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Functional characterization of interferon regulatory factor 2 and its role in the transcription of *interferon a3* in golden pompano *Trachinotus ovatus* (Linnaeus 1758)

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ABSTRACT

Similar to mammals, fish possess interferon (IFN) regulatory factor 2 (IRF2)-dependent type I IFN responses. Nevertheless, the detailed mechanism through which IRF2 regulates type I IFNa3 remains largely unknown. In the present study, we first identified two genes from golden pompano (*Trachinotus ovatus*), *IRF2* (*ToIRF2*) and *IFNa3* (*ToIFNa3*), in the IFN/IRF-based signalling pathway. The open reading frame (ORF) sequence of *ToIRF2* encoded 335 amino acids possessing four typical characteristic domains, including a conserved DNA-binding domain (DBD), an interferon association domain 2 (IAD2), a transcriptional activation domain (TAD), and a transcriptional repression domain (TRD). Furthermore, transcripts of *ToIRF2* were significantly upregulated after stimulation by polyinosinic: polycytidylic acid [poly (I:C)], lipopolysaccharide (LPS) and flagellin in immune-related tissues (blood, liver, and head-kidney). Moreover, to investigate whether ToIRF2 was a regulator of *ToIFNa3*, promoter analysis was performed. The results showed that the region from −896 bp to −200 bp is defined as the core promoter using progressive deletion mutations of *IFNa3*. Additionally, ToIRF2 overexpression led to a clear time-dependent enhancement of *ToIFNa3* promoter expression in HEK293T cells. Mutation analyses indicated that the activity of the *ToIFNa3* promoter significantly decreased after targeted mutation of M4/5 binding sites. Electrophoretic mobile shift assays (EMSAs) verified that IRF2 interacted with the binding site of the *ToIFNa3* promoter region to regulate *ToIFNa3* transcription. Last, the promoter activity of ToIFNa3-2 was more responsive to treatment with poly (I:C) than LPS and flagellin. Furthermore, overexpression of ToIRF2 in vitro obviously increased the expression of several IFN/IRF-based signalling pathway genes after poly (I:C) abduction. In conclusion, the present study provides the first evidence of the positive regulation of *ToIFNa3* transcription by ToIRF2 and contributes to a better understanding of the transcriptional mechanisms of ToIRF2 in fish.

1. Introduction

Interferon (IFN) regulatory factors (IRFs), a family of transcription factors, bind to a specific DNA motif known as the IFN-stimulated response element (ISRE) and have critical regulatory roles in the transcription of interferons (IFNs) and IFN-stimulated genes (ISGs) [1–3]. IRFs have diverse biological functions and are involved in both innate and adaptive immunity, virus-mediated signalling responses, haematopoietic cell development and tumour proliferation [1,4]. To date, nine members of the IRF family have been characterized in mammals, ten in birds, and eleven in fish [4–8]. All members of the IRF family contain a highly conserved DNA binding domain (DBD) at their N-terminus, which is characterized by five or six tryptophan residues that form a helix-turn-helix motif and is responsible for binding to the promoters of target genes [1,9], and an IRF-association domain (IAD) at their C-terminus [10]. The IAD mediates the interactions between transcription factors and other proteins to form complexes [11].

The induction and activation of IFNs are considered to be crucially important to the antiviral innate immunity of vertebrates [12,13]. Diversified IFNs are used to coordinate the antiviral immune responses in

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teleost fish [14–16]. According to the presence of conserved cysteine residues in the mature peptide, IFNs are divided into two major groups. Group I IFNs, including two cysteine residues, can be observed in all teleost fish lineages, and group II IFNs, including four cysteine residues, are confined to only a few species [16–18]. Numerous studies have shown that the IFN response is regulated by various types of IRFs. The expression of type I IFNs is controlled by two key transcription factors, the interferon regulatory factors IRF3 and IRF7 [19,20]. Moreover, IRF1/2 has been shown to control IFN-α gene expression in leukaemia patients [21]. The expression levels of IFN-α target genes are increased by the knockdown of IRF2, which has been shown to be a negative regulator of IFN-α signalling in zebrafish [22]. Dual-luciferase reporter assays indicated that IRF2 could bind to the promoter of IFN by means of its DBD and could downregulate the transcription activity of IFN in grass carp (*Ctenopharyngodon idella*) [23].

In fish, IRF2 has been characterized in rainbow trout (*Oncorhynchus mykiss*) [24], mandarin fish (*Siniperca chuatsi*) [25], snakehead (*Channa argus*) [26], eel (*Monopterus albus*) [27], paddlefish (*Polyodon spathula*) [28], and blunt snout bream (*Megalobrama amblycephala*) [29]. Furthermore, some research has shown that IRF2 can be upregulated after stimulation with different types of viruses, bacteria or polyinosinic: polycytidylic acid [poly (I:C)] in fish [24,28,29], suggesting that IRF2 plays a role in the host antiviral and antibacterial responses. Consequently, we investigated the function of golden pompano (*Trachinotus ovatus*) IRF2 (*ToIRF2*) after stimulation with poly (I:C), lipopolysaccharide (LPS) or flagellin in order to determine whether *ToIRF2* is a mediator of *ToIFNa3* in the IFN/IRF-based signalling pathway, and its sequence characterization, expression pattern and transcriptional regulation of ToIRF2 were determined. The present study regarding ToIRF2 will be useful for tracing the IFN-like system in marine fishes.

2. Materials and methods

2.1. Ethics statement

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

2.2. Fish and challenge experiments

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

The induction experiment contained four groups: the PBS group (control group) and the poly (I:C), LPS and flagellin stimulation groups. The detailed concentration of the three inducers and the experimental programme are described by Wu et al. (2018) [30]. The control group was implemented by injecting 200 μL of PBS. Moreover, the poly(I:C) stimulation group was implemented by injecting 200 μL of poly(I:C) (Sigma, USA) (200 μg/mL) in each fish. The LPS stimulation group was implemented by injecting 200 μL of LPS (Sigma, USA) (50 μg/mL) into each fish. The flagellin stimulation group was implemented by injecting 200 mL of flagellin (Sigma, USA) (1 μg/mL) in each fish. All three inducers were dissolved in phosphate buffered saline (PBS) and injected intraperitoneally. Three individuals from each group were randomly dissected at each of eight time points (0, 6, 12, 24, 36, 48, 72 and 96 h after the injection). Before dissection, fish were anaesthetized using MS222 (0.1 g L^{-1}; Sigma, Alcobendas, Spain). Three tissues (blood, liver, and head-kidney) were sampled and used for gene expression experiments.

