

Short communication

Cloning and expression analysis of interferon regulatory factor 7 (IRF-7) in turbot, *Scophthalmus maximus*

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ABSTRACT

Interferon regulatory factor (IRF) 7 is known as the master regulator of type I interferon (IFN)-dependent immune responses in mammals. In this study, the cDNA and genomic sequences of turbot (*Scophthalmus maximus*) IRF-7 (*SmIRF-7*) were cloned and found to encode a putative protein of 439 amino acids. The gene is composed of 10 exons and 9 introns similar to known IRF-7 genes of fish. The *SmIRF-7* shows the highest amino acid identity of 49.0–80.3% to fish IRF-7 and possesses a DNA-binding domain (DBD), an IRF association domain (IAD) and a serine-rich domain (SRD) of vertebrate IRF-7. In addition, the tryptophan cluster of *SmIRF-7* DBD consists of only four tryptophans, which is a characteristic unique to all fish IRF-7 members. The *SmIRF-7* transcripts were expressed constitutively in all analyzed tissues of healthy turbot, with higher levels observed in immune relevant tissues. Gene expressions of *SmIRF-7* and *Mx* were monitored over a 7-day time course by quantitative real time PCR in head kidney and muscle of turbot challenged with turbot reddish body iridovirus (TRBIV), which is a prevalent viral pathogens in farmed turbot in China. Both genes were up-regulated by TRBIV although their inducibility was much weaker in the muscle. The peak levels of *SmIRF-7* transcripts were detected at day 2 post-infection in the two organs with a 12- and 4.5-fold increase, respectively. Further, the *Mx* showed two waves of induced expression and the maximum expression of *SmIRF-7* arose earlier than the second wave of the *Mx* expression in both organs. These findings contribute to an understanding of functions of *SmIRF-7* in antiviral response.

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1. Introduction

Interferon regulatory factors (IRFs) are a family of transcription mediators involved in the transcriptional regulation of type I interferon (IFN) and IFN-stimulated genes (ISGs) (Mamane et al., 1999). IRF-7 is one of the most intensively studied IRF members due to its crucial role in innate antiviral immunity (Honda et al., 2006). 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 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 antiviral response through the TLR7/9-activated, MyD88-dependent pathway and the virus/TLR3-activated, MyD88-independent pathway, respectively (Haller et al., 2006; Takeuchi and Akira, 2007). 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- κ B. When IFNs are produced, they are secreted into neighbouring cells and trigger transcription of a set of antiviral ISGs through 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 IFNs, thus resulting in an 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 with important roles in adaptive immunity (Sgarbanti et al., 2007; Honda and Taniguchi, 2006). 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 (Honda et al., 2005).

Fish are a group of vertebrates reported to have an IFN system similar to that of mammals though much less studied (Zhang et al., 2007; Robertsen, 2008; Chaves-Pozo et al., 2010). To date, IRF-7 has been cloned and studied in crucian carp (Zhang et al.,

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2003; Jiang et al., 2009), mandarin fish (Sun et al., 2007), snakehead (Jia and Guo, 2008), rainbow trout (Holland et al., 2008), Atlantic salmon (Kileng et al., 2009; Bergan et al., 2010) and Japanese flounder (Hu et al., 2010). Interestingly, two IRF-7 isoforms, IRF-7A and B, encoded by two different genes were found in Atlantic salmon (Bergan et al., 2010). However, little is known about IRF-7 in turbot, *Scophthalmus maximus*, an important fish species cultured widely in the world. The aim of this study was to enrich the knowledge about turbot IFN system by studies of an IRF-7 gene. Here, we report its sequence, tissue distribution and expression profiles following challenge with turbot reddish body iridovirus (TRBIV), one of the most prevalent viral pathogens in farmed turbot in China (Zhang et al., 2009).

