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Molecular cloning and characterization of interferon regulatory factor 7 (IRF-7) in Japanese flounder, *Paralichthys olivaceus*

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ABSTRACT

Interferon regulatory factor (IRF) 7 in mammals is known to be a key player in regulating the type I interferon (IFN) response to viral infection as a transcription activator of IFNs and IFN-stimulated genes (ISGs). In this study, a full-length cDNA of Japanese flounder, *Paralichthys olivaceus*, (*Po*)IRF-7 was cloned and characterized. *Po*IRF-7 is 2032 bp in length, with an open reading frame (ORF) of 1293 bp that encodes 430 amino acid residues. The putative amino acid sequence shows the highest homology to fish IRF-7 with 51.5–76.3% identity and possesses a DNA-binding domain (DBD), an IRF association domain (IAD) and a serine-rich domain of vertebrate IRF-7. In addition, the tryptophan cluster of *Po*IRF-7 DBD consists of only four tryptophans, which is a characteristic unique to all fish IRF-7 members. The *Po*IRF-7 was expressed constitutively in all tested tissues of healthy flounders, with high levels in head kidney, spleen, gill, intestine and skin, and moderately expressed in FG9307 cells, a flounder gill epithelial cell line. Using a luciferase assay, *Po*IRF-7 was proved to be capable of activating fish type I IFN promoter in FG9307 cells. A quantitative real time PCR assay was employed to monitor the gene expression of *Po*IRF-7 and Mx in FG9307 cells and flounder head kidney and gill. Both genes were up-regulated by poly-inosinic:polycytidylic acid (poly I:C) and lymphocystis disease virus (LCDV) though to a much lesser extent in FG9307 cells. Further, their transcription kinetics were similar in fish organs but different in FG9307 cells. These data provide insights into the functions of *Po*IRF-7 and imply a difference in *Po*IRF-7-related signaling pathways in antiviral response between cultured cells and live fish.

1. Introduction

The interferon regulatory factors (IRFs) were originally known as transcriptional regulators of typelinterferon (IFN) and IFN-stimulated genes (ISGs) which form the first line of antiviral defence in vertebrates [1]. To date, ten IRF members, IRF-1 to -10, have been identified, with only IRF-10 not found in mammals [2]. The IRF family has been now believed to play diverse roles in a wide variety of cellular processes involving antiviral response, cytokine signaling, cell growth control and lymphocyte development [3–5]. All IRFs share extensive homology in the DNA-binding domain (DBD) covering the first about 120 amino acids of the amino terminus, in which a five tryptophan repeat cluster responsible for the binding to the promoters of target genes was located. In addition, IRFs, except IRF-1 and -2, possess an IRF association domain (IAD) at the carboxyl terminus which interacts with other proteins to form transcriptional complexes [6]. IRF-7 is one of the most intensively studied IRF members due to its crucial role in innate antiviral immunity [7].

In mammals, cellular type I IFN response is triggered through two types of pathogen-associated molecular pattern (PAMP)-recognition system. One is the Toll-like receptor (TLR)-dependent which is the unique system for plasmacytoid dendritic cells (pDCs), the other TLR-independent existing in most nucleated cells. In the two systems, IRF-7 plays a central role in driving the antiviral response through the TLR7/9-activated, MyD88-dependent pathway and the virus/TLR3-activated, MyD88-independent pathway, respectively [8,9]. Constitutively expressed IRF-7 is generally present in the cytoplasm of uninfected cells in an inactive form. After virus-mediated phosphorylation and dimerization, IRF-7 translocates to the nucleus and bind to the type I IFN promoters, activating transcription either alone or in coordination with other transcription factors such as IRF-3 and NF-κB. When IFNs are produced, they secrete into neighbouring cells and trigger transcription of a set of antiviral ISGs though the Jak-STAT pathway, leading to establishment of an antiviral state in uninfected cells. However, IRF-7 is also an ISG, which is up-regulated by early phase

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IFNs, thus resulting in a amplifier circuit in IFN signal pathway. Further, the IRF-7 bridges the innate and adaptive immunities through control of $CD8^+$ T-cell responses and activities of IRF-1 and low molecular mass polypeptide-2 (LMP-2), two proteins playing important roles in adaptive immunity [10,11]. Indeed, all elements of type I IFN responses, either the systemic production of IFN in innate immunity or the local action of IFN from pDCs adaptive immunity, are under the control of IRF-7. It is now regarded as the "master regulator" of typeI IFN-dependent immune responses [12].

Fish are a group of vertebrates reported to have an IFN system similar to that of mammals though much less studied [13–15]. To date, IRF-7 has been cloned and studied in crucian carp [16,17], mandarin fish [18], snakehead [19], rainbow trout [20] and Atlantic salmon [21,22]. Interestingly, two IRF-7 isoforms encoded putatively by two different genes were found in the last species. Fish IRF-7 exhibits lots of properties similar to those of mammalian IRF-7 in gene and promoter structures, inducibility by stimuli and regulatory activity on target promoters. In Japanese flounders, *Paralichthys olivaceus*, one of the major cultured marine fishes in China, some molecules of IFN system have been identified, such as IRF-1, IFN-γ, and two ISGs, Mx and ISG15, all of which are up-regulated by virus, polyinosinic:polycytidylic acid (poly I:C), a synthetic mimic of dsRNA, or bacterium [23–26]. Moreover, flounder IRF-1 is able to modulate the early immune response against viruses and induce an antiviral state in fish cells, a characteristic of the IFN-driven immune response described for mammals [27,28]. However, flounder IRF-7 has not yet been identified so far.

Over the past decade, flounder farming industry in China has been suffered a growing damage from various viral epidemics including lymphocystis disease caused by the infection of lymphocystis disease virus China strain (LCDV-cn) [29,30]. An understanding of the IFN system in flounder will be helpful to the control of viral diseases of this commercially important species. The aim of this study was to enrich the knowledge about flounder IFN system by studies of an IRF-7 gene in *P. olivaceus* (*Po*IRF-7). Herein, we reported sequencing and expression characterization of *Po*IRF-7 and its action on fish type I IFN promoter. In particular, we compared the transcription kinetics of *Po*IRF-7 with that of Mx, the hallmark of type I IFN response, in cultured cells and live fish upon challenge with poly I:C or LCDV-cn.

