS challenge.

3.3. Determination of full-length cDNA sequences of differential expressed genes identified by SSH

3.3.1. Sp-Cu-ZnSOD gene

The full-length cDNA of *Sp*-Cu-ZnSOD contained 1481 bp including 110 bp in the 5′ untranslated region (UTR), an open reading frame (ORF) of 621 bp, and a 750 bp in the 3′-UTR with a poly A signal (Fig. 3). The ORF of *Sp*-Cu-ZnSOD cDNA consists of 307 amino acids (aa) including a 23 aa signal peptide predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/) in the N-terminus. The calculated molecular mass of the mature protein was 20 kDa with an estimated isoelectric point (p*I*) of 6.8. The full-length cDNA sequence and its deduced amino acid sequences were deposited in the NCBI GenBank under accession number FJ774661. Analysis of amino acid sequences showed that was similar to the extracellular copper-zinc superoxide dismutase (ecCuZnSOD) from other species (66.4% homology in identity) including *S. serrata* (ABL63467), *Portunus trituberculatus* (ACI13851) and *Callinectes sapidus* (AAF74772) (the alignment data not shown). *Sp*-Cu-ZnSOD contains (1) two Cu and Zn signatures from 87 to 97 (GFHIHQWGVVD) and from 185 to 196 (GNAGSRVACCTV); (2) four copper binding sites (His89, 91, 106, and 167); (3) four zinc binding

Table 2
Primers used for RACE PCR and real-time PCR.[a]

Gene name	Primers for analysis of differential gene expression: Forward	Primers for analysis of differential gene expression: Reverse	PCR product size (bp)
Actin	GCCCTTCCTCACGCTATCCT	GCGGCAGTGGTCATCTCCT	185
Cu-ZnSOD	GGGGATGGGAAACAACTCTGGAT	GGTGCCTTGGTTAAATACACGGTGC	87
Serpin	CCAGTGACGGACCTGATGTTA	TAGCGGGTGCGAGCCTTT	103

[a] The Forward and Reverse primers were used for 3′ end and 5′ end RACE of Cu–ZnSOD or Serpin gene, respectively. The same primer pairs were also used for real-time PCR determination.