2. Materials and methods

2.1. Fish and immune challenge

Turbot (*S. maximus*) juveniles (150–165 g, $n = 18$) were purchased from a local fish farm and acclimatized in aerated seawater tanks at 16 °C for 1 week before use. TRBIV was isolated from cultured turbots with TRBIV disease as previously described (Shi et al., 2004). The viral titers were measured by a 50% tissue culture infective dose (TCID₅₀) assay according to the method of Reed and Muench (1938). Turbots were intraperitoneally (i.p.) injected with TRBIV (2×10^6 TCID₅₀/ml, 200 μ l per fish) or with a same volume of phosphate-buffered saline (PBS) as control. The head kidney and muscle were collected for quantitative real time PCR (qRT-PCR) assays at various days after injection. Untreated healthy fish were used for tissue distribution analysis.

2.2. RNA and genomic DNA extraction

Total RNA was extracted from various tissues of turbot using Isogen reagent (Nippon Gene, Tokyo, Japan). RNA samples were treated with DNase I to remove genomic DNA contamination using 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 $A_{260\text{nm}}/A_{280\text{nm}}$ ratios > 1.8 . The genomic DNA was isolated from the muscle by standard phenol/chloroform extraction (Palumbi et al., 1991).

2.3. cDNA cloning

From 1 μ g of total RNA extracted from head kidney of a turbot 4 days post injection with TRBIV, 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. An 881-bp partial cDNA of *S. maximus* IRF-7 was obtained by homology cloning, while the full-length cDNA was obtained by rapid amplification of cDNA ends (RACE) method. Primers used in cloning and expression studies are listed in Supplementary data, Table 1. The cDNA sequence of *S. maximus* IRF-7 is deposited in the GenBank database with accession number HQ424129, and is referred to as *SmIRF-7* in this paper.

2.4. Determination of gene structure of *SmIRF-7*

Based on the full-length cDNA sequence, primers were designed to obtain the full-length genomic sequence of *SmIRF-7*. PCR was performed using the pairs of primers listed in Supplementary data, Table 1. The resulting partial sequences were compiled into the full-length genomic sequence of *SmIRF-7* which is deposited in the GenBank database with accession number HQ424130. The exon/intron structure of the *SmIRF-7* gene was determined by align-

ment of the cDNA to the genomic sequence using Genetyx 7.0 software (GENETYX Corporation, Tokyo, Japan).

2.5. Sequence analysis

Sequence result of *SmIRF-7* cDNA was compared with the Genbank/EMBL database by using the BLASTX and BLASTP search programs (<http://blast.genome.ad.jp>). The cDNA sequence was translated to protein sequence using Translate Tool (<http://www.expasy.org/tools/dna.html>). Alignment of different IRF-7 protein sequences was performed by the Clustal W program (<http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html>). The phylogenetic tree was generated using the neighbor-joining algorithm within MEGA version 3.1. Bootstrap values were calculated with 1000 replications to estimate the robustness of internal branches.

2.6. RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was used to analyze tissue distribution of *SmIRF-7* mRNA in healthy fish. Total RNA at 0.2 μ g from various tissues was reverse-transcribed separately in a 20 μ l reaction system with Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). PCR was performed in a 25 μ l reaction system with 1 μ l reverse-transcribed product. For an internal control, the turbot β -actin transcript (accession no. EU686692) was amplified with primers β -actin F/ β -actin R (Supplementary Table 1).

2.7. qRT-PCR

qRT-PCR assay was employed to study gene expression of *SmIRF-7* and turbot *Mx* (AY635932) upon TRBIV challenge. 1.0 μ g of total RNA from each tissue (3 individuals for each time point) was reverse-transcribed into cDNA by random primers using Superscript First Strand Synthesis System (Invitrogen). qRT-PCR was conducted in 20 μ l volume containing $1 \times$ SYBR Green Real time PCR Mast Mix (Toyobo, Osaka, Japan), 0.2 μ M each of specific forward and reverse primers (Supplementary Table 1) and 1.0 μ l diluted cDNA (50 ng/ μ l). PCR conditions were 94 °C for 4 min, followed by 40 cycles of 94 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s. Turbot 18s rRNA (accession no. EF126038) 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. The expression levels of target genes were normalised to 18s rRNA and expressed as fold change relative to the expression level in control according to the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001).