2. Materials and methods

2.1. Fish, cells and virus

Flounders (*P. olivaceus*) (158–170 g, $n = 48$) were purchased from a local fish farm. Fish were acclimatized in aerated seawater tanks at 18 °C for one week before use. FG9307 cells, a flounder gill epithelial cell line, were maintained at 24 °C in MEM medium (GIBCO, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. LCDV-cn was isolated from tumour-carrying flounders as previously described [31]. The viral titers were estimated by a 50% tissue culture infective dose ($TCID_{50}$) assay according to the method of Reed and Muench [32]. The viruses were aliquoted in 1 ml stock and stored at −80 °C until use.

2.2. Challenge of FG9307 cells with poly I:C and LCDV

FG9307 cells were passaged to 75% confluency in 25 cm^2 plastic flasks in 5 ml complete medium and grown for 24 h prior to experimentation. The medium of cell cultures were replaced with serum-free MEM containing 50 μg/ml poly I:C (Sigma, St Louis, MO, USA) or serum-free MEM alone as control and incubated at 24 °C. In the virus infection trial, cell culture supernatants were removed, and 1 ml of LCDV-cn prepared in serum-free MEM at 10^4 $TCID_{50}$/ml or serum-free MEM alone as control were added to the surface of cellular confluency for absorption for 2 h at 24 °C, then replaced with 5 ml serum-free MEM and incubated at 24 °C. The cells were harvested for real time PCR assays at 0, 4, 8 12, 24, 36, 48, 60, 72, 84 and 96 h after treatment.

2.3. Challenge of flounders with poly I:C and LCDV

Two group of flounders were intraperitoneally (i.p.) injected with poly I:C (Sigma) (10 mg/ml, 100 μl per fish) or LCDV-cn ($10^{6.23}$ $TCID_{50}$/ml, 200 μl per fish), respectively. Control fish were injected with the same volume of phosphate-buffered saline (PBS). The head kidney and gill were collected for real time PCR assays at 0, 1, 2, 3, 4, 5, 6 and 7 day after injection.

2.4. RNA extraction

Total RNA was extracted from cells and fish tissues using Isogen (Nippon Gene, Tokyo, Japan). RNA sample were treated with DNase I to remove genomic DNA contamination using the Turbo DNA-free kit (Ambion, Austin, TX, USA). The RNA concentration was determined by measuring absorbance at 260 nm, and its quality was monitored by A_{260nm}/A_{280nm} ratios > 1.8.

2.5. cDNA cloning

From 1 μg of total RNA extracted from head kidney of a flounder 4 days post-infection with LCDV-cn, a double strand cDNA pool was produced using a SMART cDNA Library Construction Kit (Clontech, Palo Alto, CA, USA). Based on the conserved sequences of known fish IRF-7s, degenerate primers were designed. Partial cDNA was amplified with a combination of first-round and nested PCR using degenerate primer pairs IRF7-hF1/IRF7-hR1 and IRF7-hF2/IRF7-hR2, respectively. 3′-rapid amplification of cDNA ends (RACE) was performed by a first round PCR using primers IRF7-3F1 and CDS III/3′, followed by a nested PCR using IRF7-3F2 for sense primer and CDS III/3′ for antisense primer. 5′-RACE was performed by a first round PCR using 5′ UPM for sense primer and IRF7-5R1 for antisense primers, followed by a nested PCR using 5′ NUP for sense primer and IRF7-5R2 for antisense primers. The full coding region of *Po*IRF-7 was amplified using primer pair IRF7-fF/IRF7-fR with Advantage 2 Polymerase Mix (Clontech). The amplicon was cloned into EcoRI and HindIII sites of pcDNA3.1/*myc*-His(−) A vector (Invitrogen, Carlsbad, CA, USA). Primers used in the present study were shown in Table 1.

2.6. Sequence analysis

Sequence result of *Po*IRF-7 was compared with the Genbank/EMBL database by using the BLASTX and BLASTP search programs (http://blast.genome.ad.Jp). The nucleotide sequence was translated to protein sequence using Translate Tool (http://www.expasy.org/tools/dna.html). The multiple alignment of protein sequences was produced by the Clustal W program (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). The phylogenetic tree was created using the neighbor-joining algorithm within MEGA version 3.1 [33]. Bootstrap values were calculated with 1000 replications to estimate the robustness of internal branches. A bootstrap cut-off parameter of 70% was used.

2.7. Tissue distribution

Reverse transcription polymerase chain reaction (RT-PCR) was used to examine tissue distribution of *Po*IRF-7 mRNA in healthy fish and its expression in FG9307 cells. Total RNA at 0.2 μg from various

Table 1
Sequences of primers used in the present study.

Primers	Sequence (5′–3′)	Usage
IRF7-hF1	TTYMGNGTNCCNTGGAARCA	first round homology PCR
IRF7-hR1	TTYTTYTCNARNGGYTTNCC	first round homology PCR
IRF7-hF2	TGGAARACNAAYTTYMGNTGYGC	nested homology PCR
IRF7-hR2	TCNGGRAAYTTYTCNCCRAARCA	nested homology PCR
IRF7-3F1	CCAACCACAAGCTTTTACAGATCA	first round 3′-RACE PCR
IRF7-3F2	CAAATCCTACACCAGTCGTCAGTTC	nested 3′-RACE PCR
IRF7-5R1	TGTTTGTGGTCTAGCAGGCCGTCAGT	first round 5′-RACE PCR
IRF7-5R2	TCACCAGATTGTACTCATTTCCT	nested 5′-RACE PCR
IRF7-fF	TAGAATTCTGCTGAGAGATGCAAAGCCT	expression plasmid construction
IRF7-fR	GCAAGCTTTGAGATCTAATGCAGGAAGG	expression plasmid construction
IRF7-tF	TACCAGTCTCACAAACCTCCAAC	tissue PCR
IRF7-tR	CCAACCACAAGCTTTTACAGATCA	tissue PCR
β-actin F	GAAATGGGAACCGCTGCCTC	tissue PCR
β-actin R	GTTGGCGTACAGGTCCTTACG	tissue PCR
18sRNA-F	GGTGGAGCGATTTGTCTGG	real time PCR
18sRNA-R	TGGGGTTCAGCGGGTTAC	real time PCR
IFN-pF	GCTCGAGTCGATTGAGTAATTGATTG	promoter cloning
IFN-pR	GGAAGCTTAGTAGCTTTCTACACTGTCG	promoter cloning