2.3. RNA extraction and gene cloning

Total RNAs (1 μg) were extracted from pompano tissues and cells using the HiPure Fibrous RNA Plus Kit (Magen, Guangzhou, China) and were reverse transcribed into cDNA by random hexamer primers (Cloned AMV First-Strand cDNA Synthesis Kit, Invitrogen, USA). A NanoDrop 2000 spectrometer (Thermo Scientific, USA) and 1% agarose gels were used to detect the quantity and quality of the isolated RNA. According to pompano genomic data (Accession No. PRJEB22654 under ENA, Sequence Read Archive under BioProject PRJNA406847), the *IRF2* and *IFNa3* predicted sequences were acquired. Furthermore, gene-specific primers were designed to obtain the full-length sequences of two genes that were assembled by SeqMan software (Supplementary Table 1).

2.4. Bioinformatics

Mature peptides of ToIRF2 and ToIFNa3 were used as queries to search for the orthologous genes in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All available IRF2 gene sequences and structures were provided by Ensembl (http://asia.ensembl.org/) and Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat). Different IRF2 polypeptide sequences were aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Then, a maximum likelihood (ML) phylogenetic tree (LG + G model, bootstrap 1000) of IRF2 amino acid sequences was created using the MEGA 6 program [31]. A three-dimensional (3D) model of the ToIRF2 DNA-binding domain (DBD) was created using the SWISS-MODEL Protein Modelling Server. SignalP software (http://www.cbs.dtu.dk/services/SignalP/) was used to determine signal peptides, and the Compute pI/Mw software (http://web.expasy.org/protparam/) was used to calculate the molecular weights and theoretical isoelectric points.

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

Total genomic DNA was isolated from the muscle tissue of pompano, as described previously [32], and was used as a template for candidate promoter cloning. To confirm the effect of ToIRF2 on *ToIFNa3* expression, five different promoter regions from *ToIFNa3* were amplified by specific primers with *KpnI* and *XhoI* restriction sites (Supplementary Table 1). Subsequently, the five truncated fragments [denoted as pGL3-basic-IFNa3-1 (−1649 to +1), pGL3-basic-IFNa3-2 (−896 to +1), pGL3-basic-IFNa3-3 (−722 to +1), pGL3-basic-IFNa3-4 (−547 to +1) and pGL3-basic-IFNa3-5 (−200 to +1)] were subcloned into the pGL3-basic luciferase reporter plasmid (Promega, USA) with the corresponding restriction sites. Moreover, to further confirm the interaction of *ToIFNa3* with ToIRF2, the core promoter pGL3-basic-IFNa3-2 was transfected into HEK293T cells together with pcDNA3.1-IRF2 and pcDNA3.1-flag.

To confirm the potential effect of the ToIRF2 binding sites on the core *IFNa3* promoter, six predicted recombinant plasmids of mutations were constructed. To predict potential binding sites for the *ToIFNa3* promoter, the transcription factor binding site prediction (TFBS)-JASPAR database (http://jaspar.genereg.net/), TRANSFAC®, and MatInspector® were used. Moreover, the truncated mutants of the pGL3-basic-IFNa3-2 promoter were produced with Muta-direct™ site-directed mutagenesis kit (SBS Genetech, Shanghai, China) according to the manufacturer's protocol. The pGL3-basic-IFNa3-2 promoter was defined as wild-type. The six truncated mutants of the pGL3-basic-IFNa3-2 promoter had directly deleted the prediction of six binding sites,

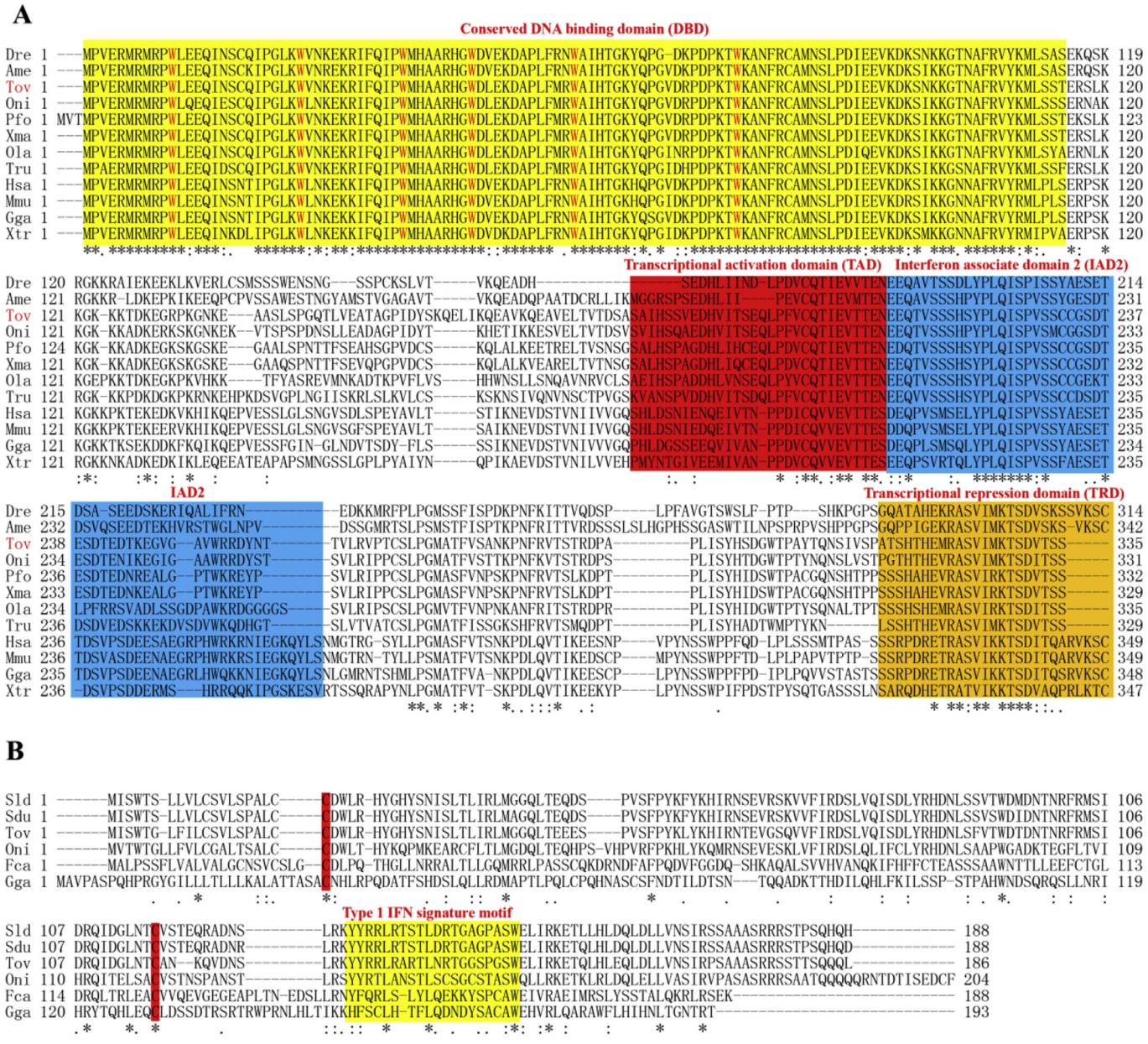

Fig. 1. Amino acid sequences of IRF2 (A) and IFNa3 (B) homologues in vertebrates. A. The conserved DNA binding domain (DBD) which contains six conserved tryptophan residues is shown with a yellow box. The transcriptional activation domain (TAD), interferon association domain 2 (IAD2), and transcriptional repression domain (TRD) are shown with red, blue, and orange boxes, respectively. B. The two highly conserved cysteine residues of group I type I IFNs (C^{20} and C^{116}) are indicated by red boxes. The signature motif of type I IFNs is marked by a yellow box. The accession numbers of the IRF2 and IFNa3 sequences used are listed in Supplementary Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

including: M1 (−799 bp to −772 bp), M2 (−768 bp to −749bp), M3 (−703 bp to −682 bp), M4 (−496 bp to −470 bp), M5 (−466 bp to −437 bp), and M6(-308 bp to −288 bp); the homologous TF binding site sequences are shown in Fig. 6A. To obtain the six mutants, the method of PCR augmentation was applied as in a previous study [33].