2.8. Statistical analysis

Statistical analysis was performed using SPSS13.0 software. Data were analyzed via one-way analysis of variance (ANOVA) when comparing the different treatments at one time point, followed by Duncan's multiple comparison test. The value of $P < 0.05$ was considered to be significant.

3. Results and discussion

The full-length *SmIRF-7* cDNA is 1976 bp long and contains a 1320-bp open reading frame (ORF) that encodes 439 amino acids. In the 643-bp 3'-UTR, two ATTTA mRNA instability motifs and two downstream overlapping polyadenylation signals (ATTAAATAAA), starting 27 and 23 bp upstream from the poly(A) tail, were found.

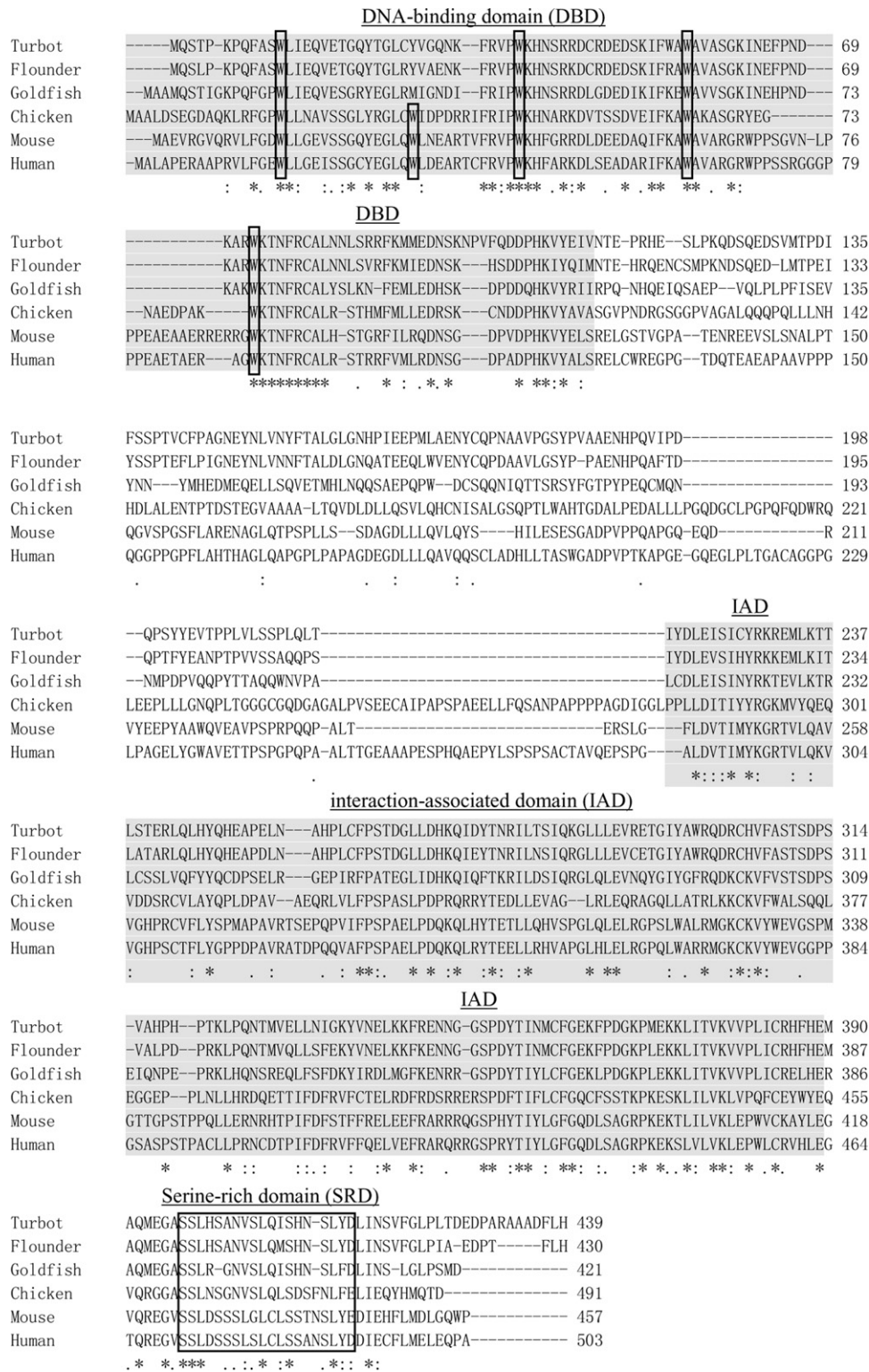


Fig. 1. Multiple alignment of *SmIRF-7* (Turbot IRF-7) amino acid sequence with other IRF-7 proteins. The putative DNA-binding domain (DBD) and IRF-associated domain (IAD) are highlighted in gray. The conserved tryptophan (W) residues composing a “tryptophan cluster” are boxed in the DBD. The predicted 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 Supplementary Table 2.

The *SmIRF-7* gene consists of 3999 bp with a same structure of 10-exon and 9-intron as other fish IRF-7 genes (Supplementary data, Fig. 1), in which all exon/intron junctions conform to the consensus ‘GT/AC’ rule (Breathnach and Chambon, 1981). The putative protein shares the highest overall sequence identity of 49.0–80.3% to fish IRF-7 by a BLASTP analysis (Supplementary data, Table 2)

and possesses three conserved domains found in all known IRF-7s: an N-terminal DNA-binding domain (DBD), a C-terminal IRF association domain (IAD) and a C-terminal serine-rich domain (SRD) (Fig. 1). 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