Note: (1) N represents all four nucleotides; M, C or A; R, G or A; Y, C or T. (2) Restriction sites are underlined.

tissues of healthy fish or untreated FG9307 cells was reverse-transcribed separately in a 20 μl reaction system with Superscript First Strand Synthesis System (Invitrogen). PCR was performed in a 25 μl reaction system with 1 μl reverse-transcribed product. Primer pair IRF7-tF/IRF7-tR (Table 1) was used for the amplification of *Po*IRF-7. For an internal control, the flounder β-actin transcript (accession number EU090804) was amplified with primers β-actin F and β-actin R (Table 1). Amplified products were electrophoresed on 1.2% agarose gels containing ethidium bromide (EtBr) and visualized under ultraviolet light.

2.8. Luciferase assay

The ability of *Po*IRF-7 to activate fish type I IFN promoter was studied using a luciferase reporter gene assay. A region of grass carp type I IFN promoter (accession number GU139255) spaning from +17 to −915 nt relative to the transcription start site was amplified with the primer pair IFN-pF/IFN-pR (Table 1) and inserted into the *Xho*I and HindIII sites of the pGL3-basic vector (Promega, Madison, WI, USA) which contains a promoterless firefly luciferase gene downstream of the multiple cloning sites. The pRL-SV40 vector (Promega) expressing renilla luciferase was used as an internal control for transfection efficiency. FG9307 cells were seeded in 24-well plates (1.5×10^5 cells per well) overnight before transfection. Cells of each well was cotransfected with 200 ng of the IFN promoter-luciferase vector, 100 ng of pRL-SV40 (Promega) and 200 ng of either empty pcDNA3.1 or pcDNA3.1-*Po*IRF-7 vector for 24 h using Lipofectamine 2000 (Invitrogen) in 500 μl serum-free MEM. Basic control cells were cotransfected only with the IFN promoter-luciferase vector and pRL-SV40. Cells were subsquently incubated in fresh MEM with 2% FBS for a further 48 h and lysed with the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity of the lysates were measured in a Junior LB9509 Luminometer (Berthold Technologies, Bad Wildbad, Germany).

2.9. Real time PCR assay

A quantitative real time PCR assay was employed to study inducible expression profile of *Po*IRF-7 and flounder Mx gene (accession number AB110446) unpon challenge with poly I:C and LCDV. 1.0 μg of total RNA from each target tissue (3 individuals each) was reverse-transcribed into cDNA by random primers using Superscript First Strand Synthesis System (Invitrogen). Real time PCR was performed on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA). The PCR mixture in 20 μl volume contained 1× SYBR Green Real time PCR Mast Mix (Toyobo, Osaka, Japan), 0.2 μM each of specific forward and reverse primers (Table 1) and 1.0 μl diluted cDNA (50 ng/μl). PCR conditions were 94 °C for 4 min, followed by 40 cycles of 94 °C for 20 s, 60 °C for 30 s, 72 °C for 30 s. Flounder 18s rRNA (accession number EF126037) was used as endogenous control. All samples were amplified in triplicates. Fluorescent detection was performed after each extension step. A dissociation protocol was performed after thermocycling to verify that a single amplicon of expected size was amplified. Expression levels of target genes were normalised to 18s rRNA and expressed as fold change relative to the expression level in control which was set to 1.

3. Results

3.1. Cloning and identification of PoIRF-7 cDNA

Using consecutive techniques of RT-PCR and RACE, a full-length cDNA (accession number GU017419) encoding IRF-like peptide was cloned from head kidney of LCDV-infected Japanese flounder. Because it exhibits the highest homology with fish IRF-7 sequences in a BLASTP analysis, we named it *Po*IRF-7. The cDNA of *Po*IRF-7 is 2032 bp long with an open reading frame (ORF) of 1293 bp encoding 430 amino acid residues. In the 655-bp 3′-UTR, three ATTTA mRNA instability motifs and one polyadenylation signal (AATAAA) 12 bp upstream of the poly(A) tail were found. The deduced amino acid sequence of *Po*IRF-7 shares identity of 51.5–76.3% to fish IRF-7, 29.5–30.5% to IRF-7 from vertebrates other than fish, and 26.2-29.6% to vertebrate IRF-3 (Table 2). The IRF-7 and -3 groups together constitute the IRF-3 subfamily. A lower identity (12.6–28.5%) was observed between *Po*IRF-7 and other IRF subfamily proteins. Although derived from a same species, *Po*IRF-7 shares an identity of only 13.8% to flounder IRF-1 (Table 2). Like

Table 2
The amino acid identities (%) and similarities (%) of *Po*IRF-7 to representive IRF proteins.

Protein	Species	Accession number	Identity	Similarity
IRF-3 subfamily				
IRF-7	Japanese flounder (*Paralichthys olivaceus*)	GU017419		
	Snakehead (*Channa argus*)	EF067848	76.3	84.3
	Rainbow trout (*Oncorhynchus mykiss*)	AJ829673	56.8	68.1
	Zebrafish (*Danio rerio*)	BC058298	51.5	66.3
	Chicken (*Gallus gallus*)	U20338	29.5	44.9
	Mouse (*Mus musculus*)	U73037	30.5	48.0
	Human (*Homo sapiens*)	U73036	30.2	44.2
IRF-3	Rainbow trout (*Oncorhynchus mykiss*)	AJ829668	26.6	40.8
	Zebrafish (*Danio rerio*)	NM_001143904	26.2	40.9
	Cow (*Bos taurus*)	AJ879589	29.6	45.6
Other subfamilies				
IRF-1	Japanese flounder (*Paralichthys olivaceus*)	AB005883	13.8	25.4
IRF-2	Atlantic salmon (*Salmo salar*)	BT044804	12.6	24.3
IRF-4	Atlantic salmon (*Salmo salar*)	BT045002	24.9	39.8
IRF-5	Atlantic salmon (*Salmo salar*)	BT044767	26.7	43.8
IRF-6	Zebrafish (*Danio rerio*)	BC056772	28.5	44.7
IRF-8	Zebrafish (*Danio rerio*)	BC075963	25.3	46.4
IRF-9	Human (*Homo sapiens*)	M87503	22.8	38.9
IRF-10	Chicken (*Gallus gallus*)	AF380350	25.2	41.4