Furthermore, to construct the expression plasmid, the ORF of *ToIRF2* was cloned into the *BamH*I and *Xho*I sites of the pCDNA3.1 vector (Invitrogen, USA). The Renilla luciferase plasmid pRL-TK (Promega, USA) was used as an internal control. Recombinant plasmids were extracted using the TransGen Plasmid Mini Kit (Beijing, China). Human embryonic kidney (HEK293T) and golden pompano *Trachinotus ovatus* snout tissue (GPS) cell cultures and transfection trials were implemented according to the methods described by Li et al. (2017) and Yu et al. (2016) [34,35].

Additionally, to further identity the regulatory role of ToIRF2, ToIRF2 was transfected into HEK293 cells together with the *ToIFNa3* gene. Twenty-four hours after transfection, the HEK293 cells were treated with PBS (control group) and poly (I:C) (5 μg/mL), LPS (2 μg/mL) or flagellin (1 μg/mL) (treated group). Then, luciferase activity was measured after 12 h of stimulation.

To clarify whether *ToIRF2* could upregulate IFNa3 in vitro, GPS cells overexpressing pcDNA3.1-IRF2 and pcDNA3.1-Flag were prepared. Moreover, to clarify whether *ToIRF2* could regulate the poly (I: C)-induced IFN/IRF-based signalling pathway, GPS cells overexpressing pcDNA3.1-IRF2 and vector (pcDNA3.1-Flag) were incubated with poly (I: C) (5 μg/mL) for 36 h.

2.6. Electrophoretic mobility shift assay (EMSA)

The experimental procedure of the EMSA was conducted as

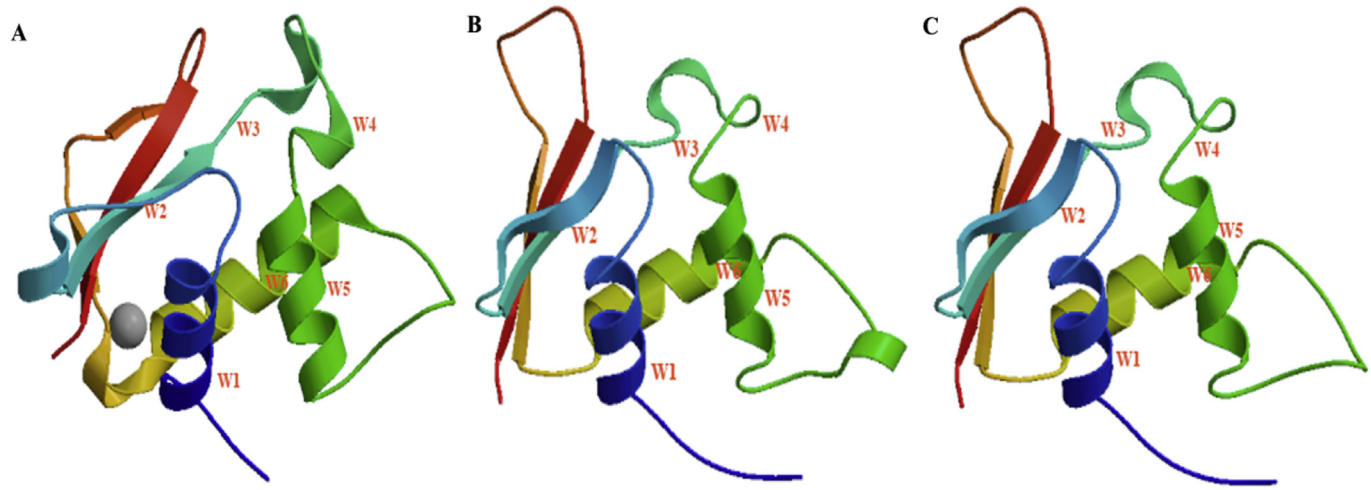

Fig. 2. Three-dimensional structures of ToIRF2 DBD in the pompano (A), zebrafish (B) and human (C). "W" indicates the six conserved tryptophan residues.

previously described [36]. Briefly, the lysates of HEK293T cells transfected with pcDNA3.1-Flag-IRF2 were prepared for DNA/protein conjugation reactions. According to the manufacturer's instructions, the EMSA Probe Biotin Labelling Kit (Beyotime, Shanghai, China) was used to label wild-type and mutated oligonucleotides (Supplementary Table 2). DNA/protein binding reactions were carried out using an EMSA/Gel-Shift Kit (Beyotime, China) at 25 °C. To determinate the specificity of the DNA/protein binding reactions, competition assays were performed with 100 × excessive unlabelled wild-type or mutated probes. Subsequently, the completed reactions were separated on nondenaturing 4% PAGE gels for 20 min. The proteins were developed by autoradiography using a LightShift® Chemiluminescent EMSA Kit (Pierce, USA).

2.7. Quantitative real-time PCR and statistical analysis

The tissue distributions of *IRF2* mRNA levels were determined by quantitative real-time polymerase chain reaction (qRT-PCR), using twelve healthy tissues and three infected tissues in pompano. Total RNA was isolated from tissues, as described above, and then subjected to qRT-PCR analysis. The specific primers for *ToIRF2*, *IFNa3*, *TRAF6*, *MXI*, *Viperin1*, *Viperin2*, *Mavs* and the housekeeping gene *EF-1α* (elongation factor 1, alpha) are displayed in Supplementary Table 1. The qRT-PCR was performed as previously described [37]. Relative expression was evaluated by the $2^{-\Delta\Delta CT}$ method [38]. Data were analysed using SPSS 19.0 software (IBM, USA). The data from different tissues and groups were analysed by the Duncan test using one-way ANOVA. Data are presented as the means of three replicates ± SE, and $p < 0.05$ indicated statistical significance.