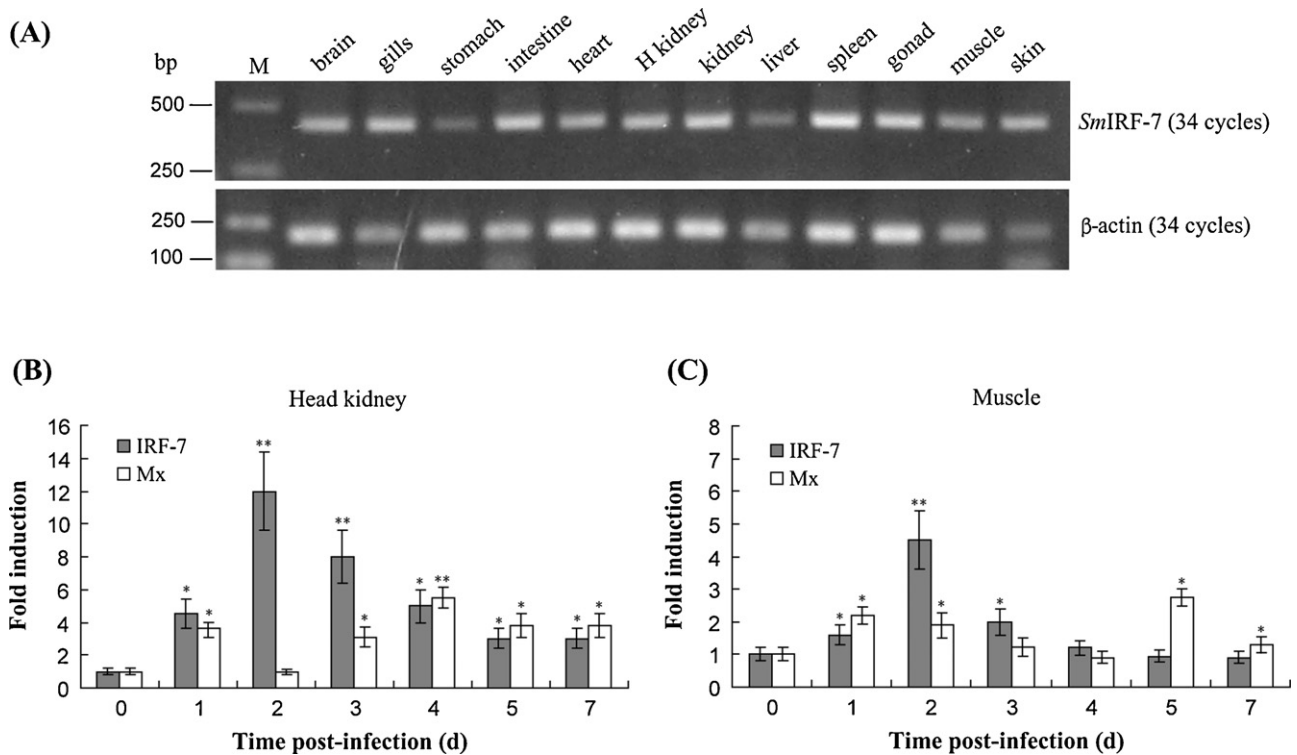


Fig. 2. (A) RT-PCR analysis of *SmIRF-7* expression in different tissues of healthy turbot. The figure shows the results from one of three fishes which had a similar tissue distribution profile. Abbreviation used: M, DL2000 marker; H kidney, head kidney. (B) Time course of *SmIRF-7* and *Mx* expression in head kidney of turbot challenged with 4×10^5 TCID₅₀/fish of TRBIV. (C) Time course of *SmIRF-7* and *Mx* expression in muscle of turbot challenged with 4×10^5 TCID₅₀/fish of TRBIV. Each data point is expressed as the mean of three replicates \pm standard error (S.E.). The level of significance of the comparison to the control is indicated by * $P < 0.05$ and ** $P < 0.01$.

response element (ISRE) and IRF regulatory element (IRF-E) consensus in target promoters (Au et al., 1995; Escalante et al., 1998). 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 (Escalante et al., 1998). In contrast to IRF-7s of vertebrates other than fish, *SmIRF-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 (Escalante et al., 1998). The IAD is another conserved domain in IRF family members except IRF-1 and -2. It is responsible for the formation of IRF homo/hetero-dimers and associations with other transcription factors (Eroshkin and Mushegian, 1999). Unlike the DBD and IAD, the SRD exists strictly in IRF-3, -5 and -7 (Holland et al., 2008; Xu et al., 2010). This domain is important for virus-induced phosphorylation and the following dimerization and exhibits a higher conservation in IRF-7 (Holland et al., 2008; Marie et al., 2000). The *SmIRF-7* SRD has all serines determined to be essential phosphoacceptor sites in human IRF-7 except for a residue corresponding to Ser⁴⁷⁷ of human sequence which is substituted by Asn⁴⁰³. It thus suggests that *SmIRF-7* is likely activated through phosphorylation of C-terminal serine residues.

In order to determine the phylogenetic position of *SmIRF-7*, overall amino acid sequences of 47 IRFs covering IRF-1 to -10 from various vertebrates were collected to construct a phylogenetic tree (Supplementary data, Fig. 2). The vertebrate IRF family is classified into four subfamilies. *SmIRF-7* was clustered into fish IRF-7 subgroup of the vertebrate IRF-7 group which together with IRF-3 group forms the IRF-3 subfamily. In the tree, *SmIRF-7* showed the closest phylogenetic distance to Japanese flounder IRF-7. This matches a close genetic relationship between the two flatfish species.