other known IRF-7 proteins, *Po*IRF-7 has a DBD at the N-terminal shared by all IRF members and an IAD and a serine-rich domain at the C-terminus (Fig. 1). The serine residues in the serine-rich domain are targeted for virus-induced phosphorylation upon which IRF-7 is activated [34]. In particular, the tryptophan cluster in the DBD of *Po*IRF-7 consists of only four of five conserved tryptophan residues with lack of the second one, a characteristic unique to fish IRF-7. The multiple alignment of IRF-7 amino acid sequences (Fig. 1) revealed that the conserved residues are basically restricted to the areas of DBD, IAD and serine–rich domain, whilst the middle part of the sequence is very diverse with several deletions found in fish lineage which accounts for a smaller size of fish IRF-7 relative to mammalian and avian sequences.

3.2. Phylogenetic analysis

In order to determine the phylogenetic position of *Po*IRF-7, overall amino acid sequences of 47 IRFs covering IRF-1 to -10 from various vertebrates were collected to construct a phylogenetic tree (Fig. 2). The vertebrate IRF family is classified into four subfamilies.

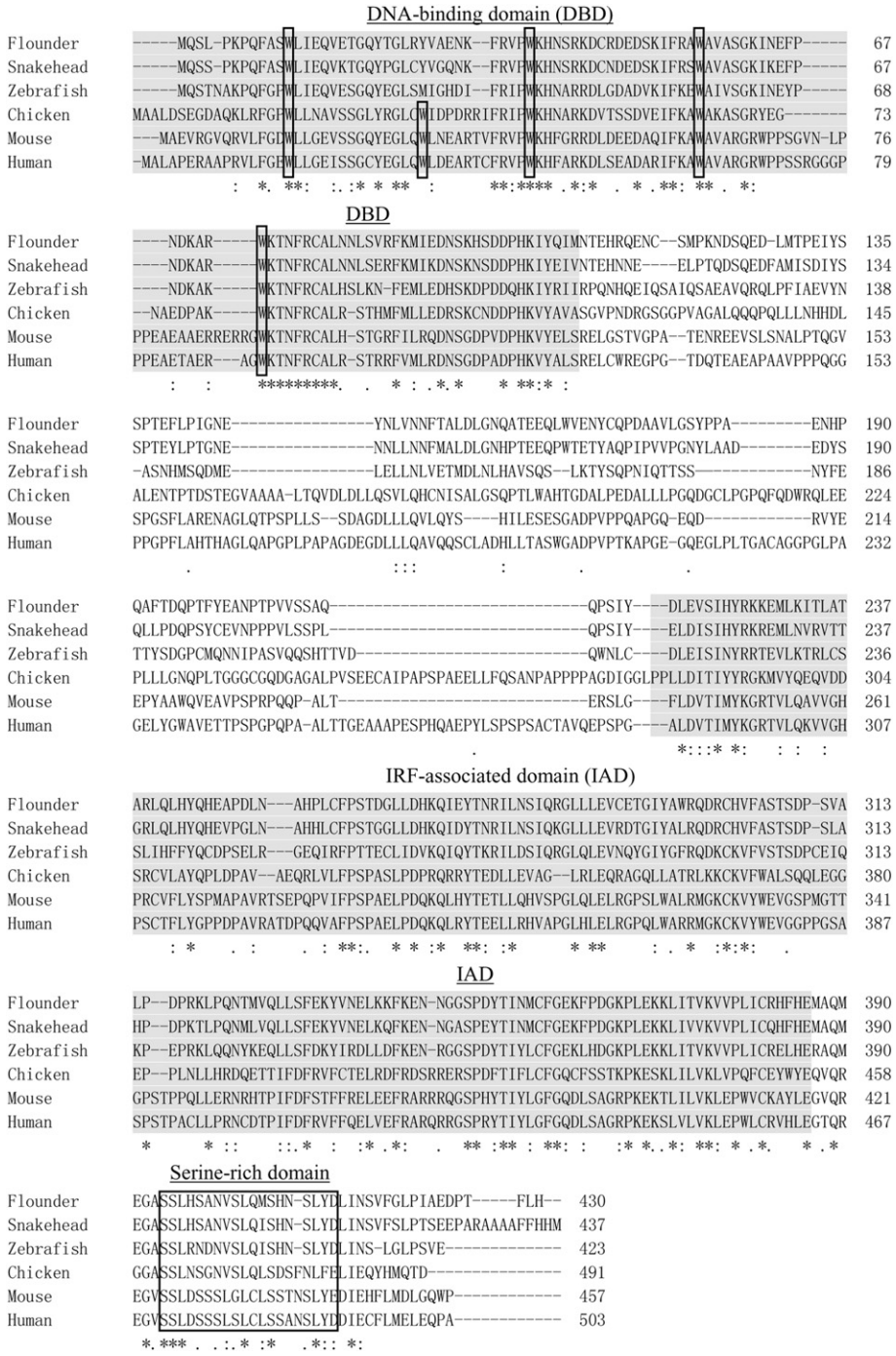

Fig. 1. Multiple alignment of *Po*IRF-7 (Japanese flounder IRF-7) amino acid sequence with other IRF-7 proteins. The putative DNA-binding domain (DBD) and IRF-associated domain (IAD) are highlighted in gray. The conserved tryptophan (W) residues composing a "tryptophan cluster" are boxed in the DBD. The predicted serine-rich domain is boxed in the C-terminal. The residues identical in all sequences are shown with asterisks (*), whereas those with strong homologies and weak similarities are marked by colons (:) and dots (.), respectively. The accession numbers of IRF-7 sequences are listed in Table 2.

