Fish and Shellfish Immunology 94 (2019) 1–9

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Functional characterization of IRF8 regulation of type II IFN in golden pompano (*Trachinotus ovatus*)

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ARTICLE INFO

Keywords:
Trachinotus ovatus
Promoter activity
Transcription factor
IRF8
IFNγ

ABSTRACT

Interferon regulatory factor 8 (IRF8) increases type I IFN transcription levels by binding to IFN promoters, thereby playing a role in innate immunity. Nevertheless, the detailed mechanism through which IRF8 regulates type II IFN in fish remains ambiguous. In the present study, two genes from the golden pompano (*Trachinotus ovatus*), *IRF8* (*ToIRF8*) and *IFN gamma* (*ToIFNγ*), were identified in the IFN/IRF-based signalling pathway. The full-length *ToIRF8* cDNA was composed of 2,141 bp and encoded a 421 amino acid polypeptide; the genomic DNA was 2,917 bp in length and consisted of 8 exons and 7 introns. The putative protein showed the highest sequence identity (90–92%) with fish IRF8 and possessed a DNA-binding domain (DBD), an IRF-association domain (IAD) and a nuclear localization signal (NLS) motif consistent with those of IRF8 in other vertebrates. Furthermore, the *ToIRF8* transcripts were expressed in all examined tissues of healthy fish, with higher levels observed in the central nervous and immune relevant tissues. They were upregulated by polyinosinic acid: polycytidylic acid [poly (I: C)], lipopolysaccharide (LPS) and flagellin treatments in the blood, liver, intestine and kidney. The results from assays of subcellular localization showed that *ToIRF8* was localized to the cytoplasm. Moreover, to investigate whether ToIRF8 was a regulator of *ToIFNγ*, a promoter analysis was performed using progressive deletion mutations of *ToIFNγ*. The results indicated that the region from −601 bp to −468 bp includes the core promoter. Mutation analyses indicated that the activity of the *ToIFNγ* promoter significantly decreased after the targeted mutation of the M1-M3 binding sites. Additionally, overexpressed *ToIRF8* in vitro notably increased the expression of several IFN/IRF-based signalling pathway genes. These results suggest that *IRF8* is vital in the defence of *T. ovatus* against bacterial infection and contributes to a better understanding of the transcriptional mechanisms of ToIRF8 on type II IFN in fish.

1. Introduction

Interferon (IFN) regulatory factors (IRFs), known as transcription factors, bind to IFN-stimulated response element (ISRE) for viral-, bacterial-, and interferon (IFN)-induced signalling pathways and are vital for antiviral defence, apoptosis, immune responses, and cell growth regulation [1–4]. To date, eleven IRFs have been identified in vertebrates [5–9]. Nine IRFs (IRF1-3, IRF4/Pip/ICSAT, IRF5-7, ICSBP/IRF8, and ISGF3g/p48/IRF-9) have been described in mammals, and IRF10 and IRF11 have been reported only in birds and fish, respectively. The first 115 amino acids (aa) constitute a DNA binding domain (DBD) in the N-terminus region of all IRFs [10]. The DBD includes a helix-loop-helix (HLH) structure and five tryptophan residues separated by 10–18 aa that bind to the promoters of target genes, such as IFN regulatory elements and IFN-stimulated response elements (ISRE). Additionally, all IRFs have an IRF-association domain (IAD), which mediates the recruitment of transcription factors and other IRFs to the target gene promoters, except IRF1 and IRF2, which are in the C-terminus region [11,12].

IRF8, also known as interferon consensus sequence-binding protein (ICSBP), is a transcription factor that plays crucial roles in the development and function of myeloid cells by activating macrophages through proinflammatory signals such as those transduced by IFN type II (interferon gamma, IFNγ) [13]. In particular, IRF8 plays a momentous role in IFNγ by mediating a second wave of IFN-γ-driven gene transcription in the cellular response [14,15]. IRF8 is known to form

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https://doi.org/10.1016/j.fsi.2019.08.060
Received 25 July 2019; Received in revised form 20 August 2019; Accepted 24 August 2019
Available online 26 August 2019

strong associations with the ISRE domains only when it forms heterodimers with other IRFs, particularly with IRF1, IRF2 or IRF4 [16,17]. Moreover, IRF8 binds to the ISRE domains and upregulates IFN type I (IFN-α/β) expression in dendritic cells [18]. Loss of *IRF1* and *IRF8* activity leads to decreased basal and IFN-γ–induced expression of different subsets of genes that play pivotal roles in macrophage activity and function in mammals [19]. In mammals, IRF8 has also been verified as a downstream target of the IFN-γ/STAT1 (signal transducer and activator of transcription 1, STAT1) signalling pathway [20,21]. IRF8-deficient mice also have pronounced susceptibility to infection with viral pathogens, which is associated with impaired dendritic cell development and defective production of IFNγ [22].

IFNs are divided into two major groups based on the conserved cysteine residues in the mature peptide. Group I IFNs, with 2 cysteine residues, have been authenticated in all teleosts. Group II IFNs, with 4 cysteine residues, are restricted to only a few species [23–25]. Many studies have suggested that the IFN response is regulated by various types of IRFs [26–30]. The expression levels of type I IFNs are controlled by key transcription factors such as IRF1, IRF2, IRF3 and IRF7 [26–30]. IRF8 is a transcription factor that has a pivotal role in the cellular response to IFNγ and is significant in myeloid cell differentiation (MCD). However, no information on type II IFNs (IFNγ) is associated with IRF8 in teleosts. Moreover, in teleosts, IRF8 has been identified in japanese flounder (*Paralichthys olivaceus*) [31], rock bream (*Oplegnathus fasciatus*) [32], rainbow trout (*Oncorhynchus mykiss*) [33], Atlantic cod (*Gadus morhua*) [34], turbot (*Scophthalmus maximus*) [35], and large yellow croaker (*Larimichthys crocea*) [36]. Furthermore, some research has indicated that *IRF8* can be upregulated after stimulation with different types of viruses, bacteria or polyinosinic acid: polycytidylic acid [poly (I: C)] in fish [31–36], suggesting that IRF8 plays a role in the host antiviral and antibacterial responses. Consequently, to determine whether *ToIRF8* is a mediator of *ToIFN*γ in the IFN/IRF-based signalling pathway, the function of IRF8 (*ToIRF8*) after poly (I: C), lipopolysaccharide (LPS) or flagellin induction was determined in golden pompano (*Trachinotus ovatus*), and the sequence characterization, expression pattern and transcriptional regulation of *ToIRF8* were also investigated. The present study regarding *ToIRF8* is expected to be useful for tracing the IFN-like system in marine fish.

