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Short communication

Expression and functional characterization of interferon regulatory factors 4, 8, and 9 in large yellow croaker (*Larimichthys crocea*)

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ABSTRACT

Interferon regulatory factor (IRF)-4, 8, and 9 are essential in host defense against pathogens. Here, the full-length coding sequence (CDS), protein structure, and immune response of IRF4/8/9 (lc IRF4/8/9) were characterized in large yellow croaker (*Larimichthys crocea*). The open reading frame of lcIRF4, lcIRF8 and lcIRF9 encoded putative proteins of 463,422 and 406 amino acids, respectively. These IRFs share high sequence homology with other vertebrate IRFs and were constitutively expressed in all examined tissues. IRFs were upregulated following stimulation with *Vibrio anguillarum* in the liver, spleen, and kidney. These results suggest that IRF4/8/9 are vital in the defense of *L. crocea* against bacterial infection and further increase our understanding of IRFs function in innate immunity in teleosts.

1. Introduction

Interferon regulatory factors (IRFs) are transcription factors for bacterial-, viral-, and interferon (IFN)-induced signaling pathways, which are vital for antiviral defense, immune responses, cell growth regulation, and apoptosis (Savitsky et al., 2010). To date, 11 IRFs have been described in vertebrates (Bei et al., 2010): nine (IRF1, IRF2, IRF3, IRF4/Pip/ICSAT, IRF5, IRF6, IRF7, ICSBP/IRF8, and ISGF3γ/p48/IRF-9) (Paun and Pitha, 2007) have been identified in mammals, IRF10 has been reported in birds, and IRF11 has only been identified in fish. Based on the C-terminus and on molecular phylogenetic analyses, the IRF family can be divided into four subfamilies: IRF1-G (IRF1, IRF2, and IRF11), IRF3-G (IRF3 and IRF7), IRF4-G (IRF4, IRF8, IRF9, and IRF10), and IRF5-G (IRF5 and IRF6) (Paun and Pitha, 2007). All IRF proteins share significant homology in their N-terminus region, which consists of a conserved DNA-binding domain (DBD) of approximately 115 amino acid residues (Liu et al., 2016). The DBD contains a helix-loop-helix structure and five conserved tryptophan residues, spaced by 10–18 amino acids, which bind to the promoters of target genes: IFN-sensitive response elements (ISRE) and IFN regulatory elements (IRF-E). In addition, all of the IRFs, except IRF1 and IRF2, contain a transcriptional activation domain named IRF-association domain (IAD) at the C-terminal (Bathige et al., 2012). The IAD mediates the formation of homo- or hetero-dimers with other IRFs and other transcription factors, which are required for accurate promoter targeting and regulation of transcription (Eroshkin and Mushegian, 1999). Despite IRFs have been extensively studied, mainly focused on IRF1-G and IRF3-G, the information of IRF4-G still remains scarce.

Among IRFs, IRF4, 8, and 9 belong to IRF4-G, which play essential roles in the innate immune system. IRF4 expression is restricted to immune cells such as T and B lymphocytes, macrophages, and dendritic cells, where it is a key factor in the regulation of differentiation and is required during the immune response for lymphocyte activation and the generation of immunoglobulin-secreting plasma cells (Haljasorg et al., 2017; Lim1, 2016; Shinnakasu and Kurosaki, 2017; Vander et al., 2017). During cell differentiation, IRF4 mediates immune responses by activating the expression of other genes (Finlay, 2014; Gao et al., 2013; Hu et al., 2002). However, its function in the myeloid lineage is not well characterized. In a recent study, IRF4 was shown to play a vital role during interleukin (IL)-21-mediated Th17 development by modulating the expression profile of the three key transcription factors associated with Th17 differentiation, namely, Foxp3, RORa, and RORgt (Ai et al., 2017; Huber et al., 2009). A subsequent study revealed a novel pathway through which the ROCK2 Rho-

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associated protein kinase phosphorylates IRF4, leading to differentiation of Th17 cells and synthesis of IL-17 (Biswas et al., 2010). The function of IRF8 in the myeloid lineage has been defined. IRF8 is required for macrophage differentiation and for stimulus-induced expression of some critical immune response genes for IL12p40 and IFN-β (Jia et al., 2017; Yoshida et al., 2014). IRF8 deficiency can lead to a pathophysiological state known as chronic myelogenous leukemia (CML) (Tamura et al., 2003). CML is characterized by uncontrolled growth of myeloid cells in the bone marrow and myeloid accumulation in the blood.

IRF9 acts as a transcription factor, and also cooperates with other transcription factors to regulate downstream gene expression (Jiang et al., 2014), such as interferon-stimulated gene factor 3 gamma (ISGF3G), which is an important mediator of immunity and inflammation. IRF9 regulates inflammatory gene expression downstream of the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway through interaction with a STAT 1 and STAT 2 heterodimer to form a transcription factor complex known as ISGF3, a potent stimulator of inflammatory gene transcription (Zhan et al., 2017). In ichthyology research, this has only been identified in zebrafish (Shi et al., 2013) and Japanese flounder (Hu et al., 2014). Although IRF10 belongs to IRF4-G, it has been seldom reported in fish.