The nodes of the phylogenetic tree perfectly reflected the known taxonomic relationship among IRF members. *Po*IRF-7 was clustered into fish IRF-7 subgroup of the vertebrate IRF-7 which together with IRF-3 group forms the IRF-3 subfamily. In the tree, *Po*IRF-7 shows the closest phylogenetic distance to snakehead IRF-7 and a similar farther distance to both mammalian and chicken IRF-7s. These data support the authenticity of the nomenclature for *Po*IRF-7. In respect to the relationship with the two isoforms of

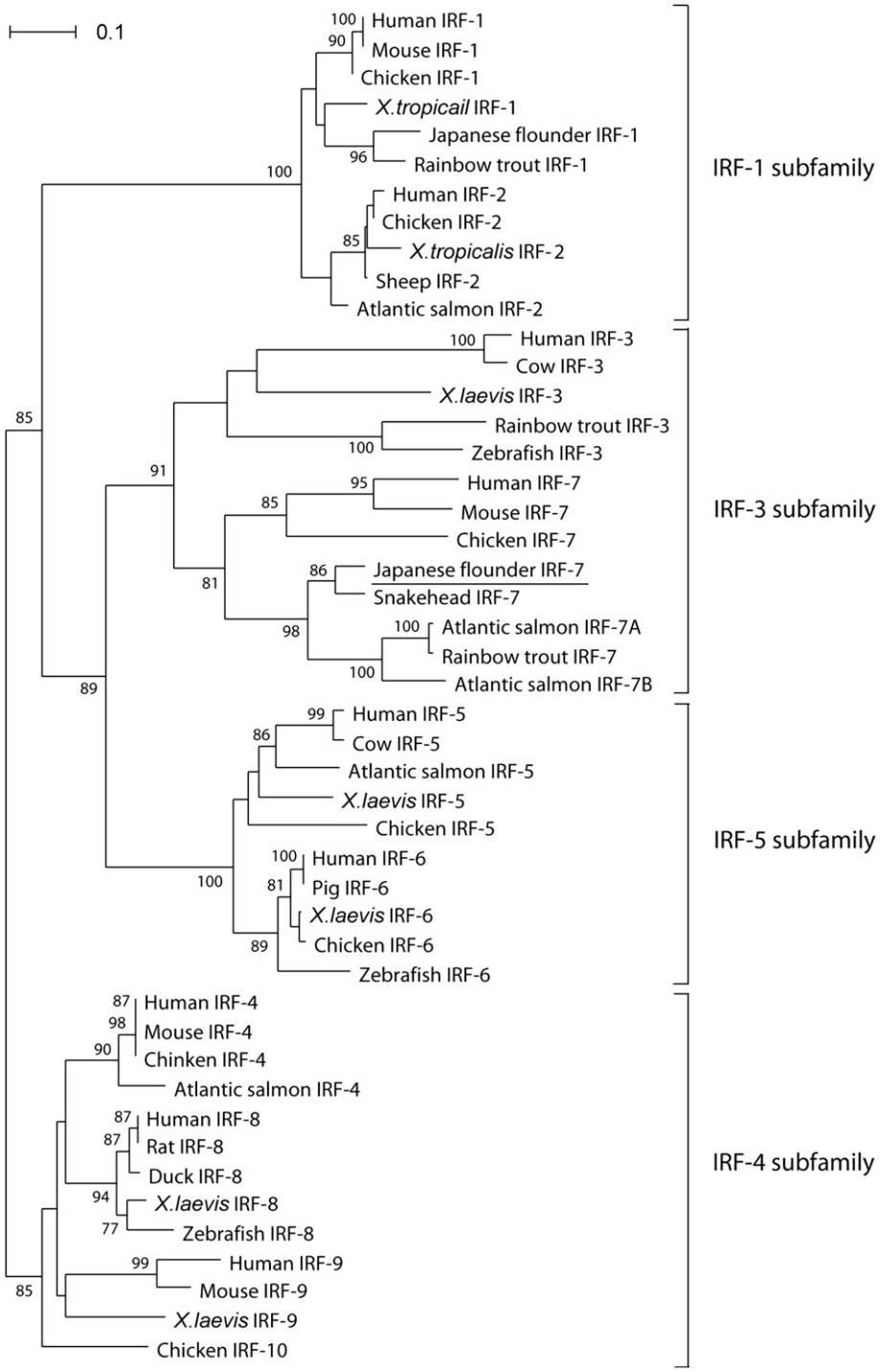

Fig. 2. Phylogenetic tree of the IRF family proteins in vertebrates. The tree was depicted on the overall sequences by neighbor-joining method. Bootstrap values larger than 70% are shown next to the branches based on 1,000 replications. The *Po*IRF-7 (Japanese flounder IRF-7) is underlined. The accession numbers of the IRF-1 sequences are: Human, X14454; Mouse, M21065; Chicken, L39766; *X. tropicalis*, BC075398; Rainbow trout, AF332147. The accession numbers of the IRF-2 sequences are: Human, X15949; Sheep, AF228445; Chicken, X95478; *X. tropicalis*, BC080892. The accession numbers of the IRF-3 sequences are: Human, BC009395; *X. laevis*, BC045003. The accession numbers of the IRF-4 sequences are: Human, U52682; Mouse, AK089319; Chinken, AF320331. The accession numbers of the IRF-5 sequences are: Human, NM_002200; Cow, BT021607; Chicken, AJ720409; *X. laevis*, BC082862. The accession numbers of the IRF-6 sequences are: Human, AF027292; Pig, AF327368; Chicken, DQ250733; *X. laevis*, BC108593. The accession numbers of the IRF-7 sequences are: Atlantic salmon-A, EU153263; Atlantic salmon-B, FJ517644. The accession numbers of the IRF-8 sequences are: Human, M91196; Rat, NM_001008722; Duck, DQ266446; *X. laevis*, BC077289. The accession numbers of the IRF-9 sequences are: Mouse, U51992; *X. laevis*, BC070535. The accession numbers of other IRF sequences are listed in Table 2.