2. Materials and methods

2.1. Ethics statement

All tests in the present study are allowed by the Animal Care and Use Committee of the South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (No. SCSFRI96-253) and performed according to the guidelines and regulations established by this committee.

2.2. Fish collection, immune challenge, and sampling

Juvenile fish (body weight = 40 ± 5 g) were clinically healthy and acquired from Linshui Marine Fish Farm (Linshui, Hainan Province, China). The fish were maintained at 28 ± 2 °C in fresh seawater, with 35% salinity, in dissolved oxygen > 6 mg/L, and fed a commercial diet (Hengxin, Zhanjiang, China, crude protein > 37%, crude fat > 7%) for 1 weeks prior to the beginning of the experiment. Under a controlled photoperiod (14 h/day and 10 h/night), all experimental fish were raised in tanks (200 L) with a recirculating aquaculture system. Moreover, tissue samples (head-kidney, gill, brain, small intestine, spleen, fin, liver, white muscle, stomach, blood, and male and female gonads) were obtained from six healthy adult fish (three male and three female), immediately frozen in liquid nitrogen, and then stored at −80 °C until use.

T. ovatus were then intraperitoneally challenged with phosphate buffered saline (PBS, 200 μL, control group) or poly (I: C) (200 μg/mL, 200 μL) or LPS (50 μg/mL, 200 μL) or flagellin (1 μg/mL, 200 μL) (stimulation groups). The detailed concentration of the three stimulants and the challenge experimental programme are referred to Wu et al. (2018) [37]. Before tissue sampling, fish were anaesthetized using MS222 (0.1 g L^{-1}; Sigma, Alcobendas, Spain) in all groups. Four tissues (blood, liver, intestine and kidney) were harvested from five fish per group at 0, 6, 12, 24, 36, 48, 72 and 96 h after the injection, immediately frozen in liquid nitrogen, and then stored at −80 °C until use.

2.3. Total RNA isolation and formation of cDNA

Total RNA was extracted from *T. ovatus* tissues and cells using Trizol kit (Promega, Madison, WI, USA). The quality and quantity (concentration) of isolated RNA was confirmed by 1% agarose gels and NANODROP 2000 spectrophotometer (ThermoScientific, Waltham, MA, USA). Furthermore, RNA (1 μg) and Olig $(dT)_{16}$ (0.5 μg) were reacted for 5 min at 70 °C. Then incubation for 2 min on ice, the mixture was reverse transcribed with M-MLV (200 units), 5 × buffer, Rnasin (25 units) and dNTPs (0.8 mM) in a total volume of 25 μL and extended for 1 h at 42 °C.

2.4. Molecular cloning and sequencing

The *IRF8*- and *IFN*γ-derived sequences were obtained based on *T. ovatus* genomic data (ENA Accession No. PRJEB22654 and Sequence Read Archive BioProject PRJNA406847). Moreover, to ascertain the full-length sequences of two genes, gene-specific primers were designed by Primer Premier 5 5 (Primer, Canada) (Table 1) and then sequenced with an ABI 3730XL automated sequencer by Sequencing Analysis 5.2 (Applied Biosystems, Foster City, CA, USA).

After original sequence assembly, a 1,266 bp fragment of the *IRF8* gene was acquired and used to clone the full-length cDNA of *IRF8* by the rapid amplification of cDNA ends (RACE) method with gene-specific primers (Table 1). According to the manufacturer's instructions, 5′-/3′-RACE was performed using the RACE cDNA amplification kit (TaKaRa, Japan). The 5′-/3′-RACE polymerase chain reaction (PCR) products were ligated into the pGEM®-T easy vector (Promega, USA).

2.5. Bioinformatics

Amino acid sequences of ToIRF8 were regarded as queries to search for the orthologous genes in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All available *IRF8* gene sequences, exon and intron structures were confirmed by Ensembl (http://asia.ensembl.org/) and Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat). Different amino acid sequences of IRF8 were aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Subsequently, the MEGA 6 program was used to structure a maximum likelihood (ML) phylogenetic tree (LG + G model, bootstrap 1000) of IRF8 aa [38]. The Compute pI/Mw software (http://web.expasy.org/protparam/) was used to calculate the molecular weights (Mw) and theoretical isoelectric points (pI).

2.6. Subcellular localization

To determine the subcellular distribution of *IRF8* in *T. ovatus* cells, the plasmid pEGFP-N3 or pEGFP-IRF8 was transfected into golden pompano *T. ovatus* snout tissue (GPS) cells as described by Yu et al. (2016) [39]. After 24 h, the cells were stimulated with poly (I: C) (5 μg/mL) for 12 h. Then, the cells were fixed with 4% paraformaldehyde. The cells were stained with 4,6-diamidino-2-phenylindole (DAPI) and observed under fluorescence microscopy (Leica, Switzerland).

2.7. Plasmid construction, cell culture and dual-luciferase reporter assays

Total genomic DNA was isolated from muscle tissue of *T. ovatus*, as described previously [40], and was regarded as a template for cloning

Table 1
Primers used for sequence cloning, deletion mutant construction, mRNA construction and qRT-PCR.

Subject and Primers	Nucleotide sequence
Primers for sequence cloning	
IRF8-ORF-F	CGCGGATCCATGTCAAACTCGGGAGGT
IRF8-ORF-R	CCGCTCGAGTCAGGCAGTGATAGGCATGT
IRF8-genome-F	GGCTTGGTCAACGGGAATC
IRF8-genome-R	TCAGGTTTACTTTCGTTG
IRF8-3′RACE-outer	AAACACGCAGGGAAACAG
IRF8-3′RACE-inner	ACTTTGAGGAGGTGACGG
IRF8-5′RACE-outer	TTCTGTTCTTCTTCGGGC
IRF8-5′RACE-inner	CCGTCACCTCCTCAAAGT
IFNγ-ORF-F	ATGGTTGCAGCAGCGAGGG
IFNγ-ORF-R	TCAGGCTCTCAGACTAGTT
Deletion mutant construction	
IFNγ-pF1	CGGGGTACCTTCATCTTTTCATTGGATGT
IFNγ-pF2	CGGGGTACCTCAGTTAAAATCACCAAACC
IFNγ-pF3	CGGGGTACCATCTAATGATTTCCGACGCA
IFNγ-pF4	CGGGGTACCCCCAGTATGACCAGTAAAG
IFNγ-pF5	CGGGGTACCCAGTTTGAGCCAACTTCAG
IFNγ-pR	CCGCTCGAGATAGTGCTGCAGCAGGTTGCTG
Primers for qRT-PCR	
qRT-IRF8-F	AAACACGCAGGGAAACAG
qRT-IRF8-R	CCGTCACCTCCTCAAAGT
qRT-IFNγ-F	CCAGATGGATAACCTCGTCGT
qRT-IFNγ-R	AGCAGTGAACTCGCCTCGG
qRT-IFP35-F	GAATCCAGGCTTCAGTCG
qRT-IFP35-R	CACCTTCAGTGCGTCCAG
qRT-TRAF6-F	CCCTAAAGCACCCATCGC
qRT-TRAF6-R	AAGGTCACGCAGGAACTCAG
qRT-MXI-F	CATACCCTTGGGACCTGA
qRT-MXI-R	TGCTTTGGCTTTGTTGAGT
qRT-ISG15-F	TACGCTGAGTGAGACCCG
qRT-ISG15-R	GGAGGAACACCTGGATGG
qRT-Viperin1-F	GACCCGTCCAAGTCCATC
qRT-Viperin1-R	CAAAGCCACTGAAGCAAAT
qRT-Viperin2-F	CCCGAGTCCAATGAGAAGA
qRT-Viperin2-R	CGAAGCCACTAAAGCAGATG
qRT-Mavs-F	GTTTGGAGGTGCGGATGA
qRT-Mavs-R	CCTTTTCGGCTTTGCTGTA
EF1α-F	AAGCCAGGTATGGTTGTCAACTTT
EF1α-R	CGTGGTGCATCTCCACAGACT