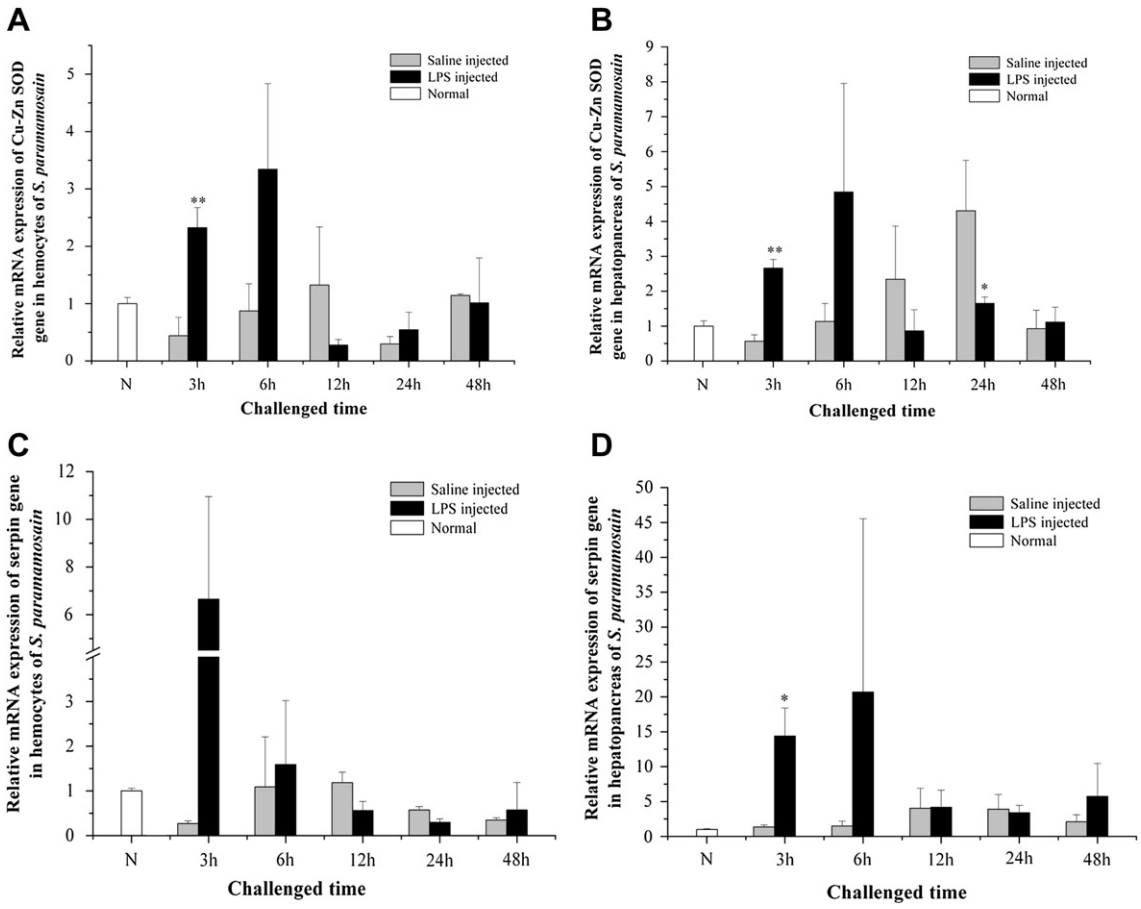

Fig. 2. Gene expressions in hemocytes and hepatopancreas of *S. paramomosain* with LPS challenge. A and B showed the relative mRNA expression of SOD gene in hemocytes and hepatopancreas, respectively. C and D showed the relative mRNA expression of SPI gene in hemocytes and hepatopancreas, respectively. SOD: *Sp*-Cu-ZnSOD; SPI: *Sp*-serpin. * and ** indicated statistically significant at $P < 0.05$ and $P < 0.01$, respectively.

sites (His106, 114, 123, and 126); (4) two cysteines (cys100 and cys193) that form a disulfide bridge; (5) a putative N-linked glycosylation site (NVTG, same as ICSOD from *Crassostrea gigas* AJ496219) [14] and (6) a signal peptide (Fig. 3).

3.3.2. *Sp-serpin like serine protease inhibitor gene*

The full-length cDNA of *Sp*-serpin gene was composed of 1792 bp with an ORF of 1122 bp encoding 374 amino acid residues. Analysis of amino acid sequences indicated that *Sp*-serpin shares low identity (36.7%) with the serpins from other species such as *Tachypleus tridentatus* (BAA03374), *Pacifastacus leniusculus* (CAA57964), *Penaeus monodon* (AAP92780), and *Fenneropenaeus chinensis* (ABC33916) (the alignment data not shown). There was no signal peptide present in this sequence suggesting that this *Sp*-serpin is a non-secretory protein. The ORF of *Sp*-serpin contained a modified serpin signature of FYVNQPSIIFIR (amino acid residue 342–353) and a serpin motif EEGTK (amino acid residue 320–324) (Fig. 4). Between these two serpin motifs, a reactive site loop with a stretch of 17 amino acid residues containing the cleavable bait attacked by proteinase was observed. The calculated molecular mass of the mature protein was 41 kDa with an estimated pI of 6.6. The full-length sequence was deposited in the NCBI GenBank under accession number FJ774918.