Atlantic salmon IRF-7, *Po*IRF-7 has a little bit closer relatedness with IRF-7A.

3.3. Tissue distribution of PoIRF-7 mRNA

The mRNA expression of *Po*IRF-7 in various tissues and FG9307 cells was examined by RT-PCR (Fig. 3). The results showed that *Po*IRF-7 was constitutively expressed in all tested tissues including brain, gill, esophagus, stomach, anterior and posterior intestines, head kidney, kidney, spleen, liver, heart, gonad, skin and muscle of healthy flounders. The strong expression of *Po*IRF-7 was detected in the head kidney, kidney, spleen, anterior and posterior intestines and skin, while a weaker expression detected in the brain, gonad, esophagus and liver. In untreated FG9307 cells, a moderate expression was observed.

3.4. Activation of fish type I IFN promoter by PoIRF-7

As shown in Fig. 4, cells of basic control and empty vector control had a similar luciferase level. In contrast, cells transfected with *Po*IRF-7 construct showed a higher (2.2-fold increase) luciferase level than the empty vector control. This thus suggests an activation of the type I IFN promoter by over-expressed *Po*IRF-7.

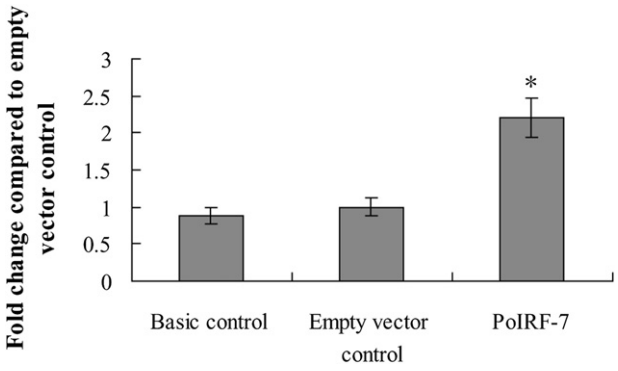

Fig. 4. Effect of *Po*IRF-7 on the grass carp type I IFN promoter in FG9307 cells. Cells were cotransfected with one of following vector combinations for 24 h: (1) the IFN promoter-luciferase vector and pRL-SV40 as basic control, (2) the IFN promoter-luciferase vector, pRL-SV40 and empty pcDNA3.1 as empty vector control and (3) the IFN promoter-luciferase vector, pRL-SV40 and pcDNA3.1-*Po*IRF-7 vector as *Po*IRF-7 transfection treatment. Luciferase activities were measured after a further 48 h incubation of cells in fresh medium. The data are presented as the fold change of luciferase activity relative to the empty pcDNA3.1 control after normalized against the secreted renilla luciferase activity. The mean of three replicates is shown ± S.D. Asterisk indicates a significant difference ($p < 0.05$) from pcDNA3.1 by Student's *t* test.

3.5. Induction of PoIRF-7 and Mx transcripts in FG9307 cells by poly I:C and LCDV

In order to obtain further insights into the roles of *Po*IRF-7 in antiviral response, we compared expression profile of *Po*IRF-7 and Mx genes in FG9307 cells challenged with poly I:C or LCDV. Both genes were up-regulated by poly I:C or LCDV, though a lower inducibility was seen for *Po*IRF-7 and a weaker action for LCDV (Fig. 5). It is noteworthy that the two genes differed in expression time course. In poly I:C stimulated cells, *Po*IRF-7 transcripts reached peak levels (6-fold increase) at 24 h post-stimulation. In contrast, the highest level (11-fold increase) of Mx transcripts appeared at 48 h. In LCDV-infected cells, both *Po*IRF-7 and Mx levels increased slower and more weakly. Peak levels were observed at 48 h post-infection with a 2.8-fold increase for *Po*IRF-7, whereas, 84 h post-infection with a 6-fold increase for Mx.

3.6. Induction of PoIRF-7 and Mx transcripts in flounder organs by poly I:C and LCDV

The expression profile of *Po*IRF-7 and Mx upon challenge with poly I:C or LCDV was next studied *in vivo*. Because head kidney is an important immune organ in teleost and an organ where *Po*IRF-7 was isolated, and gill is the original tissue from which FG9307 cell line was derived, they were selected for the study. Therefore, four sets of expression kinetics curves were obtained. As shown in Fig. 6, in all cases, *Po*IRF-7 had a transcription kinetics similar to Mx although it responded to the stimuli more weakly. Both *Po*IRF-7 and Mx were strongly induced by poly I:C in head kidney with peak transcript levels of 25- and 53-fold over control, respectively, at day 3 post-injection (Fig. 6A), while they were more weakly induced by LCDV in head kidney (12.6- and 26-fold over control, respectively) with a later peak time of day 4 post-injection (Fig. 6B). In gill, both *Po*IRF-7 and Mx showed a less inducible expression than that in head kidney either by poly I:C (Fig. 6C) or by LCDV (Fig. 6D) with lower peak levels (13- and 39-fold over control, respectively, upon poly I:C stimulation and 8.7- and 18-fold over control, respectively, upon LCDV-infection) and later peak times (4 days post poly I:C-injection and 5 days post LCDV-infection). However, their inducible expressions in gill are much stronger than those in FG9307 cells. As observed in FG9307 cells and head kidney, poly I:C exhibited a more potent and rapid inductive action than LCDV in gill.

