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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