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Short communication

Identification of differentially expressed genes in the spleens of polyriboinosinic polyribocytidylic acid (poly I:C)-stimulated yellow catfish *Pelteobagrus fulvidraco*

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ABSTRACT

The yellow catfish, *Pelteobagrus fulvidraco* (Siluriformes: Bagridae) is an economically important fish in China. However, genomic research and resources on this species are largely unavailable and still in infancy. In the present study, we constructed a cDNA library following poly I:C injection to screen for immune response genes in the spleens of *P. fulvidraco* using suppression subtractive hybridization (SSH). A total of 420 putative expressed sequence tag (EST) clones were identified at 24 h post-injection, which contain 103 genes consisting of 25 immune response genes, 12 cytoskeleton genes, 7 cell cycle and apoptosis genes, 7 respiration and energy metabolism genes, 7 transport genes, 26 metabolism genes, 10 stress response genes, 9 translational regulation genes, and 71 unknown genes. Real-time quantitative reverse transcription-PCR (qRT-PCR) results revealed that a set of randomly selected immune response genes were identified to be up-regulated after 24 h of poly I:C stimulation compared to controls. Our study provides an annotation of immune genes in detail and insight into fish immunity.

1. Introduction

The adaptive immune response in fish is less developed than that observed in higher vertebrates [1]. Therefore, the innate immune system in fish is believed to be the first line of host defense to kill invaders and prevent infection, in addition to its instructive role in the adaptive immune system of higher vertebrates [2]. Fish act as a bridge between innate and adaptive immunity, and are considered to be an important model in comparative immunology studies [3]. With the increasing release of the whole genome sequences of fish, genome-wide identification and characterization of immune related genes in fish by computational approaches have become possible, including *Danio rerio* [4,5], *Cyprinus carpio* [6], *Larimichthys crocea* [7], and other species. Research into the immune system of fish can provide novel insight into the early events in the development of the adaptive immune system, and are helpful to understand the evolutionary history of the vertebrate immune system.

A species of yellow catfish, *Pelteobagrus fulvidraco*, is an omnivorous freshwater fish that has been widely cultured in Asian countries, especially in China because of its delicious meat and high market value. Recently, the rapid development of the *P. fulvidraco* farming industry has led to increasingly severe outbreaks of infectious disease caused by parasites, bacteria, and viruses, which have resulted in great economic losses [8]. However, little is known about the molecular mechanisms underlying the immune response to such pathogens in this fish species; thereby hindering the establishment of effective measures in disease control. Polyinosinic–polycytidylic acid (Poly I:C) is a double-stranded homopolymer used as a model RNA to study the immune response against viral pathogens [9]. EST analysis is an effective approach for the identification of novel gene functions, homologous gene comparison, and transcription profiles [10,11]. SSH is a much simpler and more powerful technique, and has proven to be useful for studies of immunity in other species of fish [12–15]. In the present study, to further investigate the mechanism of the immune response of *P. fulvidraco* to infection, we constructed a subtractive

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cDNA library following poly I:C administration to screen for genes involved in the immune response using SSH. qRT-PCR was used to investigate the expression patterns of a few randomly selected genes.

2. Materials and methods

2.1. Experimental fish and poly I:C injection

Yellow catfish (200 g ± 10 g) were obtained from Yancheng (Jiangsu province, China) on April 2015 and were acclimated at 24 °C before the experiment commenced. The fish were fed to satiation daily and acclimated for two weeks before use in experiments. Five control fish were injected with 100 μL PBS, taken as controls. Five fish were also injected intraperitoneally with poly I:C and were regarded as the testers. The spleens were collected at 24 h after injection, frozen in liquid nitrogen, and then stored at −80 °C until further use.

2.2. RNA extraction and cDNA synthesis

The total RNA was extracted using Trizol reagent (Aidlab, China) according to the manufacturer's instructions, and treated with RNase-Free DNase (Promega, USA). The RNA integrity was evaluated by gel electrophoresis on denaturing formaldehyde agarose. The mRNA was purified from the total RNA using Dynabeads mRNA Purification kit (Invitrogen, USA) as described in the manufacturer's procedures. The mRNA quantity was determined spectrophotometrically at A 260/280. For double-stranded cDNA synthesis, we used the Super SMART PCR cDNA Synthesis Kit (Clontech, USA) according to the manufacturer's instruction.