4. Discussion

In the present study, we cloned a full-length cDNA homologus to known IRF-7 genes from head kidney of Japanese flounder. Based on the IRF-like structure of the inferred peptide and homology with fish IRF-7 orthologs, the cloned sequence was identified as *Po*IRF-7. The phylogenetic analysis further confirmed the authenticity of the nomenclature for *Po*IRF-7 by clustering it into the group of vertebrate IRF-7. Like other fish IRF-7 gene, *Po*IRF-7 contains the ATTTA

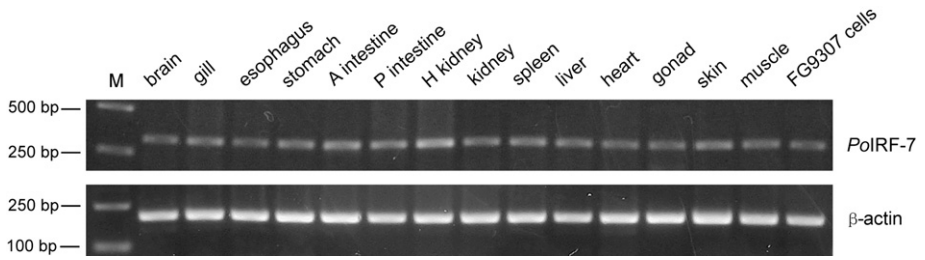

Fig. 3. RT-PCR analysis of *Po*IRF-7 expression in various tissues and FG9307 cells. Transcripts were amplified by RT-PCR with the PCR primer sets presented in Table 1 and electrophoresed on 1.2% agarose gels containing EtBr. Japanese flounder β-actin was used as an internal control. This figure shows the results from one of three fishes which had a similar tissue distribution profile. Abbreviation used: M, DL2000 marker; H kidney, head kidney; A intestine, anterior intestine; P intestine, posterior intestine.

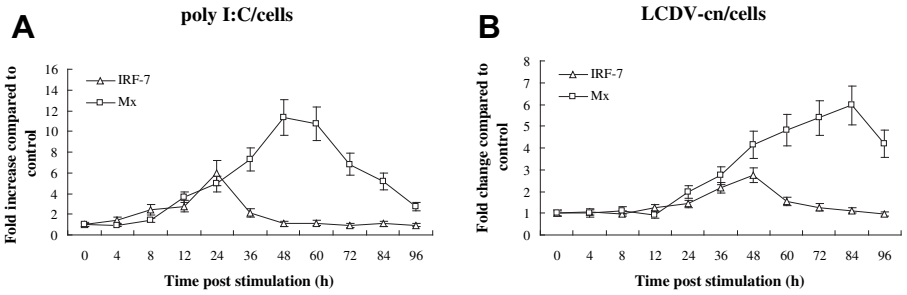

Fig. 5. Expression kinetics of *Po*IRF-7 and Mx in FG9307 cells challenged with 50 μg/ml of poly I:C (A) or 10^4 $TCID_{50}$/1 × 10^6 cells of LCDV (B). Each data point is expressed as the mean of three replicates ± S.D.

instability motif in the 3′-UTR, which is a characteristic of inflammatory mediator genes [35]. The putative *Po*IRF-7 protein possesses three conserved domains found in all known IRF-7s: an N-terminal DBD domain, a C-terminal IAD domain and a C-terminal serine-rich domain. The DBD is typical of all IRF members, characterized by a cluster of five well-spaced tryptophan residues. This region forms a helix-turn-helix motif that binds to the interferon stimulating response element (ISRE) and IRF regulatory element (IRF-E) consensuses in target promoters [36,37]. Studies revealed that three of the five tryptophans are crucial to DNA-protein interactions, which orientate and stabilize amino acid contacts in the IRF molecule with GAAA core sequences of the ISRE/IRF-E consensus [37]. In contrast to IRF-7s in vertebrates other than fish, *Po*IRF-7 and other fish IRF-7s lack the second residue of the tryptophan cluster. Nevertheless, they remain tryptophans at similar positions as the DNA-binding tryptophans of human IRF-1 [37]. The IAD is another conserved domain in IRF family members except IRF-1 and -2. It's responsible for the formation of IRF homo/hetero-dimers and associations with other transcription factors [6]. Unlike the DBD and IAD, the serine rich domain exists straitly in IRF-3, -5 and -7 [20,38]. This domain is important for virus-induced phosphorylation and the following dimerization and exhibits a higher conservation in IRF-7 [20,39]. The *Po*IRF-7 serine rich domain has all serines determined to be essential phosphoacceptor sites in human IRF-7 except for a residue corresponding to Ser^{477} of human sequence which is substituted by Asn^{400}. This thus suggests that *Po*IRF-7 is likely activated through phosphorylation of C-terminal serine residues. Collectively, the structural characteristics of *Po*IRF-7 reveal that it may have a similar function in host's antiviral response as mammalian IRF-7.

In mammals, IRF-7 is usually expressed in immune tissues, especially in plasmacytoid dendritic cells (pDCs), whereas IRF-3 is constitutively expressed in most tissue and cell types and not transcriptionally induced by stimuli [40]. Constitutively expressed IRF-7 in immune cells is crucial for driving the early and late IFN production, the latter driven via IFN-mediated IRF-7 induction [7,11]. In contrast to mammals, fishes including crucian carp, mandarin fish, snakehead and Atlantic salmon express IRF-7 constitutively in an extremely wide variety of tissue types although the tissue types abundant in IRF-7 transcripts were not completely overlapped among various species [16,18,19,22]. However, rainbow trout is an exception since no constitutive expression of IRF-7 was detected in its tissues [20]. In this study, the constitutive expression of *Po*IRF-7 was found ubiquitously in all tested tissues and FG9307 cells. The tissues abundant in *Po*IRF-7 transcripts included head kidney, spleen, kidney, intestine and skin. In agreement with observations in other fish [16,18,19], all these tissues are either