of candidate promoter sequences. To investigate the regulatory function of ToIRF8 on *ToIFN*γ expression, five different promoter regions from *ToIFN*γ were amplified by specific primers with *KpnI* and *XhoI* restriction sites (Table 1). Subsequently, the five truncated fragments [denoted as pGL3-basic-IFNγ-1 (−1817 to +120), pGL3-basic-IFNγ-2 (−989 to +120), pGL3-basic-IFNγ-3 (−601 to +120), pGL3-basic-IFNγ-4 (−468 to +120) and pGL3-basic-IFNγ-5 (−189 to +120)] were inserted into the pGL3-basic luciferase reporter plasmid (Promega, USA).

To understand the potential effect of the ToIRF8 binding sites on the core *IFN*γ promoter, three deductive mutations were designed. To predict potential binding sites for the *ToIFN*γ promoter, the transcription factor binding site prediction (TFBS)-JASPAR database (http://jaspar.genereg.net/), TRANSFAC®, and MatInspector® software were utilized. Furthermore, according to the manufacturer's protocol, truncated mutants of the pGL3-basic-IFNγ-3 promoter were structured by Muta-direct™ site-directed mutagenesis kit (SBS Genetech, Shanghai, China), and the pGL3-basic-IFNγ-3 promoter was defined as wild-type. Three binding sites, including: M1 (−595 bp to −577 bp), M2 (−353 bp to −323 bp), and M3 (−220 bp to −200 bp), were directly deleted in pGL3-basic-IFNγ-3 promoter [41]; the corresponding TF binding site sequences are shown in Fig. 6A.

For subcellular localization, the ORF of *ToIRF8* was cloned into the pEGFP-N3 (Clontech, USA) vector. Moreover, to construct the expression plasmid, the ORF of *ToIRF8* was inserted into the pCDNA3.1 vector (Invitrogen, USA) at the *BamH*I and *Xho*I sites (Table 1). Renilla luciferase plasmid pRL-TK (Promega, USA) was used as an internal control. The TransGen Plasmid Mini kit (Beijing, China) was used to extract recombinant plasmids. Human embryonic kidney (HEK293T) (GeneCreate, Wuhan, China) and golden pompano *T. ovatus* snout tissue (GPS) cell cultures and transfection trials were implemented according to the methods described by Li et al. (2017) [42] and Yu et al. (2016) [39]. Additionally, to further clarify whether *ToIRF8* could upregulate IFNγ in vitro, GPS cells overexpressing pcDNA3.1-IRF8 or pcDNA3.1-Flag were prepared. After 48 h post-transfection, the GPS cells were collected.

2.8. Quantitative real-time PCR and statistical analysis

The tissue expression pattern of *IRF8* was ascertained by quantitative real-time polymerase chain reaction (qRT-PCR), using twelve healthy tissues and four infected tissues in *T. ovatus*. Total RNA was isolated from tissues and cells, as described above, and then subjected to qRT-PCR analysis. The specific primers for *ToIRF8*, *IFN*γ, *IFP35*, *TRAF6*, *MXI*, *ISG15*, *Viperin1*, *Viperin2*, *Mavs* and the housekeeping gene *EF-1α* (elongation factor 1, alpha) were listed in Table 1 [43]. The qRT-PCR was performed as previously described [44]. Relative expression was evaluated by the $2^{-\Delta\Delta CT}$ method [45]. SPSS 19.0 software (IBM, USA) was used to analyse data. The data from different groups and tissues were analysed by the Duncan test by one-way ANOVA. Data were provided as the means of three replicates ± SE, and $p < 0.05$ indicated statistical significance.

3. Results

3.1. Sequence characterization of ToIRF8 and ToIFNγ

The genomic sequence of *ToIRF8* is 2,917 bp, including 8 exons and 7 introns, producing a 2,141 bp cDNA sequence (GenBank accession number: MN186880; Supplementary Figs. 1–2). The sequence consisted of a 162 bp 5′-untranslated region (5′-UTR), a 713 bp 3′-UTR, and a 1,266 bp (421 aa) ORF with a predicted molecular weight of 47.86 kDa and a theoretical isoelectric point of 5.98. In the 3′-UTR, the two common polyadenylation signal sequences (AAUAAA) are located 159 and 35 bp upstream of the poly (A) tail, and the mRNA instability motif (ATTTA) is found 122 bp upstream of the poly (A) tail. The multiple alignment analysis indicated that the putative protein had a winged-helix conserved DNA binding domain (DBD) (Met^{1}–Pro^{113}) that spanned the N-terminal region and had five representative tryptophan residues (Trp^{12}, Trp^{27}, Trp^{39}, Trp^{60}, and Trp^{78}). The nuclear localization signal (NLS) sequence (“KGKFK” and “KTRLR”) was also located in the N-terminal region, while $AA^{188-371}$ made up the interferon association domain (IAD) of ToIRF8 in the C-terminal region (Fig. 1). Moreover, the putative ToIRF8 protein sequence shared high similarity with turbot (*Scophthalmus maximus*) IRF8 (92%), grouper (*Epinephelus coioides*) IRF8 (91%), large yellow croaker (*Larimichthys crocea*) IRF8 (90%), and rock bream (*Oplegnathus fasciatus*) IRF8 (90%) and shared lower similarity with chicken (*Gallus gallus*) IRF8 (52%), human (*Homo sapiens*) IRF8 (52%), mouse (*Mus musculus*) IRF8 (52%), and Xenopus (*Xenopus tropicalis*) IRF8 (51%) (Supplementary Table 1).