4. Discussion

LPS is a structural component of the outer membrane of Gram-negative bacteria and in vertebrates has strong innate immune stimulatory properties by triggering the release of proinflammatory cytokines from various target cells [15,16]. So far, the LPS stimulation pathway in the crab *S. paramamosain* is not yet clear. In order to find differentially expressed genes and to search further for new genes in LPS-challenged crabs, a forward subtracted cDNA library was constructed from the hemocytes of the LPS-challenged *S. paramamosain* using SSH and the potential ESTs or gene sequences were randomly screened on the middle sequencing scale (721 clones). Importantly, many genes related to LPS stimulation responses revealed in mammals like chaperones, transport proteins, and clathrin, have also been identified from our SSH library. This strongly suggests that a successful induction of LPS-induced immune response in crab *S. paramamosain* has been made. These induced genes encode proteins involved in cellular metabolic processes; cytoskeletal component organizations; signal transductions; immune defenses; responses to stimuli and other unknown functions. In general, our investigation of *S. paramamosain* to LPS challenge revealed a robust innate immune response in the crab.

4.1. *Cellular metabolic process*

Some up-regulated genes related to protein translation/folding and ubiquitin dependent protein catabolic process were isolated from the SSH library, suggesting a strong metabolic activity occurred under LPS stimulation in *S. paramamosain*. It is well-known that, in mammals, ubiquitin and ubiquitin-like proteins play important roles in multiple biological processes including the cell cycle,