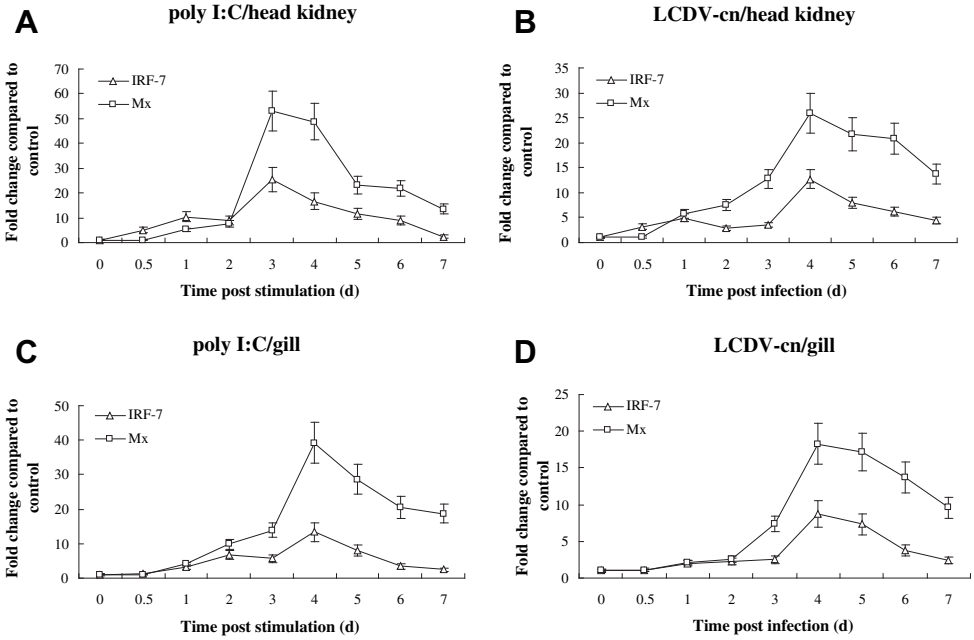

Fig. 6. Expression kinetics of *Po*IRF-7 and Mx in head kidney (A, B) and gill (C, D) challenged with 1.0 mg/fish of poly I:C or 10^6 $TCID_{50}$/fish of LCDV. Each data point is expressed as the mean of three replicates ± S.D.

immune-related or serve as portals for microbes to enter into fish body, where the immune response is easily initiated. Therefore, these data imply that *Po*IRF-7 may play an important role in fish immune system. The constitutive expression of *Po*IRF-7 in FG9307 cells may account for its nature of branchial origin.

We observed that over-expressed *Po*IRF-7 had an ability to elicit a low level activation of fish type I IFN promoter in untreated FG9307 cells. This result is in line with previous studies of other IRF-7s from mammals [41–43], chicken [44] and other fishes [20–22], which are capable of activating type I IFN and ISG promoters either or not challenged with poly I:C or virus. However, the two Atlantic salmon IRF-7 isoforms have different abilities to induce type I IFN promoter [22]. In this regard, the *Po*IRF-7 is much more similar with Atlantic salmon IRF-7A than IRF-7B. The previous studies also demonstrated an enhanced activity of over-expressed IRF-7 following poly I:C or virus challenge. In our preliminary studies, the impact of both poly I:C or virus on the activity of *Po*IRF-7 was inconsistent in FG9307 cells (data not shown). Thus, the action of *Po*IRF-7 on luciferase activity was studied in their absences, which may reflect better any effect of over-expressed *Po*IRF-7. Since promoter sequences of flounder type I IFN, Mx and ISG15 have not yet been identified, we used a grass carp type I IFN promoter to test *Po*IRF-7′ activity. The increased luciferase level seen in *Po*IRF-7 construct transfected cells provides an evidence supporting the induction of grass carp type I IFN promoter by *Po*IRF-7, which is likely mediated by the binding of *Po*IRF-7 to the IRF-E consensus in the promoter, a mechanism as reported for mammals [37].

*Po*IRF-7 was found to be up-regulated unpon challenge with poly I:C and LCDV. In other fish species, the inductions of IRF-7s by treatment with poly I:C or viruses were also confirmed [16,18–21]. These results suggest that the expression of fish IRF-7 gene is regulated in a similar fashion to mammalian IRF-7. Poly I:C, a synthetic mimic of dsRNA, is a well established inducer of fish type I IFNs and ISGs [45]. In mammals, it stimulates the IFN response mainly through the RIG-1 and TLR3 pathways, which implicates activation of the promoter by IRF-3, IRF-7 and NF-κB [46]. In contrast, LCDV is a double-stranded DNA virus which doesn't produce the PAMP of dsRNA over its replication cycle. Thus, it may activate host's antiviral response via a different mechanism. In pDCs of mammals, CpG-rich viral DNA trigger the IFN response through the TLR9-MyD88 pathway [11]. Further, the TLR9 and MyD88 homologs were found in flounders as well as other fish species and capable of responding to CpG oligodeoxynucleotide (ODN), bacterium or virus stimulation [47–50]. Thus, this pathway was likely activated in response of flounder cells to the LDCV infection. Anyway, our study demonstrated a more rapid and stronger induction of *Po*IRF-7 and Mx by poly I:C than LCDV, suggesting that the two stimuli differ in their abilities to activate various signaling pathways. The expression of *Po*IRF-7 was further compared to Mx expression in FG9307 cells and *in vivo*, which may help us to better understand the function of *Po*IRF-7. We used Mx protein as a reference since it is a known ISG with antiviral activity and looked as an indicator of type I IFN response in vertebrates [51]. Both *Po*IRF-7 and Mx genes were shown to be up-regulated slightly by poly I:C or LCDV in FG9307 cells. However, The expression of Mx was induced and reached peak levels at a later stage with a more persistent enhancement, thus suggesting that their transcription kinetics were different. In contrast, the two genes had a similar expression profile in flounder head kidney and gill despite that Mx showed a much higher inducibility by poly I:C or LCDV. The consistency in expression time course of *Po*IRF-7 and Mx may reflect their common property as ISGs. Further, the two genes were induced more strongly in live fish than in cultured cells. Taken together, the discrepancies in gene expression profile between *in vitro* and *in vivo* indicate that the signaling pathways mediating the antiviral response of cultured cells are different from those in live fish. A comparison of the gene expression profile in FG9307 cells with that in the gill provide a more solid evidence supporting this speculation because the cellular differences have been reduced to the most extent in this case. The altered signaling pathways in FG9307 cells may be attributed to their genetic changes in the IFN system and/or lack of signals from the multicellular lymphoid system. The weaker antiviral response in FG9307 cells also accounts in part for why they are highly susceptible to LCDV while *in vivo* infection such as lymphocystis disease in flounders are usually recovered by self-healing [31].

In conclusion, this study demonstrates the present of an IRF-7 gene in Japanese flounder. Its expression profiles and effect on fish type I IFN promoter were also reported. The data indicate that the *Po*IRF-7-related signaling pathways are different between FG9307 cells and live flounders. Further studies of this phenomenon will benefit the elucidation of these signaling pathways important for antiviral response in flounders.

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