The genomic sequence of *ToIFN*γ is 4,599 bp and consists of 4 exons and 3 introns with a 600 bp ORF, which encodes a polypeptide of 199 amino acids (GenBank accession number: MN186881) with a predicted Mw of 22.63 kDa and a theoretical pI of 10.07.

3.2. ToIRF8 structural and phylogenetic analysis

The genomic structural features and phylogenetic relationship of IRF8 were determined in vertebrates (Fig. 2A). All the 5′ and 3′ ends of the introns showed typical sequence characteristics (GT/introns/AG) [46]. The lengths and distribution patterns of the exons and introns of vertebrate *IRF8* genes are shown in Supplementary Table 2. Generally, *IRF8* had a similar exon-intron organization in vertebrates from fish to

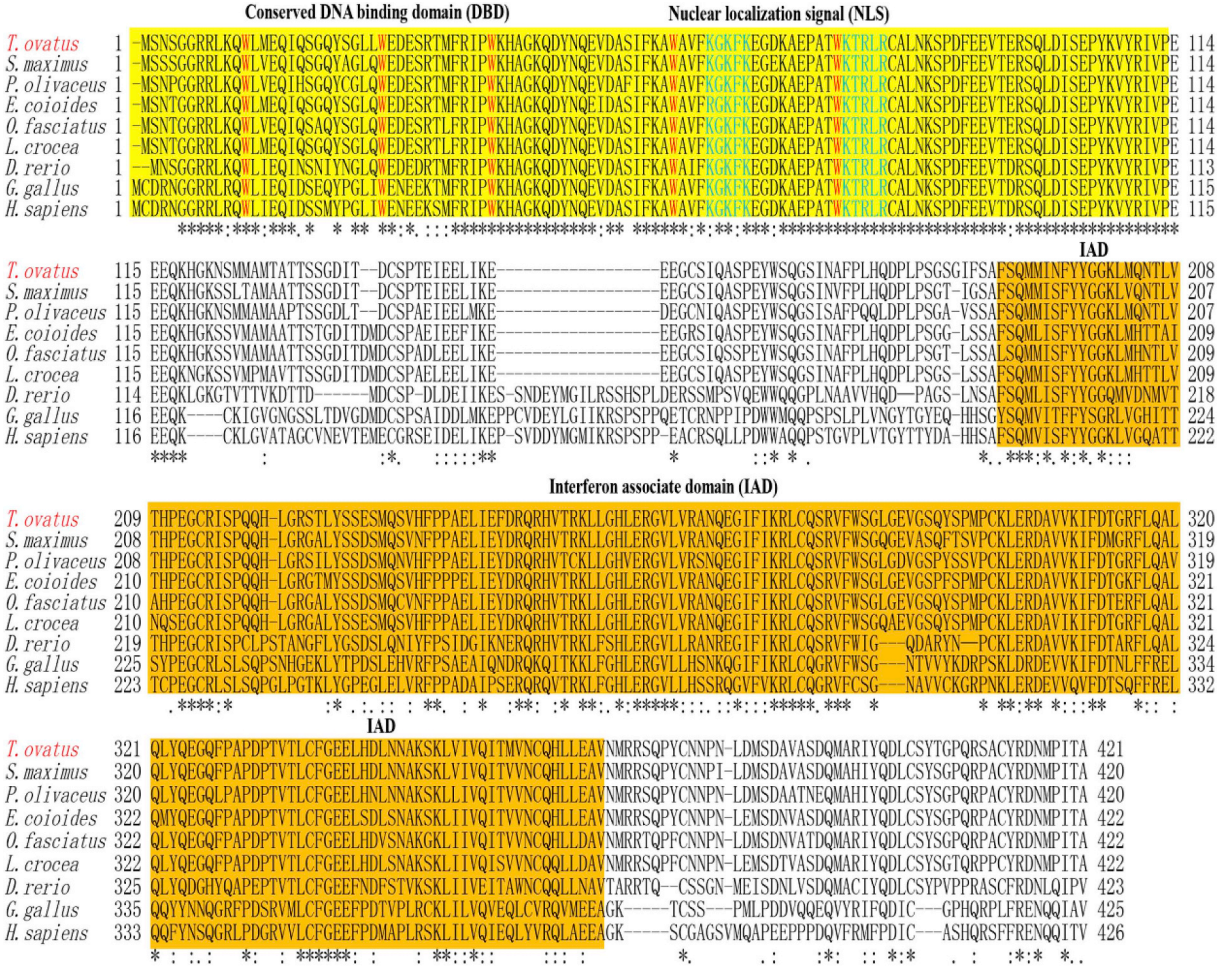

Fig. 1. Amino acid sequences of IRF8 homologues in vertebrates. The conserved DNA binding domain (DBD) which contains five conserved tryptophan residues is shown with a yellow box. The nuclear localization signal (NLS) ("KGKFK" and "KTRLR") and interferon association domain (IAD) are shown with green and orange boxes, respectively. The accession numbers of the IRF8 sequences used are listed in Supplementary Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mammals, with eight exons and seven introns. Additionally, *ToIRF8* was grouped together with stickleback (*Gasterosteus aculeatus*). The topology suggested that the homology of ToIRF8, from distant to close, was with Mammalia, Aves, Amphibia, and other Osteichthyes.

3.3. Expression levels of ToIRF8 by tissue

To confirm the role of *ToIRF8* in healthy fish, we investigated the mRNA expression levels in twelve tissues by qRT-PCR (Fig. 2B). *ToIRF8* was constitutively expressed in all the tissues analysed with varied