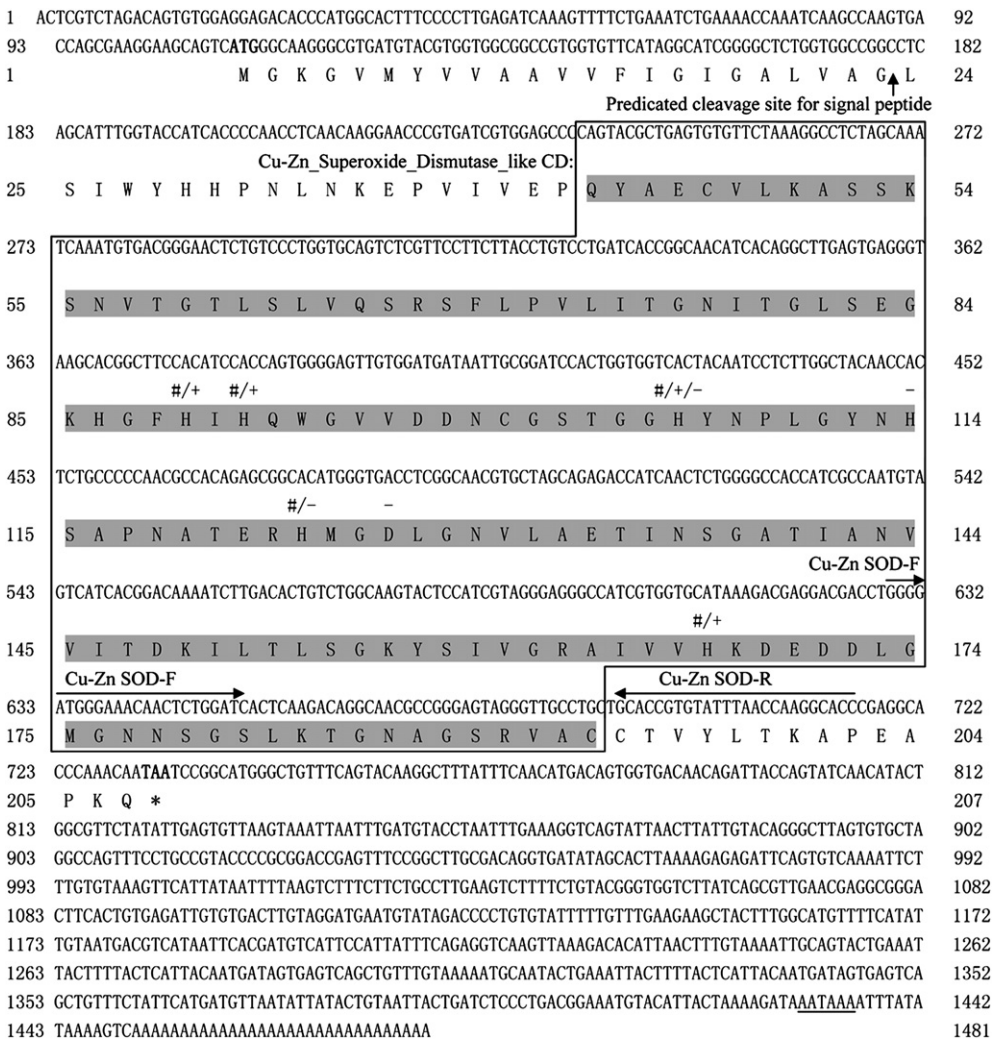

Fig. 3. Complementary DNA and predicted amino acid sequences of *Sp*-Cu-ZnSOD (FJ774661) from *S. paramamosain*. The polyadenylation signal was underlined and the stop codon was indicated by asterisks (*). Primers for 5′ end and 3′ end RACE were shown with arrows (5′–3′). The same primer pairs (Cu-ZnSOD-F and Cu-ZnSOD-R) were also used for the mRNA transcripts detection by real-time PCR. The organization of the predicted conserved domains (CD) using the CD-Search service were framed. #: active sites; +: Cu^{2+} binding sites; −: Zn^{2+} binding sites.

development and the immune response. A series of three enzymes known as E1 (activating), E2 (conjugating), and E3 (ligating) involved in these biological reactions have been well studied. The gene encoding ufc1 exhibits the characteristic E2-like catalytic core domain which enables classification of ufc1 as an E2-like enzyme [17]. Ufc1 acts in the recently discovered ufm1-uba5-ufc1 ubiquination pathway which is found in metazoan organisms [18]. A gene encoding *Sp*-ufc1 was described for the first time from a crustacean in this study. The increased gene expression of *Sp*-ufc1 suggests that *Sp*-ufc1 is related to the immune response in *S. paramamosain* against LPS challenge.

4.2. Cytoskeletal component organization

A number of genes related to cytokinesis, such as septin-2, Rab35, profilin and suppressor of profilin, were found in the SSH library. These genes may play critical roles in cytokinesis and endocytosis since they were up-regulated by LPS challenge in the crab.

Septins are a conserved GTPase family and are incorporated into distinct structures during cell division and differentiation. Mammalian septins show diverse roles in protein folding, cytokinesis, and vesicle trafficking [19–21]. They exhibit diverse effects on the actin cytosketeton, and microtubules if there was loss of or a reduction in the expression of specific septins. A septin-2 like gene was isolated for the first time from a crustacean in our SSH library, indicating a putative role of this molecule in the response to LPS in the crab.

Rab GTPases are key intracellular transport regulators in eukaryotes and they act in vesicle formation, motility, docking, and fusion. For instance, Rab35 regulates endocytic recycling together with its regulator receptor-mediated endocytosis in *Caenorhabditis elegans* [22]. In addition, Rab35 was found on the cell surface and endosomes, where it was proposed to play a role in recycling of transferrin and other receptors [23,24]. Recent studies have characterized that Rab35 and Rab11 in the endocytic pathway and as a regulator of cytokinesis [23,25]. From our SSH library, several Rab family protein genes like Rab35, Rab11 and Rab1A showed up-regulation, implying some roles of these molecules in the host defenses against LPS challenge.

Profilin acts by sequestering actin monomers and inhibiting actin polymerization. It plays important roles in membrane trafficking, small GTPase signaling, nuclear activities, neurological diseases and tumor generation [26]. Recent studies demonstrated that the human profilin-1 works as suppressor of tumorigenicity of