In mammals, IRF-7 is usually expressed in immune tissues, especially in pDCs and induced transcriptionally by viruses, poly I:C, type I IFNs, lipopolysaccharide and tumor necrosis factor- α (Ning et al., 2005; Honda et al., 2006; Paun and Pitha, 2007). 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 (Honda et al., 2006; Honda and Taniguchi, 2006). In contrast to mammals, fish express IRF-7 constitutively in an extremely wide variety of tissue types (Zhang et al., 2003; Sun et al., 2007; Jia and Guo, 2008; Bergan et al., 2010; Hu et al., 2010). The wider tissue distribution of fish IRF-7 suggests that it may have a diverse role not just limited to the immune system. In this study, the constitutive expression of *SmIRF-7* was found ubiquitously in all tested tissues, with higher levels observed in the immune relevant ones including the spleen, kidney, head kidney, gills and intestine (Fig. 2A). This result indicates an important role of *SmIRF-7* in fish immune system.

To explore the potential role of *SmIRF-7* in antiviral response, its expression profile in organs of TRDIV-injected turbot was studied over a 7-day time course and compared with that of *Mx* gene, the expression of which is usually regarded as a hallmark of type I IFN response in vertebrates (Collet et al., 2004). We selected head kidney and muscle to conduct this study as they represent immune and non-immune organs of fish, respectively. Upon challenge with TRDIV, both genes were up-regulated in the head kidney (12- and 5.5-fold increase at peak level for *SmIRF-7* and *Mx*, respectively, Fig. 2B) and muscle (4.5- and 2.8-fold increase, respectively, Fig. 2C). The weak inducibility found in the muscle suggests a weak IFN response taking place in this tissue where the lymphoid and myeloid cells are absent. It is noteworthy that the *SmIRF-7* reached the maximum expression (at day 2 post-infection in both organs) earlier than the *Mx* (at days 4 and 5 post-infection in head kidney and muscle, respectively), which points toward a potential role

for *SmIRF-7* in activating the downstream cascade of the IFNs-ISGs producing signalling mediated by the PAMP-recognition receptors in turbot. In addition, we detected a minor expression peak for the Mx at day 1 post-infection in both organs. Such an early phase induction of the Mx was likely generated via an IFN-independent way as reported previously (Bazzigher et al., 1992; Kileng et al., 2007). In contrast to some of other IRF-7s (Bergan et al., 2010; Hu et al., 2010), *SmIRF-7* exhibited a higher inducibility than the Mx, which can be explained by a high base-line level of constitutive expression of the Mx in turbot (data not shown). Nevertheless, the weak induction of Mx may represent a sign of IFN suppression caused by TRDIV. In other fish species, the inductions of IRF-7s by treatment with viruses or poly I:C were also confirmed (Zhang et al., 2003; Sun et al., 2007; Jia and Guo, 2008; Holland et al., 2008; Bergan et al., 2010; Hu et al., 2010). These results suggest that the mode of IRF-7 gene regulation is conserved between fish and mammals. TRDIV is a double-stranded DNA virus and expected to trigger host's antiviral response by a mechanism different from that the poly I:C employs since it possesses potentially both DNA (CpG DNA) and RNA (intermediate single- and double-stranded RNAs) PAMPs. In pDCs of mammals, CpG-rich viral DNA trigger the IFN response through the TLR9-MyD88 pathway (Honda and Taniguchi, 2006). Further, the TLR9 and MyD88 homologs were found in other fish species and capable of responding to CpG oligodeoxynucleotide, bacterium or virus stimulation (Takano et al., 2007, 2006; Skjaeveland et al., 2008, 2009). Thus, this pathway was likely activated in response of turbot to TRDIV infection in addition to the pathway activated by the definite PAMP of single-stranded RNA. Together, the structural and expression properties of *SmIRF-7* suggest that it may play a similar role in antiviral defence response as its mammalian counterparts. This, however, remains to be verified by further studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2010.12.004.

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