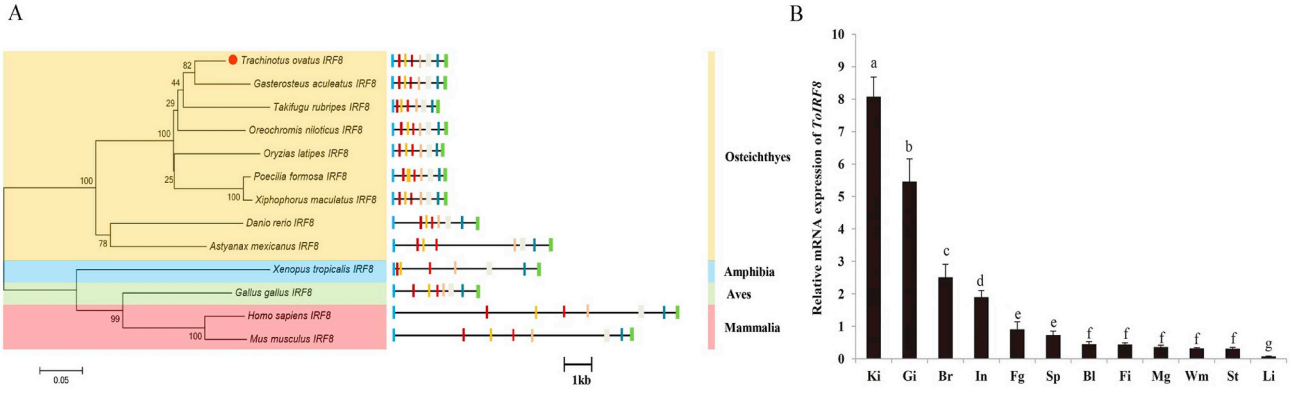

Fig. 2. The structure and tissue expression of the *ToIRF8* gene. A. Genome structure analysis of *IRF8* genes according to their phylogenetic relationship. Lengths of exons and introns of each *IRF8* gene are displayed proportionally. Different colour boxes and lines represent exons and introns, respectively. The identical colour boxes represent homologous sequences. B. Gene transcription of *ToIRF8* in various tissues. The twelve tissues are small intestine (In), head-kidney (Ki), white muscle (Wm), stomach (St), female gonad (Fg), male gonad (Mg), brain (Br), liver (Li), gill (Gi), spleen (Sp), fin (Fi), and blood (Bl). Different letters indicate significant differences ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

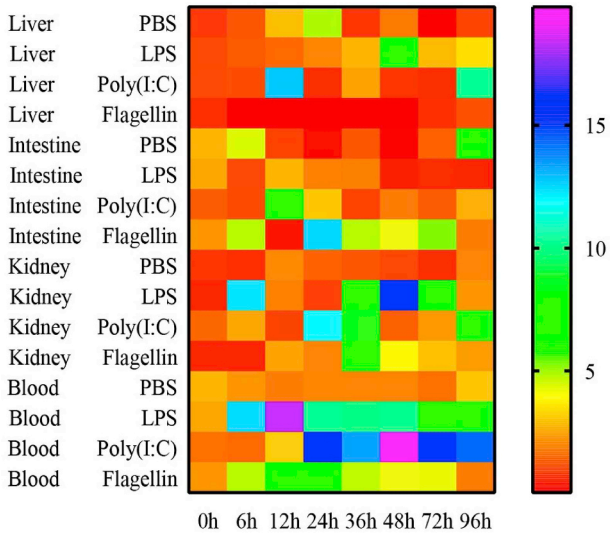

Fig. 3. The mRNA levels of ToIRF8 (relative to EF-1α) in different tissues (liver, intestine, kidney, and blood) were detected after PBS (control), poly(I:C), LPS or flagellin challenges (0, 6, 12, 24, 36, 48, 72 and 96 hpi). The heatmap was constructed by Graphpad Prism software.

expression levels observed. *ToIRF8* was highly expressed in the kidney, followed by the gill, brain, and intestine, with lower expression levels in the liver ($P < 0.05$).

To further identify the role of *ToIRF8* in the immune response, gene expression changes in response to poly (I: C), LPS and flagellin challenges were detected (Fig. 3). In comparison to the control group, the mRNA levels of *ToIRF8* were markedly increased in response to poly (I: C), LPS and flagellin challenges in the blood, liver, intestine and kidney. As shown in Fig. 3, *ToIRF8* expression was upregulated by LPS and poly (I: C) in the four tissues. Interestingly, *ToIRF8* transcripts were upregulated to a higher level in blood than they were in the other three tissues, showing a remarkable increase of 7.1-fold and 11.0-fold in the blood after challenge with LPS and poly (I: C), respectively, compared to the control (data not shown). Furthermore, *ToIRF8* expression was also upregulated in vivo in response to stimulation with flagellin in the four tissues. However, *ToIRF8* was more responsive in the intestine than in the other three tissues. The highest levels were observed in the intestine at 24 hpi (5.3-fold).

3.4. Cytoplasm localization of ToIRF8

Fluorescence microscopy was used to investigate the subcellular localization of *ToIRF8* with or without poly (I: C) stimulation. Poly (I: C), an RNA virus mimic, could induce significant IFN expression. GPS cells transfected with pEGFP-N3 were used as a control group. The fluorescence signals of pEGFP-N3 were distributed in the cytosol and the nucleus with or without poly (I: C) stimulation (Fig. 4A). Compared with the controls without stimulation, the green signal of *ToIRF8* was detected in the cytosol but not in the nucleus. Green fluorescence signals were also observed only in the cytosol after poly (I: C) stimulation (Fig. 4B). Taken together, these findings suggested that *ToIRF8* was localized in the cytoplasm.

3.5. ToIRF8 promotes ToIFNγ expression

A total of 1,937 bp of the 5' sequence flanking the *ToIFN*γ gene was cloned and regarded as the candidate promoter. To determine the promoter activity of *ToIFN*γ in response to the transcription factor ToIRF8 in HEK293T cells, a series of progressive deletion constructs were generated (Fig. 5A). Compared with the activity of the promoter candidate (IFNγ-3), a deletion of a fragment from −601 bp to −468 bp (IFNγ-4) showed decreased promoter activity in response to ToIRF8. The expression levels of IFNγ-3 were 2.14-fold higher than those of IFNγ-4 in response to ToIRF8 (Fig. 5B), indicating that the core promoter region was located at −601 bp to −468 bp, which included the IRF8 binding sites.

To further confirm the ToIRF8 binding sites in the *ToIFN*γ promoter, the binding sites were predicted and mutated (Fig. 6A, Table 2). HEK293T cells were co-transfected with ToIRF8 together with each mutant plasmid (M1, M2, and M3) or the empty vector (pGL3-basic). The results showed that mutations in the M1 (−595 bp to −577 bp), M2 (−353 bp to −323 bp), and M3 (−220 bp to −200 bp) binding sites caused obvious dramatic reductions in promoter activity (Fig. 6B), suggesting that all three mutations affecting the ToIRF8 binding sites in the *ToIFN*γ promoter were necessary for triggering *ToIFN*γ expression by ToIRF8.