Large yellow croaker (*Larimichthys crocea*) is one of the most important maricultured species in Southeast China. In particular, and its aquaculture has suffered from serious disease in recent years, including parasitic, bacterial, and viral infection. However, compared with other high economically important fish, details on the large yellow croaker immune response are lacking. Bacterial burden is an important indicator for innate host immunity in response to infection. *Vibrio anguillarum* is a common pathogenic bacteria in marine and fresh/brackish water fish, which widely distributed in the coastal and estuarine waters, on the body surface and in the intestinal tract of diverse marine organisms, and has caused serious economic losses owing to high morbidity and mortality rates. Typical external clinical signs of vibriosis include weight loss, lethargy, red spots on the ventral, lateral areas of the fish, swollen and dark skin lesions. In recent years, the research of immune genes showed its importance in studying the disease defense system in teleosts. To gain an overview of the *L. crocea* IRF4-G system, we selected three IRF genes belonging to IRF4-G, all of which are involved in the positive feedback regulation of IFNs in other vertebrates: IRF4, IRF8, and IRF9. We have detected, sequenced, and characterized these three genes, as well as their putative peptide products. Furthermore, we have studied their expression in order to demonstrate their role in the immune response against invading bacteria in *L. crocea*. These results will contribute to our understanding of regulation associated with the interferon immune system in fish and help to establish efficient treatment strategies in farmed *L. crocea*.

2. Materials and methods

2.1. Fish collection, immune challenge, and sampling

The fish examined (weight 80 ± 15 g) were clinically healthy and obtained from Zhejiang Dahaiyang Technology Co. Ltd (Zhoushan, Zhejiang Province, China). The fish were maintained at 25 °C in an aerated seawater tank and fed a commercial diet for 2 weeks prior to the beginning of the experiment. The water in the tank was changed daily. For the tissue specific expression analysis, spleen, liver, kidney, heart, gill, brain, intestine, muscle and skin were isolated from six unchallenged *L. crocea.* Immediately following tissue excision, samples were placed into 1 mL of TRIzol reagent and homogenised. Tissue homogenates were stored at −80 °C prior to RNA extraction. After acclimation, three groups of 100 individuals were randomly chosen for challenge experiments. *L. crocea* were then intraperitoneally challenged with *V. anguillarum* (1×10^8 CFU/mL, re-suspended in sterile phosphate buffer saline (PBS), pH 7.4), polyinosinic:polycytidylic acid (Poly I:C), and PBS (as control) (both 300 μL/200 g). Animals in all groups were anesthetized by immersion in MS222 before tissue sampling, as required. The liver, kidney, and spleen tissues were harvested from five fish per group at 0, 6, 12, 24, 36, 48, and 72 h after injection. All procedures were performed in accordance with the guidelines of the Regulations for the Administration of Laboratory Animals (Decree No. 2 of the State Science and Technology Commission of the People's Republic of China, November 14, 1988), and were approved by the Animal Ethics Committee of Zhejiang Ocean University (Zhoushan, China).

2.2. Molecular cloning of the complete L. crocea IRF4, IRF8, and IRF9 ORF

The CDS of large yellow croaker IRF4, IRF8, and IRF9 (lcIRF4, lcIRF8, and lcIRF9) was obtained from whole-genome data of *L. crocea* (Wu et al., 2014). Total RNA was isolated from livers, kidneys, and spleens using the TRIzol Total RNA Kit (Invitrogen, USA), following the manufacturer's instructions. cDNA synthesis was performed with the M-MLV RTase cDNA Synthesis Kit (TaKaRa, Japan). The reactions were performed according to the manufacturer's instructions. PCR amplification was conducted using a Thermal Cycler (Bio-Rad, USA), with the following amplification conditions: 4 min at 94 °C, followed by 35 cycles of 60 s at 94 °C, 30 s at 60–65 °C, and 2 min at 72 °C, with a final extension of 5 min at 72 °C.

2.3. Genomic and putative protein structure analysis and phylogenetic tree construction

Similarity with other IRF homologs available in the GenBank database was determined using BLASTX and BLASTP available from the NCBI (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi/). The putative amino acid sequence was predicted using the NCBI's ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and Expasy's Translate Tool (http://web.expasy.org/translate/). Physicochemical properties of the putative IRF polypeptides were determined by Expasy's ProtParam prediction server (http://web.expasy.org/protparam/). The signal peptide sequence was identified by SignalP v4.0 server (http://www.cbs.dtu.dk/services/SignalP-4.0/). Secondary and tertiary structures of the protein domains were predicted using NPS at (https://npsaprabi.ibcp.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_server.html), SMART, SWISS-MODEL. The protein transmembrane domain was identified using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/). Multiple sequence alignment of different IRF polypeptide sequences was performed by ClustalW2 web tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Zhan et al., 2017). A phylogenetic tree was generated based on the predicted amino acid sequence using the maximum likelihood method in the Mega 7.0 program.

2.4. Quantitative real-time PCR (qPCR) and statistical analyses

qPCR was used to investigate the mRNA expression patterns of *L. crocea* IRFs in different tissues and at different time points following *V. anguillarum* and poly I:C challenge. qRT-PCR was performed in a reaction mixture of 20 μL, containing 0.8 μL primer-F (10 μmol/L), 0.8 μL primer-R (10 μmol/L), 10 μL 2 × SYBR® Premix Ex Taq™ II, 0.8 μL cDNA sample (100 ng/μL), 0.4 μL ROX II, and 7.2 μL ddH_2O (for reagent concentrations, refer to the manufacturer's