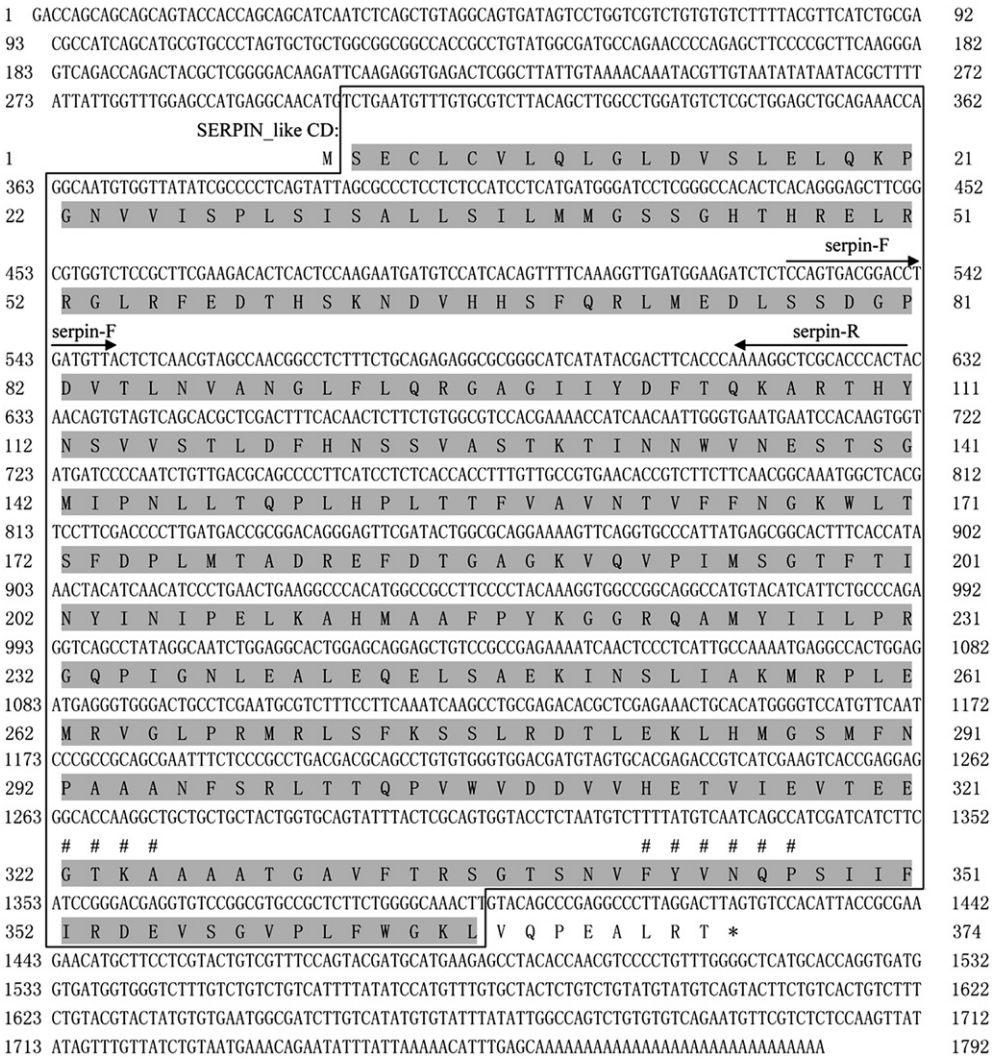

Fig. 4. Complementary DNA and predicted amino acid sequences of *Sp*-serpin (FJ774918) from *S. paramamosain*. The stop codon was indicated by asterisks (*). Primers for 5′ end and 3′ end RACE were shown with arrows (5′–3′). The same primer pairs (Serpin-F and Serpin-R) were also used for the mRNA transcripts detection by real-time PCR. The organization of the predicted CD using the CD-Search service was framed. #: reactive center loop.

breast cancer cells [27]. In shrimps, profilin showed up-regulation after *Vibrio* challenge [28] and WSSV challenge [29]. The increase of profilin gene transcripts after LPS challenge might suggest a putative role of profilin in the immune defense against bacterial infection in crab. Above all, the up-regulation of several genes involved in actin microfilament formation such as Rab35, profilin, gelsolin-like gene, and thymosin may also affect the capacity of hemocytes to phagocytose invading bacteria. Future work is still needed to verify this hypothesis.

