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Immune gene discovery in the crucian carp *Carassius auratus*

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ABSTRACT

The crucian carp *Carassius auratus* (Cyprinidae) is one of the important fish species in aquaculture. Although the crucian carp has several economic benefits, their immune system and gene information have not been investigated in depth as yet. Here, we performed the transcriptome analysis of *C. auratus* using the pyrosequencing method and selected several immune-related genes. Of unigenes obtained in this species, we identified a number of immune system-related genes (e.g. adhesive protein, antimicrobial protein, apoptosis- and cell cycle-related protein, cellular defense effector, immune regulator, pattern recognition protein, protease, protease inhibitor, reduction/oxidation-related protein, signal transduction-related protein and stress protein) that are potentially useful for studies on fish immunity. To be of public and practical use, we designed primer pairs of each gene from the crucian carp for real-time RT-PCR application and tested the amplicon identity of entire gene sets with the total RNA sample. For comparative analysis, we measured tissue-preferential transcript profiles of selected genes. This study will be helpful to extend our knowledge on the immune system of the crucian carp in comparative aspects and to develop the crucian carp as a potential model organism for aquatic quality monitoring in fish farming.

1. Introduction

The crucian carp *Carassius auratus* is a member of the family Cyprinidae (Teleostei). These fishes are world-wide distributed in freshwater systems such as river, lake, and pond. Previously, *C. auratus* was highlighted as one of most important commercial species in aquaculture including several marine fish and crustaceans as their large production, for their excellent growth traits, good taste, and suitability in culture systems (FAO; Food and Agriculture Organization of the United Nations). Although the crucian carp is highly susceptible to bacterial and pathogen challenges, exploration of immunity at the molecular level is dominated by several aquaculture model animals by diverse experimental approaches with little attention to the crucian carp *C. auratus*. To date, in crucian carp, several immunity-relevant factors such as interferon regulatory factor 7 [63], *CD8α* [46], interferon-stimulated gene ISG15 [64], *GATA3* [49], MHC class I and β-2 microglobulin [52], and *IRF9* [45] were cloned and characterized but have not been reported as yet for the identification of extensive immune genes and their employment for immunity. Therefore, investigation of key immune components and characterization of gene/protein expression of immunity-relevant genes would have priority in the immune research of crucian carp.

Molecular genomic and proteomic approaches are able to provide a better understanding on the intracellular mechanism on the immune system [14,47]. Recently, several Next Generation Sequencing (NGS) technologies enable us to obtain the massive complementary DNA (cDNA) or genomic DNA (gDNA) information, and thus to date the extensive sequence information of important immune-relevant genes has been identified in several fish species and employed for further understanding of immune system [2,14,47,55]. On the usefulness of the crucian carp as a potential model species for aquaculture monitoring, we sequenced transcriptomes for the gene/protein information-based application to

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detect early molecular biomarker genes of the disease induction or pollution in crucian carp.

In this study, we obtained expressed cDNA information by pyrosequencing in the crucian carp, *C. auratus*. Our results are the first report on the extensive identification of various immune mechanism related genes in this species. To employ transcriptional profiling for aquaculture monitoring, we designed primer sets for entire immune-relevant genes of the crucian carp and tested amplicon identity for public and practical use in immunity research. Finally, we supposed that these results would be useful to obtain a better understanding of the mode of action of disease induction by pathogens in the crucian carp, *C. auratus*.

2. Materials and methods

2.1. Fish

The crucian carp *C. auratus* were reared and maintained at the National Institute of Environmental Research (Incheon, South Korea). The fish were maintained at 25 °C with 12 h/12 h light/darkness. The automated water changing system was set for constant flow-through and water quality (pH, salinity, and temperature) was recorded using various instruments (5.71 ± 0.19 mg O_2/L). Experimental fish were anaesthetized on ice and sacrificed by decapitation.