3.6. Overexpression of ToIRF8 positively regulates the IFN/IRF-based signalling pathway

To confirm that ToIRF8 could upregulate *IFNa3* in endogenous cells,

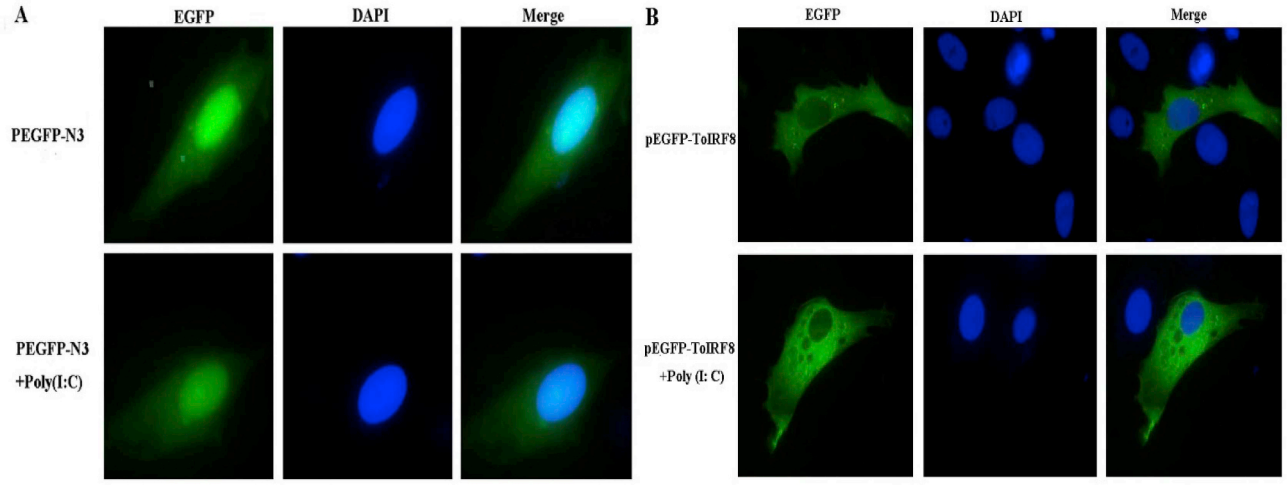

Fig. 4. Subcellular localization of *ToIRF8* in pompano cells. GPS cells seeded onto microscopy cover glass in 6-well plates were transfected with 2 μg pEGFP-ToIRF8 or pEGFP-N3 plasmid. After 24 h, the cells were stimulated with poly (I: C) (5 μg/mL) for 12 h, then the cells were fixed and subjected to confocal microscopy analysis. Green staining represents the ToIRF8 protein signal, and blue staining indicates the nucleus region. All experiments were repeated at least three times, with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

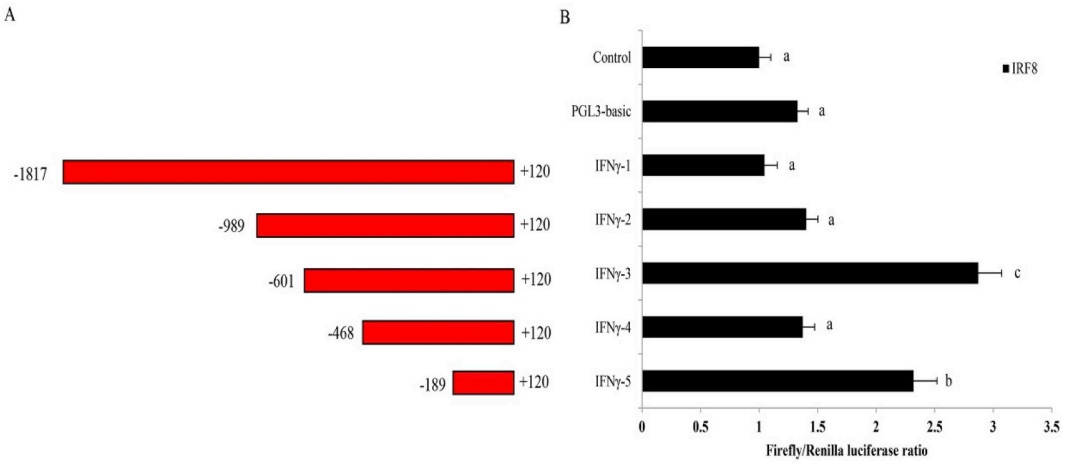

Fig. 5. Promoter activity analysis of the *ToIFN*γ gene. (A) The structure and transcriptional activity of the *ToIFN*γ promoter. Five recombinant plasmids, denoted IFNγ-1 (−1817 to +120), IFNγ-2 (−989 to +120), IFNγ-3 (−601 to +120), IFNγ-4 (−468 to +120) and IFNγ-5 (−189 to +120) were constructed and transfected, along with the transcription factor ToIRF8, into HEK 293T cells. (B) Dual-luciferase activity was driven by the ToIFNγ-3 core promoter upon the transfection of pcDNA3.1-IRF8 and pcDNA3.1 into HEK 293T cells. Data are presented as the means of three replicates ± SE. Different letters indicate significant differences ($p < 0.05$).

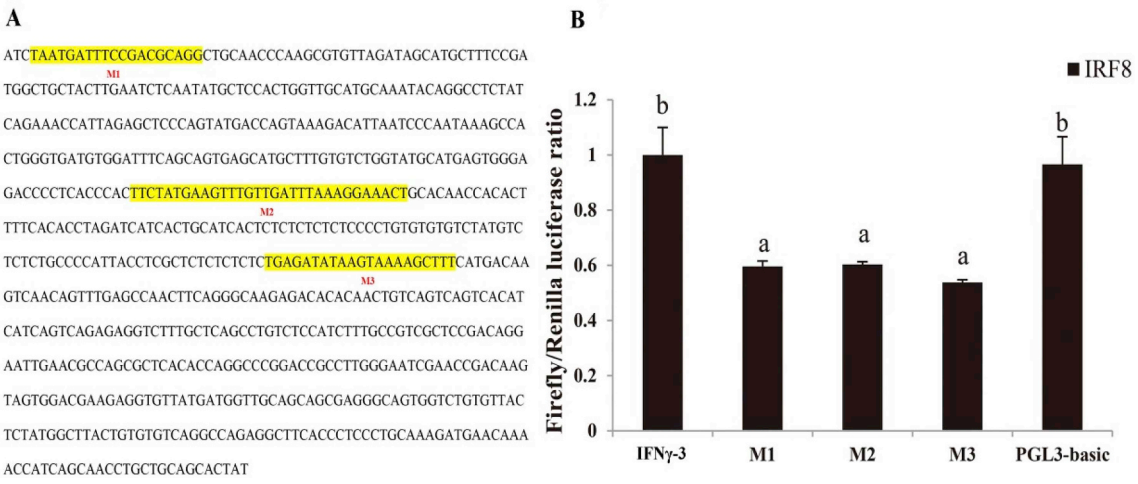

Fig. 6. Construction of truncated mutants for the identification of predicted transcription factor (TF) binding sites in the *ToIFN*γ promoter. (A) The nucleotide sequence and predicted binding sites in the core region of the *ToIFN*γ*-3* promoter. (B) Effects of three mutants on *ToIFN*γ*-3* promoter activity. Binding sites are shown with boxes. Mutations of promoter sequences are listed in Table 2. Data are presented as the means of three replicates ± SE. Different letters indicate significant differences ($p < 0.05$).