3. Results

3.1. Sequence characterization of ToIRF2 and ToIFNa3

The genomic sequence of *ToIRF2* is 7581 bp, including 8 exons and 7 introns containing 146 bp of the 5′ untranslated region (5′-UTR) and a 1008 bp ORF, which encodes a polypeptide of 335 amino acids (GenBank accession number: MK034134; Supplementary Fig. 1) with a predicted molecular weight of 37.48 kDa and a theoretical isoelectric point of 7.64. Additionally, similar to IRF2 in teleosts and mammals, multiple sequence alignments indicated that there is a winged-helix conserved DNA binding domain (DBD) (Met^{1}–Thr^{116}) in the ToIRF2 N-terminal region. The DBD of ToIRF2 also contains six conserved tryptophan residues (Trp^{11}, Trp^{26}, Trp^{38}, Trp^{46}, Trp^{58}, and Trp^{77}). Similar to other IRF2 proteins, a transcriptional activation domain (TAD), an interferon association domain 2 (IAD2), and a transcriptional repression domain (TRD) were identified in the C-terminal region (Fig. 1A) [28]. Moreover, the putative ToIRF2 protein sequence shared high identity with tilapia (*Oreochromis niloticus*) IRF2 (82%), Amazon medaka (*Poecilia formosa*) IRF2 (80%), and platyfish (*Xiphophorus maculatus*) IRF2 (79%) and shared lower identities with Xenopus (*Xenopus tropicalis*) IRF2 (53%), chicken (*Gallus gallus*) IRF2 (52%), mouse (*Mus musculus*) IRF2 (52%), and human (*Homo sapiens*) IRF2 (52%) (Supplementary Table 3). Moreover, pairwise alignments of the DBD regions among metazoans showed that the IRF2 DBD exhibited higher identity levels (87–99%) than the complete amino acid sequences (52–82%) (Supplementary Table 3).

The genomic sequence of *ToIFNa3* is a 2459 bp sequence, including 5 exons and 4 introns containing a 561 bp ORF, which encodes a polypeptide of 186 amino acids (GenBank accession number: MK034135; Supplementary Fig. 2) with a predicted molecular weight of 21.48 kDa and a theoretical isoelectric point of 9.35. The predicted signal peptide is 21 aa. Although the deduced ToIFNa3 protein contains four cysteine residues, only two conserved cysteine residues (C^{20} and C^{116}) are found in fish and Aves type I IFNs (Fig. 1B). ToIFNa3 also possesses typical features of type I IFNs in teleost fish, including a distinctive family signature motif ([FYH]-[FY]-X-[GNRCDS]-[LIVM]-X2-[FYL]-L-X7-[CY]-[AT]-W) in the C-terminal domain. Furthermore, the putative ToIFNa3 protein sequence shared high identities with yellowtail amberjack (*Seriola lalandi dorsalis*) IFNa3 (84%) and greater amberjack (*Seriola dumerili*) IFNa3 (83%) and lower identities with cat (*Felis catus*) IFNa3 (24%), and chicken IFNa3 (23%) (Supplementary Table 3).

3.2. ToIRF2 structural analyses

In general, the 3D structure of the ToIRF2 DBD was highly similar to that of the zebrafish (*Danio rerio*) and human homologs, with six conserved tryptophan residues (W1-6) (Fig. 2). Furthermore, the genomic structural features and phylogenetic relationship of IRF2 were determined and constructed in metazoans (Fig. 3A). The lengths and distributions of the exons and introns of metazoan *IRF2* genes are shown in Supplementary Table 4. Eight exons and seven introns were identified in *IRF2* gene sequences, except for medaka (*Oryzias latipes*) *IRF2*, which only possessed seven exons and six introns. Furthermore, the sizes of the exon sequences revealed that there is nearly no diversity among species, except for the medaka exons, while the number of homologous intron sequences was distinctive. Additionally, ToIRF2 was grouped together with other Perciformes, such as tilapia. The topology indicated that the homology with ToIRF2, from distant to close, was

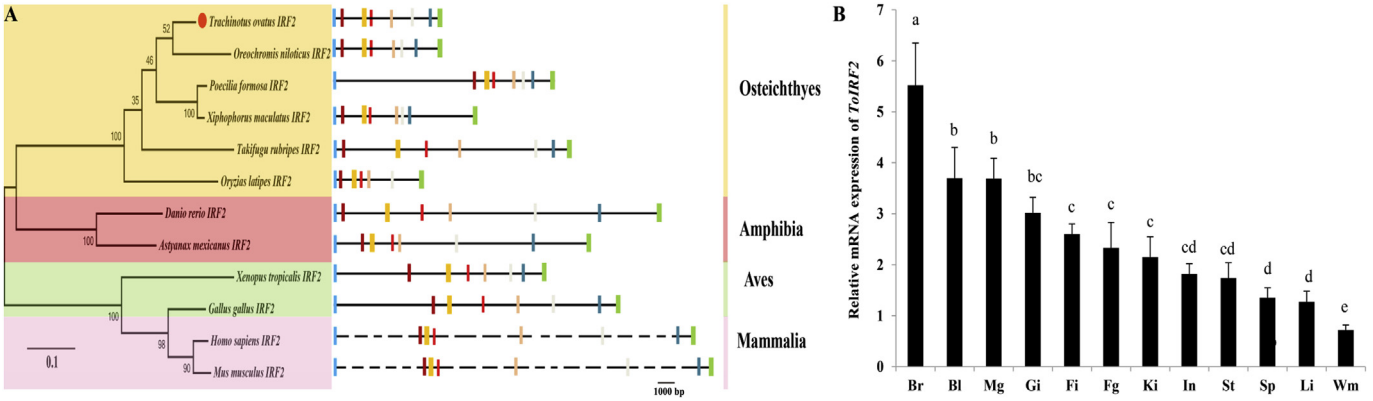

Fig. 3. The structure and tissue expression of the *ToIRF2* gene. A. Genome structure analysis of *IRF2* genes according to their phylogenetic relationship. Lengths of exons and introns of each *IRF2* gene are displayed proportionally. Different colour boxes and lines represent exons and introns, respectively. The identical colour boxes represent homologous sequences. B. Gene transcription of *ToIRF2* 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Mammalia, Aves, Amphibia, and other Osteichthyes.

3.3. Tissue expression of ToIRF2

To confirm the role of *ToIRF2* in healthy fish, qRT-PCR was used to detect the mRNA expression levels in twelve tissues (Fig. 3B). *ToIRF2* was constitutively expressed in all tissues analysed, with varied expression levels observed. *ToIRF2* is highly expressed in the brain, followed by the blood, male gonad, gill, fin, female gonad, and kidney, with lower expression levels in the white muscle ($P < 0.05$).