4.3. Signal transduction

Several genes encoding proteins (like ERM-homology, G protein family, and MAP kinase) involved in the process of signal transductions showed up-regulation against LPS challenge in the SSH library. The ERM family belongs to cytoskeletal proteins but recently was found to participate in signaling T cell function, including formation of the immunological synapse and spreading of the T cell membrane adjacent to APC [30]. The initiation and maintenance of the immunosynapse needs the interaction of surface molecules with the cytoskeleton. The ERM family of cytoskeletal proteins plays a crucial role in this remodeling, especially for the exclusion of glycoproteins like CD43 from the synapse area [31]. These transitions are likely to involve the dephosphorylation and rephosphorylation of ERM proteins and physical separation of ERM away from the F-actin network in areas of microclusters and at the T cell APC interaction area [32]. To our knowledge, no report of ERM family has been available from any crustacean, and the role of ERM family remains unknown in crustaceans. The up-regulation of *Sp*-ERM family homology might indicate a possible important role of this family involved in anti-stress or antimicrobial infection. It would be interesting to do further experiments for identifying the putative role of ERM family in crustaceans.

The G proteins function as molecular switches and their activities are controlled by the particular form of guanosine nucleotide cofactors to which they are bound. One form of small G proteins is the Rab GTPase family and several members, including Rab1A, Rab5, Rab11a and Rab35, were isolated from our SSH library. The Rab35 has been discussed in the cytokinesis above and here we focus on the role of Rab5 in signaling transduction and infection defense. As shown earlier there is a tight crosstalk between receptor signaling and membrane trafficking [33]. Activation of many hormone and growth factor receptors results in their trafficking to lysosomes in which they are degraded, and several

signaling pathways need translocation of receptors from the plasma membrane into endosomes. Rab GTPases are one of the various regulators that relay receptor signaling to the endosomal membrane trafficking machinery. Rab5, one of the endocytic GTPases, mediates cargo sequestration into and budding of endocytic vesicles, the uncoating of clathrin-coated vesicles, vesicle motility along microtubules and the tethering of vesicles to acceptor membranes. It is a hub for crosstalk between signaling and trafficking. Activation of the epidermal growth factor (EGF) receptor leads to conversion of Rab5 into its active GTP bound form, thereby stimulating trafficking of the receptor along the early endocytic pathway [34]. Activation of Rab5 downstream of the EGF receptors will result in both facilitating receptor trafficking by the stimulation of endosomal fusion and transmitting signals for cytoskeletal regulation. Importantly, Rab GTPases like Rab5 also play crucial roles in pathogen infection. It is necessary for the group B coxsackievirus to enter epithelia through the internalization of tight junctions [35]. Meanwhile, Rab5 is in particular required in the early phagosome necessary for successful phagocytosis [36]. However, the distinct roles of the Rab5 and other Rab GTPase members, up-regulated in the crab after LPS challenge remain to be elucidated.

Interaction of innate immune cells with microbial components results in the activation of multiple signaling pathways, including the family of IRAK and PI3 kinase, finally leading to the activation of MAP kinase pathway and multiple transcription factors. One of these critical transcription factors is nuclear factor (NF)-κB, which binds to the promoter regions of many cytokine and chemokine genes and initiates their transcription [37]. MAP kinases play critical roles in this process. In mammalian macrophages, MAP kinase was suggested to be activated by several external stimuli like LPS and *E. coli* [38]. MAP kinase is also involved in the uptake of bacteria, latex beads and LPS in medfly [39]. Further, MAP kinases control gene expression by facilitation of chromatin remodeling and activation of large numbers of transcription factors like activating protein [40]. Therefore, MAP kinases play a crucial role in multiple cellular processes such as cell proliferation, differentiation, stress response, apoptosis and host immune defense. In this study, several clones related to the serine/threonine kinases and MAP kinases were also isolated from the SSH library, suggesting the putative important roles of those factors involved in antibacterial infection in a crustacean.