2.3. Subtracted cDNA library construction

We used the PCR-Select cDNA Subtraction Kit (Clontech, USA) to generate a cDNA library of the genes differently expressed following poly I:C infection. In brief, we defined the infected sample as the "tester" and control sample as the "driver." We digested the tester and driver cDNA with *Rsa* I endonuclease to yield blunt ends appropriate for ligation. We subdivided the digested tester sample into two portions and ligated each with a different adaptor (Adaptor 1 or 2R). We then performed two hybridizations: 1) we added an excess of the driver sample to two separate tester samples; and 2) we hybridized the sample again by adding a fresh volume of the driver. Since the cDNA presented in both the tester and driver populations reannealed to form double-stranded cDNA, any remaining single-stranded molecules should represent uniquely expressed genes. This single-stranded tester cDNA was then targeted for PCR enrichment via adaptor-mediated priming. We subsequently cloned the PCR products by ligating the amplified DNA fragments into a pMD-19 T vector (TaKaRa, China).

2.4. Expressed sequence tags sequencing and annotation

EST clones were selected randomly from the cDNA library. We then sequenced an initial 420 clones from the M13 forward primer using an Applied Biosystems 3730 analyzer (Sunbiotech, China). Raw sequences were first trimmed to remove the vector sequence and low-quality sequences using the "Crossmatch" program. ESTs with a length less than 60 bp were also discarded. The high-quality sequences were assembled and clustered using the CAP3 program with the default options (http://pbil.univ-lyon1.fr/cap3.php). EST sequences annotation were performed using the blast tools on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast) and DNAStar package (DNAStar Inc. Madison, USA).

2.5. qRT-PCR of candidate gene expression

We used qRT-PCR to verify the expression levels of the most promising candidate immunity genes. The synthesized cDNA was also used as a template for qRT-PCR by the TUREscript cDNA Synthesize Kit (Aidlab, China) following the manufacturer's instructions. The primers were derived from the cDNA sequence using Primer Premier 5.0 software. The sequences of PCR primer pairs for each immune-related gene are presented in Table 1. The gene for β-actin was used as an internal reference and the primers for qPCR were designed using Primer Premier 5.0 software. The qPCR was performed using a Mastercycler ep realplex (Eppendorf, Germany) with the 2 × SYBR Green qPCR Mix Kit (Aidlab, China). Reaction mixtures (20 μL) contained 10 μL 2 × SYBR® Premix Ex Taq™ buffer, 1 μL forward and reverse primers, 1 μL cDNA, and 7 μL RNase-free H_2O. The PCR procedure was as follows: 95 °C for 10 s followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s. At the end of the reaction, a melting curve was produced by monitoring the fluorescence continuously while slowly heating the sample from 60 °C to 95 °C. Each independent experiment was conducted in triplicate, and the relative expression level of a gene was determined using the method described in Ref. [16]. The data were presented as the mean ± standard error (standard error of the mean, SEM). Statistical analysis was performed and considered statistically significant when the value of P was less than 0.05 and highly significant when the P value was less than 0.01.

3. Results and discussion

3.1. Construction of SSH cDNA library and sequencing

A total of 420 clones were randomly selected for isolation and sequencing from the SSH cDNA library, of which 298 EST sequences were obtained with sequence quality acceptable for further analysis. The high-quality sequences were assembled and clustered using the CAP3 program with the default options. A total of 103 unigene annotations were performed using Blastn of NCBI (Table 2), which were then assorted into nine groups. These groups included 25 immune response genes, 12 cytoskeleton genes, 7 cell cycle and apoptosis genes, 7 respiration and energy metabolism genes, 7 transport genes, 26 metabolism genes, 10 stress response genes, 9 translational regulation genes, and 71 unknown genes that were enriched for differentially expressed transcripts (Fig. 1A; Fig. 1B). The EST sequences have been deposited into GenBank under accession no. from JZ923675–JZ923777. Thus, we successfully constructed an SSH cDNA library from yellow catfish spleens, providing a useful resource for characterizing the innate immunity of *P. fulvidraco*.

3.2. Identification of immune response-related genes

3.2.1. Antimicrobial peptides (AMPs)

AMPs are a group of immune response and evolutionarily conserved proteins that play key roles in eliminating infections from various bacteria, fungi, viruses, and protozoa, and regulate the mammalian host physiological processes (e.g., inflammation, angiogenesis, and wound healing) [17]. Recent studies show that fish AMPs (e.g., defensins [18], natural resistance-associated macrophage protein (Nramp) [19], NK-lysin [20], and hepcidin) are important components of the innate immune system [21]. In *P. fulvidraco*, a potential AMP, Nramp was identified. Nramp is an integral membrane molecule similar to that of transporters in vertebrates, is involved in the inhibition of pathogen invasion [22], and has been isolated from *Cyprinus carpio* [19], *Paralychthys olivaceus* [22], and *Scophthalmus maximus* [23]. Nramp also plays an