Table 2
Sequences of putative binding sites on *ToIFN*γ*-3* promoter.

Putative binding sites	Nucleotide sequence	Mutated pattern
M1	TAATGATTTCCGACGCAGG	deletion
M2	TTCTATGAAGTTTGTTGATTTAAAGGAAACT	deletion
M3	TGAGATATAAGTAAAAGCTTT	deletion

GPS cells that overexpressed pcDNA-IRF8 or the empty vector (pcDNA3.1-Flag) were prepared. The results showed that ToIRF8 also upregulated *ToIFN*γ expression in homologous GPS cells (Fig. 7A–B). Moreover, to demonstrate the potential regulatory role of the *ToIRF8* gene on the IFN/IRF-based signalling pathway, the expression levels of IFN/IRF-based signalling pathway genes, such as interferon-induced protein 35 (*IFP35*), TNF receptor-associated factor 6 (*TRAF6*), MAX interactor 1 (*MXI*), interferon-stimulated gene (*ISG15*), *Viperin1*, *Viperin2* and mitochondrial antiviral signalling protein (*Mavs*) were detected by qRT-PCR. The results showed that overexpression of *ToIRF8* significantly increased the expression of the IFN/IRF-based signalling pathway genes (Fig. 7C–I) and upregulated the mRNA level of *TRAF6* such that it was higher than that of other genes.

4. Discussion

IRFs are a class of extensively verified transcription factors that play dominant roles in the immune response, particularly in resisting viral infections that affect cell apoptosis or growth [3]. IRF8 has been widely characterized in mammals and has been shown to monitor antiviral and antibacterial activity. Nevertheless, information related to the functions of IRF8 in fish is very limited. It is well known that the IFN system, including the IRF genes, is highly conserved in fish. To comprehend the molecular level of *IRF8* action in teleosts, we first identified the genome structures and mRNA expression of IRF8 from *T. ovatus*.

Three conserved domains are found in all known IRF8 isoforms: an N-terminal DBD, a C-terminal IAD and an NLS domain. The DBD is representative of all IRF members and is characterized by a cluster of five well-spaced tryptophan residues [11,31–36], which are located at

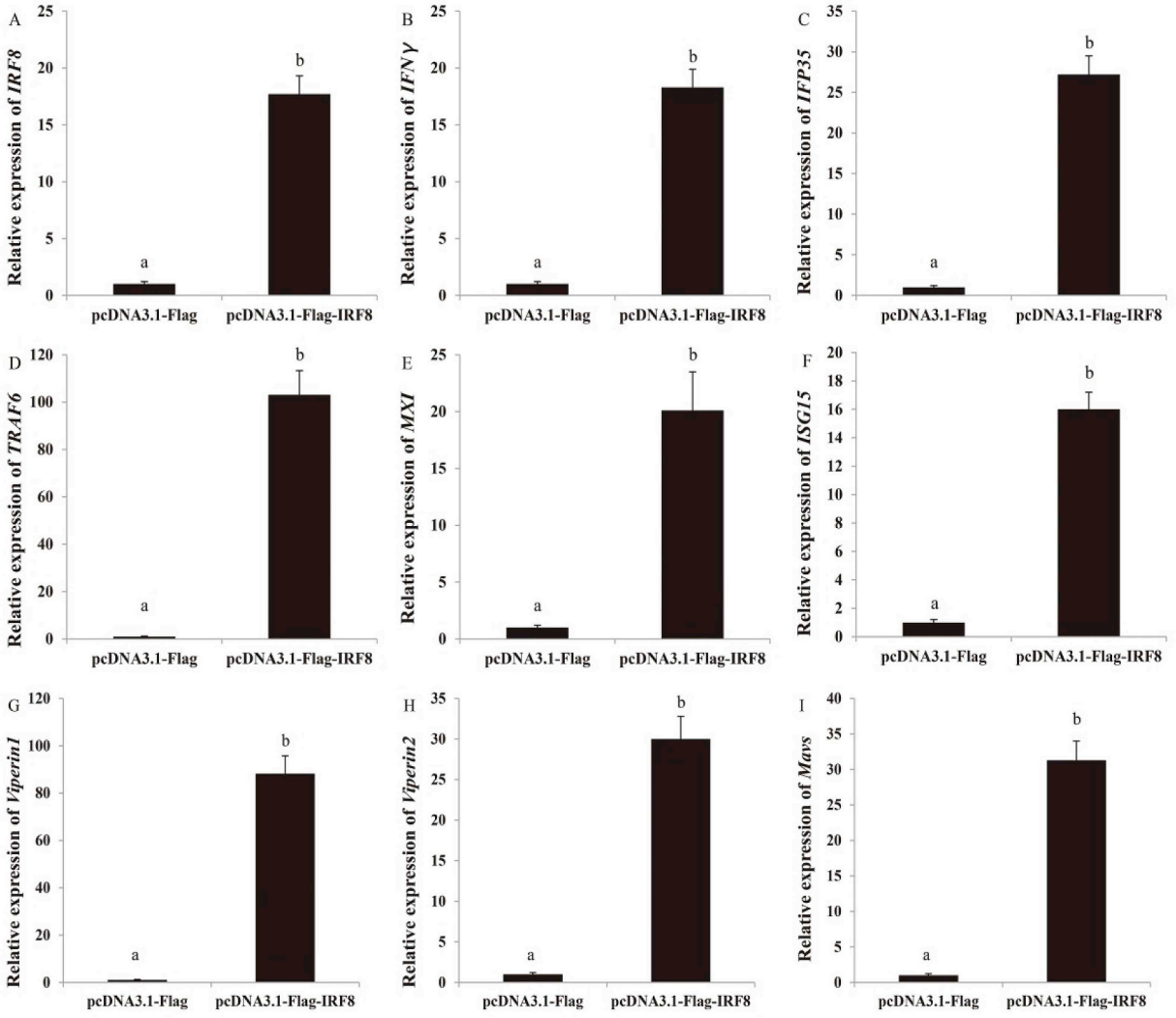

Fig. 7. Overexpression of ToIRF8 altered the expression levels of IFN/IRF-based signalling pathway genes in GPS cells for 36 h. The relative expression levels of *IRF8* (A), *IFN*γ (B), *IFP35* (C), *TRAF6* (D), *MXI* (E), *ISG15* (F), *Viperin1* (G), *Viperin2* (H) and *Mavs* (I) were determined using qRT-PCR analysis. The Y-axis represents the fold change of target gene expression in each group relative to that in control group (control vector cells). Different letters indicate significant differences ($p < 0.05$).