4.4. Immune defense

Transcripts of proteins involved in invertebrate immunity like integrin, serpin, proteinase inhibitor, Cu-ZnSOD, flotillins and ferritin genes were also screened from the SSH library.

Phagocytosis is one of the earlier immune responses in invertebrates. This response is an evolutionarily conserved complex process based on recognition, engulfment and intracellular destruction of invading bacteria. Phagocytosis is triggered by the attachment of the phagocyte to the target particle and followed by cytoskeleton modification, internalization and destruction of the engulfed target within a phagosome [41]. The integrin family proteins are critical phagocytic receptors both in invertebrate hemocytes and in mammalian phagocytes. Many integrins contain α and β subunits which serve as heterodimeric transmembrane receptors recognizing the RGD peptide as a binding site involved in bacterial phagocytosis and encapsulation [42]. In arthropods, integrins have been shown to have important functions including binding to peroxinectin and involvement in the cell adhesion process [43], regulating bacterial phagocytosis in medfly hemocytes [44], and WSSV infection in shrimp [45]. The enhanced gene transcripts of *Sp*-integrin suggest that it might also play an important role in the host defense against bacterial infection in crab.

Proteinase inhibitors play crucial roles in host defense systems including blood coagulation, prophenoloxidase activation, pathogen digestion, apoptosis, complement system and cellular remodeling. There is a relatively high concentration of serine proteinase inhibitors, like Kazal-type inhibitors (KPIs), Kunitz-type inhibitors, the α-macroglobulins and serpins, in the haemolymph of insects and other arthropods [46]. The KPIs family has been studied in a various range of organisms from protozoans to mammals. A recent study showed that a KPI was characterized as a specific protein marker for the semigranule cells involved in the hematopoiesis in freshwater crayfish [47]. Serpins are a family of both extracellular and intracellular proteins that inhibit irreversibly against S1 (IPR001254), S8 (IPR000209), and C14 (IPR002398) peptidases. Recently, many functional studies on serpins have been carried out like in the Toll signaling pathway activation and immune response in *Drosophila* [48], regulation of coagulation in *T. tridentatus* [49], and proPO activation regulations in *Manduca sexta* [50], *Anopheles gambiae* [51], and *P. leniusculus* [52]. Interestingly, the KPIs and serpins have also been shown to participate in bacterial [53] and viral infections [54–56] in crustaceans, demonstrating a key role of proteinase inhibitors in the host immune defenses against pathogen infection. The increased gene expression of KPI and serpin from *S. paramamosain* SSH library after LPS challenge is likely to indicate that these two proteinase inhibitors may function in protecting the host from bacterial infection. Further functional work will be focused on elucidating this hypothesis.

The superoxide dismutase (SOD) catalyzes the conversion of superoxide radicals to molecular oxygen and hydrogen peroxide. Three evolutionarily distinct SOD families are characterized from eukaryotes which are distinguished by the metallic ions presenting at the active site: copper-zinc (Cu-ZnSOD), manganese SOD (MnSOD) and iron SOD (FeSOD) [57]. It has been previously shown that the hyaline cells of shore crab, *Carcinus maenas*, could produce superoxide anions when stimulated by LPS [58]. In the present study, the LPS stimulus increased the SOD gene expression which might enhance the host resistance to a pathogen infection. Moreover, the ecCuZnSOD transcript in hemocytes was significantly increased by injection of β-glucan or peptidoglycan in *S. serrata*, suggesting that ecCuZnSOD gene expression is involved in the immune system against challenge [59]. Further, ecCuZnSOD transcripts/activities have also been described to increase under different challenge conditions using different treatments like dsRNA or lower ambient ammonia in Chinese mitten crab *Eriocheir sinensis* [60,61], Cd in marine crab *Charybdis japonica* [62], β-glucan in white shrimp *Litopenaeus vannamei* [63]/freshwater prawn *Macrobrachium rosenbergii* [64], laminarin or LPS in freshwater crayfish *P. leniusculus* [47], and β-1, 3-glucan or other stimuli in shrimps [65–68]. The induction of ecCuZnSOD expression involves several regulation pathways such as AP1 and NF-κB which are activated by ROS participating in the up-regulation of Cu-ZnSOD [69]. In this study, the *Sp*-Cu-ZnSOD transcript was significantly increased between 3 and 6 h after LPS challenge both in hemocytes and hepatopancreas (Fig. 2A and B). This result suggests that LPS may activate the immune system of *S. paramamosain* followed by increase of SOD activity for self-protection.