To further understand the role of *ToIRF2* in the immune response, qRT-PCR was used to investigate gene expression in response to poly (I:C), LPS and flagellin challenges (Fig. 4). In comparison to the control group, the mRNA levels of *ToIRF2* were markedly increased in response to poly (I:C), LPS and flagellin challenges in blood, liver, and head-kidney. As shown in Fig. 4, *ToIRF2* expression was upregulated by poly (I:C) and LPS in the three tissues. Notably, *ToIRF2* was more responsive in the liver than in the other two tissues, showing a remarkable increase of 5.8-fold and 3.8-fold in the liver after challenge with poly(I:C) and LPS, respectively, compared to the control (data not shown). Moreover, *ToIRF2* expression was also upregulated in vivo in response to stimulation with flagellin in the three tissues. However, *ToIRF2* was more responsive in the blood than in the other two tissues. The highest levels were observed in the blood for all time points, especially at 24 hpi (7.6-fold). The expression profile of *ToIRF2* in the liver was similar to that of *ToIRF2* in head-kidney following treatment with flagellin for all time points.

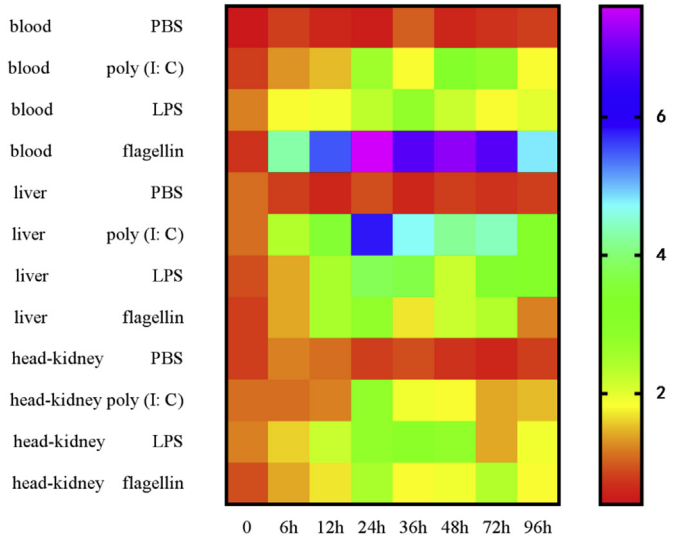

Fig. 4. Temporal mRNA expression analyses of *ToIRF2* in different tissues (blood, liver, and head-kidney) after PBS (control), poly(I:C), LPS or flagellin challenges (0, 6, 12, 24, 36, 48, 72 and 96 hpi).

3.4. ToIRF2 positively promotes ToIFNa3 expression

A total of 1649 bp of the 5' flanking sequence of the *IFNa3* gene was cloned and defined as the candidate promoter. To determine the promoter activity of *ToIFNa3* in response to the transcription factor ToIRF2 in HEK293T cells, a series of progressive deletion constructs were made (Fig. 5A). Compared with the activity of the promoter candidate (IFNa3-2), a deletion of a fragment from −896 bp to −200 bp (IFNa3-5) showed decreased promoter activity in response to ToIRF2. The expression levels of IFNa3-2 were 3.24-fold greater than those of IFNa3-5 in response to ToIRF2 (Fig. 5A), suggesting that the core promoter region was located at −896 bp to −200 bp, which contained the IRF2 binding sites. To further confirm the interaction of ToIRF2 with *ToIFNa3*, the influence of ToIRF2 overexpression on *ToIFNa3* transcription was determined. ToIRF2 overexpression increased the promoter activity of ToIFNa3-2 at all tested time points in heterologous HEK293T cells except for the 3 h time point, and the maximum difference occurred at 24 h posttransfection, which was detected as 2.1-fold higher in ToIRF2-overexpressing cells than in controls (Fig. 5B). These results indicated that constitutively expressed ToIRF2 positively regulated *ToIFNa3* expression in HEK293T cells. Moreover, to clarify whether ToIRF2 could upregulate IFNa3, GPS cells that over-expressed pcDNA-IRF2 and the vector (pcDNA3.1-Flag) were prepared. The results showed that ToIRF2 also upregulated *ToIFNa3* expression in homologous GPS cells (Supplementary Fig. 3).

To investigate the ToIRF2 binding sites in the *ToIFNa3* promoter, the binding sites were predicted and mutated (Fig. 6A, Table 1). HEK293 cells were co-transfected with ToIRF2 together with each mutant plasmid or the empty vector. The results showed that mutation of the M4 (−496 bp to −470 bp) and M5 (−466 bp to −437 bp) binding sites caused dramatic reductions in promoter activity (Fig. 6B), suggesting that both M4 and M5 were ToIRF2 binding sites in the *ToIFNa3* promoter. Obviously, the four other predicted mutant plasmids did not induce luciferase activity in response to ToIRF2, indicating that these four sites were not necessary for triggering *ToIFNa3* expression by ToIRF2.

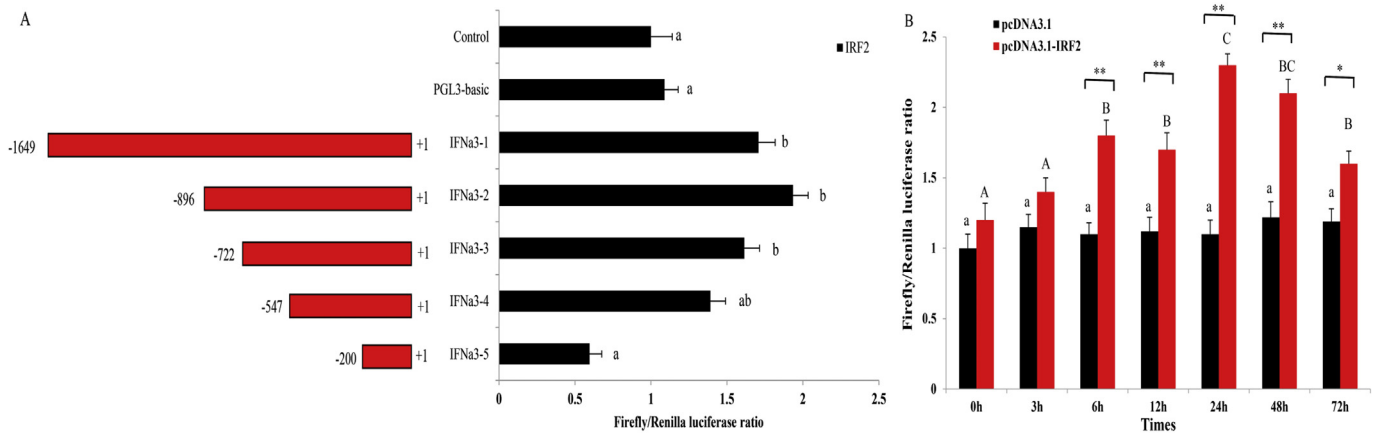

Fig. 5. Promoter activity analysis of the *ToIFNa3* gene. (A) The structure and transcriptional activity of the *ToIFNa3* promoter. Five recombinant plasmids, denoted IFNa3-1 (−1649 to +1), IFNa3-2 (−896 to +1), IFNa3-3 (−722 to +1), IFNa3-4 (−547 to +1) and IFNa3-5 (−200 to +1) were constructed and transfected, along with the transcription factor ToIRF2, into HEK293T cells. (B) Dual-luciferase activity was driven by the ToIFNa3-2 core promoter upon the transfection of pcDNA3.1-IRF2 and pcDNA3.1 into HEK293T cells. Data are presented as the means of three replicates ± SE. Asterisks indicate that the values are significantly different from the individual controls (*$p < 0.05$ and **$p < 0.01$). Bars on the same group with different letters are significantly different from one another ($p < 0.05$).