the 12, 27, 39, 60 and 78 positions of ToIRF8. The tryptophan pentad-repeat forms a helix–turn–helix motif that binds to interferon regulatory factor element (IRF-E) or ISRE consensus sequences found in the target promoters [2,6,10]. The IAD domain is another conserved region in IRFs through which IRF8 interacts with IRF1, IRF2 or IRF4, and especially with PU.1/Spi-1, to form a heterodimer with a high DNA binding affinity that promotes or suppresses gene transcription [14]. Studies have shown that Y^{211} within the human IRF8 IAD domain is a target of the phosphorylation mediated by TRAF6 stimulation [14,47]. Moreover, IRFs, except IRF6 and IRF10, have 1 or 2 nuclear localization signals (NLSs) related to nuclear preservation and translocation of IRFs in terminal regions [48]. In the present study, 2 NLSs were located in the DBD region, similar to those of PoIRF8 [31]. In addition, the genomic sequences of *IRF1/2/6/8* have conserved structures in terms of exon-intron organization and in the distribution of intron phases [7]. The *ToIRF8* gene includes the conserved 8-exon and 7-intron structure as found in other vertebrate IRF8s [7]. Apparently, exon sizes in the *IRF8* ORF are also conserved in vertebrates. Because of these properties, IRF8 is regarded as a good model to study evolutionarily conserved functions in living vertebrates. Furthermore, the result of the evolutionary relationship was consistent with the findings of conventional taxonomy, showing that *ToIRF8* has a close genetic relationship with Perciformes [31–36].

In the present study, *ToIRF8* transcripts were enriched in known lymphomyeloid-rich tissues, such as the kidney, gill, brain, and intestine, and sparse in the liver, a finding that is in accordance with the overall expression profile of IRF8 in other teleosts [32,33,35]. The expression level of *ToIRF8* was relatively high in the kidney, gill, brain, and intestine, suggesting that *ToIRF8* plays a significant role in the vertebrate central nervous system and immune system. Moreover, previous studies have demonstrated that viral (turbot reddish body iridovirus (TRBIV), lymphocystis disease virus (LCDV), and rock bream iridovirus), bacterial (*Edwardsiella tarda*, *Streptococcus iniae* and *Vibrio anguillarum*), and poly (I: C) can cause antiviral and antibacterial responses in teleosts and that *IRF8* plays a role [31–36]. Using gene expression analysis in this study, we investigated whether *ToIRF8* is involved in this process after induction by poly (I: C), LPS and flagellin. The results indicated that *ToIRF8* expression was enhanced by three inducers in the immune tissues. The induction by poly (I: C) and LPS was most rapid in blood, suggesting that it directly activates the host's immune response as a pathogen-associated molecular pattern. This result was consistent with the expression levels of *PoIRF8* [31], *OmIRF8* [33], and *SmIRF8* [35].

Under steady conditions, *IRF8* is expressed at low levels in the cytoplasm. In response to exposure to IFNγ or microbial products, *IRF8* expression is induced, and the protein is recruited to the nucleus

[49,50]. In the present study, *ToIRF8* was located in the cytoplasm with or without poly (I: C) induction, indicating that *IRF8* is mainly activated downstream of the signal pathway in the cytoplasm and that *IRF8* is regulated in the cytoplasm by upstream molecules. These data showed that the cytoplasmic localization of *IRF8* was responsible for its activation and function in *T. ovatus*.

In teleosts, two subfamilies of IFNs have been identified based on differential structural and functional properties: type I and type II IFNs [51]. The type I IFN subfamily consists of a group of classic antiviral proteins, and numerous type I IFN family members were described in teleost species [51]. Type II IFN (IFNγ) is a markedly different IFN than the INFs in the type I subfamily and can intervene, to some extent, during viral infections but is mainly an immunomodulatory molecule [52,53]. IRF8 upregulates type I IFN expression by binding to type I IFN promoters, thereby playing a role in innate immunity. Whether IRF8 regulates the expression of type II IFN remains in question. In the present study, a positive regulatory role of ToIRF8 in *ToIFN*γ transcription in *T. ovatus* was shown, suggesting that *ToIRF8* increases type II IFN transcription in exogenous cells. The results of the luciferase reporter assay also indicated that the region between −601 bp and −468 bp is the core regulatory region of the *ToIFN*γ promoter and that it has potential binding sites for ToIRF8. The deletion of the ToIRF8 binding sites M1, M2, and M3 resulted in significantly reduced promoter activity of *ToIFN*γ (Fig. 6B). Obviously, IRF8 played a key regulatory role in the IRF8-dependent type II IFN responses through the ToIRF8 M1-M3 binding sites. These results showed that *ToIFN*γ expression is controlled by ToIRF8.

These results provide the first evidence of the involvement of IRF8 in the expression of type II IFN in fish. ToIRF8 also increased type II IFN transcription in endogenous cells (Fig. 7A–B). Moreover, the mRNA levels of the IFN/IRF-based signalling pathway genes, *IFP35*, *TRAF6*, *MXI*, *ISG15*, *Viperin1*, *Viperin2* and *Mavs*, were also promoted by overexpression of IRF8 (Fig. 7C–I), suggesting that *IRF8* might positively upregulate the IFN/IRF-based signalling pathway. We speculated that the positive control of the IFN/IRF-based signalling pathway by *ToIRF8* might contribute directly to the enhancement of pro-inflammatory and immune responses [53].

In conclusion, the expression characteristics and regulatory function of *ToIRF8* were reported. We demonstrated that *ToIRF8* transcripts were expressed abundantly in lymphomyeloid-rich tissues and were stimulated by three molecules. Moreover, the positive regulatory function of ToIRF8 on *ToIFN*γ expression was also determined. Our findings might help further understanding of the regulatory functions of *IRF8* in fish.

Conflicts of interest

The authors declare no competing interests.

Acknowledgments

This work was supported by the National Key R&D Program of China (2018YFD0900301), China Agriculture Research System (CARS-47), Guangdong Provincial Special Fund for Modern Agriculture Industry Technology Innovation Teams, the National Infrastructure of Fishery Germplasm Resources Project (2019DKA30470) and the Science and Technology Infrastructure Construction Project of Guangdong Province (2019B030316030).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.060.

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