2.2. Total RNA extraction

The main objective of this study was the mining of expressed transcripts that provide a potentially immune-relevant gene pool of the crucian carp for public use in diverse immune research. Therefore, to enrich transcriptome information, we sampled different total RNA pools from immunized or non-immunized tissues. In particular tissues collected from brain, eye, gill, heart, head kidney, intestine, kidney, liver, muscle, skin, spleen, and stomach of 10 fish at the adult stage (≈8 month) of both sexes. Each pooled tissues were homogenized in 3 volumes of TRIZOL® reagent (Molecular Research Center, Inc., Cincinnati, OH) with a tissue grinder and stored at −80 °C until use. Total RNAs were isolated from each pooled tissues according to the manufacturer's instructions. DNA digestion was performed using DNase I (Sigma, St. Louis, Mo). After DNase treatment, the total RNAs were purified using RNeasy Mini Spin Column (Qiagen), and were quantified by absorption of light at 230, 260, and 280 nm (A230/260, A260/280) using a spectrophotometer (Ultrospec 2100 pro, Amersham Bioscience). To check the genomic DNA contamination, we loaded the total RNAs in a 1% agarose gel which contained ethidium bromide (EtBr) and visualized on a UV transilluminator (Wealtec Corp., Sparks, NV). Also, to verify the total RNA quality, we loaded the total RNAs in a 1% formaldehyde/agarose gel with EtBr staining and checked the *18/28S* ribosomal RNAs integrity. After RNA quality was determined, single-strand cDNA was synthesized from total RNA using an oligo$(dT)_{20}$ primer for reverse transcription (SuperScript™ III RT kit, Invitrogen, Carlsbad, CA).

2.3. cDNA library preparation

cDNA library was constructed according to the manufacturer's instructions (Roche Applied Science, Genome Sequencer 20™ System). Briefly, cDNA was fractionated into smaller fragments (300–500 base pairs) that were subsequently blunt-ended using Fragment End Polishing kit supplied by manufacture (Roche Applied Science). Short Adaptors (Two adaptors, A and B, were provided in the GS-20 Library Preparation Kit) were then ligated onto the ends of the fragments. These adaptors were provided priming for both amplification and sequencing of the sample-library fragments. Adaptor B contained a 5′-biotin tag that enables immobilization of the library onto streptavidin-coated beads. After fill-in reaction for nicks, the non-biotinylated strand was used to isolate a single-stranded template DNA (sstDNA) library. The sstDNA library was assessed for its quality and the optimal amount (DNA copies per bead) needed for emulsion PCR (emPCR) was determined by titration.

2.4. Pyrosequencing

Expressed cDNA sequencing was performed with GS 20 sequencer. Constructed sstDNA library was clonally amplified via emPCR, thereby generating millions of copies of templates per bead. The DNA beads were then distributed into picolitre-sized wells on a fiber-optic slide (PicoTiter-Plate™) along with a mixture of smaller beads coated with the enzymes required for the pyrosequencing reaction including the firefly enzyme luciferase. The four DNA nucleotides were then flushed sequentially over the plate. Light signals released upon base incorporation were captured by a CCD camera, and the sequence of bases incorporated per well was stored as a read.

2.5. Assembly, gene annotation, and GO analysis

For assembly, we used the Newbler Assembler software (454 Life Sciences, Roche Diagnostics company) in the assembly stage 1, and the assembled EST translations were aligned with the GenBank NR (non-redundant; including all GenBank, EMBL, DDBJ, and PDB sequence except EST, STS, GSS, or HTGS) amino acid sequence database using BLASTx. The aligned data were arranged according to read length, gene annotation, GenBank number, *E*-value, species, and its accession number. All the bacterial clones were eliminated from the annotated genes. The Gene ontology (GO) functional annotations were assigned by Blast2GO automated sequence annotation tool ([11]; http://www.blast2go.org). Three main categories for biological process, cellular component, and molecular function were obtained by using default parameters. Assembly, gene annotation, and GO analysis were performed at the National Instrumentation Center for Environmental Management (NICEM), Seoul National University (Seoul, South Korea).

2.6. Immune-relevant gene mining

In each section of GO terms, immune-relevant gene mining was performed manually within the expected value threshold of 1.00E-05 that has potential immune-relevant function. Selected genes were arranged with read length, gene annotation, GenBank number, *E*-value, and species with its accession number.

2.7. Primer design and real-time RT-PCR

Primers were designed after comparing exon/intron boundary to genomic DNA using GENRUNNER software (Hastings Software, Inc. N. Y. USA) and confirmed by Primer 3 program (Whitehead Institute/MIT center for Genome Research). To determine the amplicon identity, all the PCR products were cloned into pCR2.1 TA vector, and sequenced with an ABI 3700 DNA analyzer (Bionics Co., Seoul, South Korea). To analyze transcriptional expression patterns in different tissues of the crucian carp, several immune-relevant genes were collected from the assembled EST database, and their transcript abundances were analyzed with real-time RT-PCR. Template cDNA was made by pooled total RNA of each tissue from 10 adult fish of both sexes. Each reaction included 1 μl of cDNA and 0.2 μM primer (real-time RT-F/R or *18S rRNA* RT-F/R). Optimized