Flotillins is a family of raft-associated integral membrane proteins which belongs to a larger class of integral membrane proteins carrying an evolutionarily conserved domain called the prohibitin homology (PHB) [70]. Recent data have provided novel insights into the functions of flotillin and other PHB domain proteins. For example, Flotillin-1 has been implicated in numerous cellular processes including phagocytosis, cell proliferation,

neuronal regeneration, and progression of diseases like Alzheimer's disease [70]. We found two genes belonged to the flotillin family, including *Sp*-flotillin and *Sp*-prohibitin (discussed in the stress section) from SSH library. The *Sp*-flotillin is reported for the first time from a crustacean. Taken together, the enhanced gene expression of *Sp*-flotillin suggested some important roles exerted by them in the immune responses against LPS challenge and further putative functions in antimicrobial infections.

Ferritin is an iron storage protein playing key roles in the metabolism of iron as well as in the detoxification of this metal. The *Sp*-ferritin showed increased expression after LPS challenge in the crab. It has been found in several studies in which ferritin is also involved in viral infection in shrimps [29,71,72] and clotting in fly [73], implying the crucial role of ferritin in the host defense reactions.

4.5. Stress-related genes responding to stimuli

Several genes related to stress such as prohibitin and oncoprotein nm23 were also found in the SSH library. Prohibitin gene has been previously identified in shrimp and its gene expression was induced to the highest level in gills after 24 h of WSSV challenge [29]. It was originally described as an inhibitor of cell proliferation, and is a highly conserved protein family [74]. Known as a mitochondrial inner-membrane protein, it has been suggested to be a chaperone protein involved in the stabilization of mitochondrial proteins [75]. Prohibitin is also thought to function in cell proliferation and senescence, stomatin involved in ion channel regulation and plant defenses of hypersensitive-induced reaction family, playing a role in resistance to pathogen attack by hypersensitive responses culminating in cell death [76]. Prohibitin may target to lipid rafts in addition to act as chaperone molecules in the mitochondria. It functions in the negative regulation of transcription and recent data suggest that human prohibitins are localized in the nucleus and can regulate transcription activity via interaction with various transcription factors like the steroid hormone receptors [77].

4.6. Unknown-function genes

Ninety two genes with unknown functions were obtained from the SSH library. These up-regulated genes are of particular interest because of their potential functions probably associated with crab immune defense. To further confirm the up-regulated gene expression from the SSH library, two up-regulated genes, *Sp*-Cu-ZnSOD and *Sp*-serpin screened from this library were randomly selected to test their expression patterns after LPS challenge for 48 h. Both genes were dramatically induced to express at 3 and 6 h after LPS challenge showing an early response to LPS and demonstrating that a useful SSH library was constructed in the study.

In conclusion, an up-regulated SSH library was constructed successfully from the hemocytes of *S. paramamosain* challenged with LPS. The ESTs screened from the library encode various molecules potentially associated with different biological processes, which are involved in cellular metabolic processes, cytokinesis and biogenesis, signal transduction and biological regulation, immune defense, response to stimuli, and other functions and unknown functions. The information obtained from this study is expected to provide new insights into the immune mechanisms of *S. paramamosain* and focus on some target genes in response against specific invading microorganisms in future studies. Taken together, our data provide novel insights into the innate immune responses against LPS challenge in a crustacean *S. paramamosain*.

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