3.5. Binding of ToIRF2 to ToIFNa3 promoters

To confirm the ToIRF2 binding motif in the *ToIFNa3* promoter, an EMSA assay was performed. Based on the predicted ToIRF2 binding site, oligonucleotide probes were synthesized (Supplementary Table 2) and incubated with HEK293T cell lysates including recombinant IRF2, in vitro. Recombinant IRF2 bound to the oligonucleotide probes of the predicted IRF2 binding site in the *ToIFNa3* promoter. However, mutations in the IRF2 binding site resulted in the dissociation of the DNA-rIRF2 complex (Fig. 7), suggesting that IRF2 was specifically interacting with the M4 and M5 motif in the *ToIFNa3* promoter. The formation of the DNA-rIRF2 complex was specific, as it could only be blocked by excessive unlabelled control probes (100 ×).

Table 1
Site-directed mutations of putative binding sites on *ToIFNa3* promoter.

Putative binding sites	Nucleotide sequence	Mutated pattern
M1	GTTTCCATAACAACCAGAAAATGAATGT	deletion
M2	GCCTGAAAGCGAAAACTGTG	deletion
M3	CCTTCAAAGTAAAAGTTTAAAA	deletion
M4	GTTTAGAAAATGAAAGTGAAGGGACTT	deletion
M5	CAGCAGAAATCCACTGAGCGGGAAAAATAT	deletion
M6	AGGTACTTTTACTTCCATGTT	deletion

3.6. Overexpression of ToIRF2 positively regulated the IFN/IRF-based signalling pathway

To demonstrate the regulatory role of the *ToIRF2* gene on the IFN/IRF-based signalling pathway, ToIRF2 and ToIFNa3-2 plasmids were

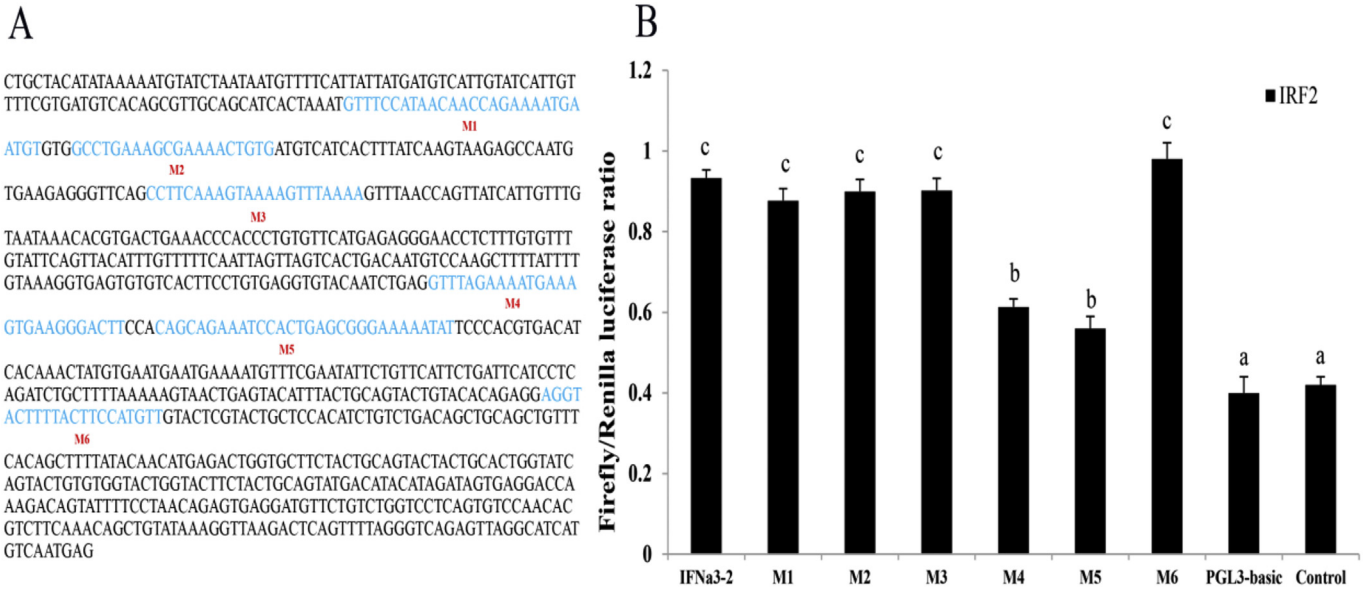

Fig. 6. Construction of truncated mutants for the identification of predicted transcription factor (TF) binding sites in the *ToIFNa3* promoter. (A) The nucleotide sequence and predicted binding sites in the core region of the *ToIFNa3* promoter. (B) Effects of six mutants on *ToIFNa3-2* promoter activity. Binding sites are shown with boxes. Mutations of promoter sequences are listed in Table 1. Data are presented as the means of three replicates ± SE. Different letters indicate significant differences ($p < 0.05$).

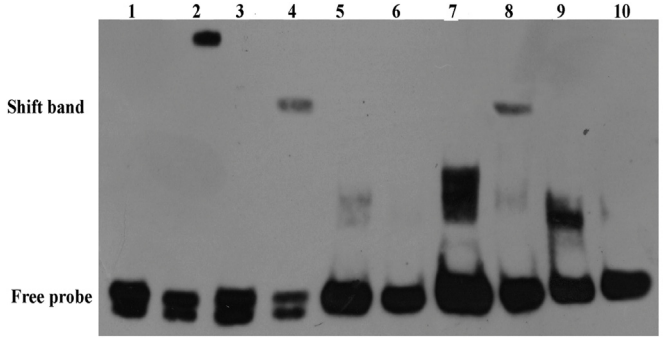

Fig. 7. Binding reactions of ToIRF2 and *ToIFNa3* promoters. Biotin-labelled EMSA probes were incubated with lysates of HEK293T cells containing IRF2 protein. WT, wild-type probe; MT: mutated probe. 1, negative control; 2, positive control; 3, plus IFNa3-P2-WT4; 4, IFNa3-P2-WT4 plus IRF2-flag; 5, plus IFNa3-P2-MUT4; 6, IFNa3-P2-MUT4 plus IRF2-flag; 7, plus IFNa3-P2-WT5; 8, IFNa3-P2-WT5 plus IRF2-flag; 9, plus IFNa3-P2-MUT5; 10, IFNa3-P2-MUT5 plus IRF2-flag.

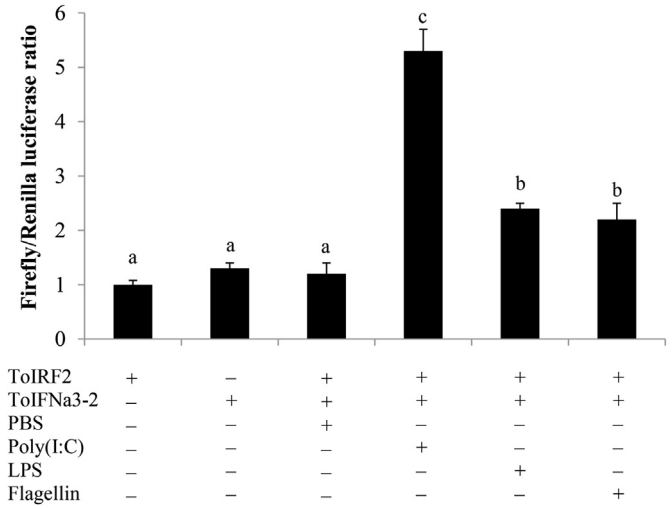

Fig. 8. Effects of ToIRF2 on induction of *ToIFNa3* promoter activity by treatment with three stimulants. HEK293 cells were co-transfected with ToIRF2 and ToIFNa3-2; after 24 h, the cells were stimulated with poly (I: C) (5 μg/mL), LPS (2 μg/mL) or flagellin (1 μg/mL) for 12 h prior to measuring the luciferase activity. Different letters indicate significant differences ($p < 0.05$).

transfected into HEK293 cells together with stimulation using three pathogen-associated molecular patterns (poly (I:C), LPS and flagellin) after 24 h transfection. The results indicated that the promoter activity of ToIFNa3-2 was dramatically upregulated in response to the three inductor challenges in the IFN/IRF-based signalling pathway (Fig. 8). Notably, the promoter activity of ToIFNa3-2 was more responsive to treatment with poly (I:C) than to treatment with the other two immunopotentiators (Fig. 8).

To further clarify whether ToIRF2 could regulate the poly (I: C)-induced IFN/IRF-based signalling pathway, GPS cells overexpressing pcDNA-IRF2 and pcDNA3.1-Flag were incubated with poly (I: C) for 36 h. As shown in Fig. 9, overexpression of ToIRF2 significantly increased the expression of IFN/IRF-based signalling pathway genes, such as IFNa3, TNF receptor associated factor 6 (TRAF6), MAX interactor 1 (MXI), Viperin1, Viperin2 and mitochondrial antiviral signalling protein (Mavs).

4. Discussion

To prevent pathogen invasion, innate immunity systems have developed in lower vertebrate teleosts. Some key cytokines, transcription factors, and pattern recognition receptors (PRRs) have been compensated for in fish, which involve adjustments to the classic NF-kB, JAK-STAT, and IFN signalling pathways of the innate immunity systems [39]. In mammals, type I IFNs are prevailingly regulated by transcription factors of the IRF family during innate immunity. Notably, IRF2 has been shown to decrease the IFN1 immune responses and, thus, further induce cell phenotypes and the differentiation of haematopoietic stem cells [40]. In this study, to confirm the regulatory function of *ToIRF2* on type I *ToIFNa3* in teleosts, we identified the complete coding sequences of *ToIRF2* and *ToIFNa3* in pompano.

Similar to other teleost IRF2 proteins, the isolated ToIRF2 protein possesses four typical characteristic domains, including a conserved DBD, an IAD2, a TAD, and a TRD. In the N-terminus, the DBD region, which is important for the recognition of the IFN-stimulated response element (ISRE) sequence and the regulation of type I IFN secretion [4], is highly conserved and functionally important in metazoans [6,26,28,29]. Moreover, most mammalian IRFs contain five conserved tryptophan residues, while fish IRF2 appears to exhibit more variation, with the ToIRF2 containing six conserved tryptophan residues in the DBD [28,29]. These conserved tryptophan residues are essential for the binding of IRFs to the promoter elements of genes in mammals [2]. In addition to the DBD, ToIRF2 also possesses a classic IAD region in the C-terminus, which is involved in its interaction with other IRF family members, the interactions between transcription factors and promoters of downstream genes, and the activation of the double-stranded RNA activated factor 1 as defences against viral invasion [2,41–43]. All of these characterizations indicate that ToIRF2 may potentially play key roles in IFN activation.

The tissue expression profile of *ToIRF2* mRNA supports previous studies of IRF2 in fish, including blunt snout bream and paddlefish, which have generally shown similar patterns of constitutive expression in most tissues, with higher expression in blood and gill [28,29]. Constitutive IRF2 expression was also observed in mandarin fish, grouper fish (*Epinephelus coioides*) and rainbow trout cells and tissues [24,25,44]. High mRNA levels of *ToIRF2* were detected in immune organs (blood and gill) and non-immune organs (brain) [28,29]. In most fish species, IRF2 appears to exhibit a high level of constitutive expression in the spleen, while low mRNA levels of *ToIRF2* were detected, suggesting that these differences could be due to differences between different species.

Previous studies have demonstrated that poly (I:C), LPS and flagellin can cause defence responses against pathogens in pompano [30,45–48]. Many studies have found that poly I:C and LPS can induce the expression of IRF genes in the kidney, liver and blood. After injection with poly (I:C), LPS or flagellin, *ToIRF2* expression was upregulated in the tissues of the immune system, especially in the blood and liver, which is consistent with *IRF2* expression patterns in other fish, such as mandarin fish, grouper fish, paddlefish and blunt snout bream [28,29,31,44].

Mammalian IRFs have been demonstrated to activate the mRNA transcription of type I IFN genes and ISGs by binding to the ISRE in their promoters [1–3]. IRF2 is regarded as an important transcriptional factor in the induction of the type I IFN signalling pathway, which is necessary for immune responses to defend against DNA or RNA viral invasions [39]. Fish IRF2 was recently proven to play crucial roles in the IFN response, as the overexpression of fish IRF2 reduced the activity of the IFN promoter by binding to the IRF2 DBD [23]. In this study, a positive regulatory role of ToIRF2 on *ToIFNa3* transcription in pompano was demonstrated. Conversely, it is well-known that IRF2 is a negative regulator of type I IFN [49]. We concluded that no study has been conducted on the function of *IFNa3*, and some different functions involving other type I IFNs might not be known.

The corresponding luciferase reporter assay indicated that *ToIFNa3* expression was regulated by ToIRF2 in pompano (Fig. 5). The region between −896 bp to −200 bp was identified as the core regulatory region of the *ToIFNa3* promoter, suggesting that it is reasonable to

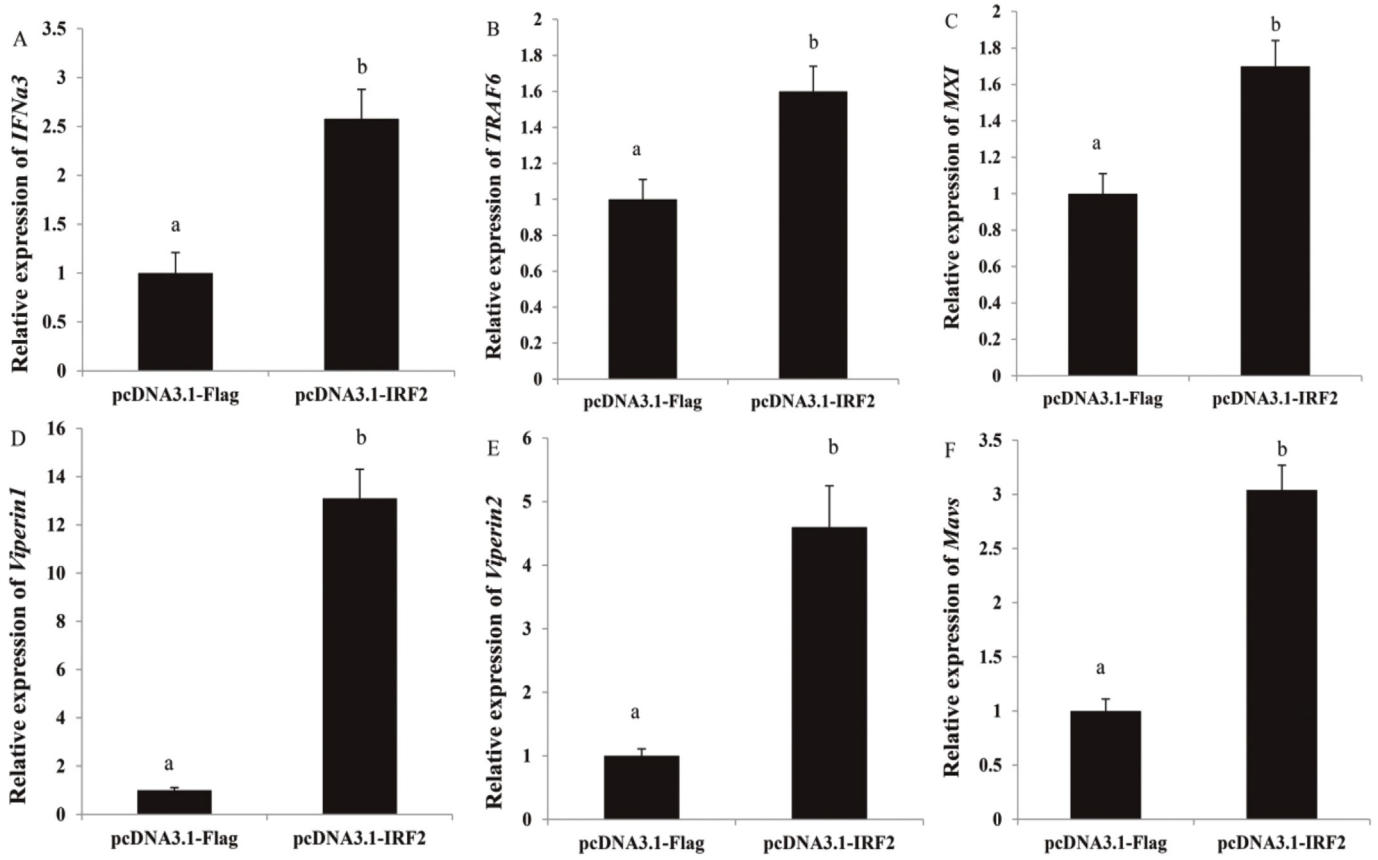

Fig. 9. Overexpression of ToIRF2 altered the expression levels of IFN/IRF-based signalling pathway genes after induction by poly (I: C) (5 μg/mL) in GPS cells for 36 h. The relative expression levels of IFNa3 (A), TRAF6 (B), MXI (C), Viperin1 (D), Viperin2 (E) and Mavs (F) were determined using qRT-PCR 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.

speculate that ToIRF2 has the potential to bind to the *ToIFNa3* promoter and stimulate its activity. We found that the deletion of the ToIRF2 M4(-496 bp to −470 bp) and M5 (−466 bp to −437 bp) binding sites results in a significantly reduced promoter activity of *ToIFNa3* (Fig. 6). Obviously, IRF2 plays a key regulatory role in the IRF2-dependent type I IFN responses through the ToIRF2 M4/5 binding sites. These results showed that *ToIFNa3* expression was controlled by ToIRF2. The EMSA assay further demonstrated that IRF2 specifically bound to the *ToIFNa3* promoter at the M4/5 binding site (Fig. 7).

These results provide the first evidence of the involvement of IRF2 in the expression of type I IFNα. *ToIFNa3* transcription showed increasing levels in heterologous HEK293T cells. Moreover, the promoter activity of *ToIFNa3-2* was observably upregulated by all three pathogen-associated molecular patterns, especially by poly (I:C) stimulation (Fig. 8). The mRNA levels of poly (I:C)-induced IFN/IRF-based signalling pathway genes, IFNa3, TRAF6, MXI, Viperin1, Viperin2 and Mavs, were also upregulated by overexpression of IRF2 after poly (I:C) stimulation (Fig. 9). These results suggested that IRF2 might positively promote the poly (I:C)-induced IFN/IRF-based signalling pathway. We speculated that the positive regulation of the poly (I:C)-induced IFN/IRF-based signalling pathway by IRF2 might contribute directly to its enhancing effect on immune responses.

In general, structural complexity can be caused by intron gains or losses, which is a core evolutionary mechanism in most gene families [50]. An exon-intron structure analysis of the *ToIRF2* gene indicated that all metazoan *IRF2* genes had eight exons, while *IRF2* had seven exons in medaka. These findings might represent introns gained or lost during evolution and may also suggest that the metazoan *IRF2* genes consist of highly conserved numbers of exons and introns. Moreover, the results of the phylogenetic analysis were consistent with the findings of conventional taxonomy, suggesting that *ToIRF2* exhibited a closer genetic relationship with Perciformes, such as tilapia *IRF2*.

In summary, *ToIRF2* mRNAs were prominently upregulated in immune-related tissues under poly(I:C), LPS or flagellin induction, demonstrating a possible role for *ToIRF2* in the defence against pathogenic microbes. Furthermore, we demonstrated clear associations between ToIRF2 and the *ToIFNa3* promoter, as well as the positive regulatory functions of ToIRF2 on *ToIFNa3* transcription in pompano. The present study provides the first evidence of IRF2 as a positive regulator of *IFNa3* transcription. It would be interesting to further clarify the interactions between IRF2 and the proposed cooperative companions to better comprehend the mechanisms underlying the IRF-mediated regulation of type I IFN transcription. Furthermore, the specific mechanism of IRF2 in the regulation of *ToIFNa3* and whether it binds directly with other proteins still requires further investigation.

Competing Interests

The authors declare no competing interests.

Acknowledgments

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(2019B030316030).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.045.

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