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# Cloning and expression analyses of interferon regulatory factor (IRF) 3 and 7 genes in European eel, *Anguilla anguilla* with the identification of genes involved in IFN production

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ABSTRACT

Interferon regulatory factor (IRF) 3 and IRF7 have been identified as regulators of type I interferon (IFN) gene expression in mammals. In the present study, the two genes were cloned and characterized in the European eel, *Anguilla anguilla*. The full-length cDNA sequence of IRF3 and IRF7 in the European eel, named as AaIRF3 and AaIRF7 consists of 2879 and 2419 bp respectively. Multiple alignments showed that the two IRFs have a highly conserved DNA binding domain (DBD) in the N terminus, with the characteristic motif containing five tryptophan residues, which is a feature present in their mammalian homologues. But, IRF7 has only four of the five residues in other species of fish. The expression of AaIRF3 and AaIRF7 both displayed an obvious dose-dependent manner following polyinosinic:polycytidylic acid (PolyI:C) challenge. *In vivo* expression analysis showed that the mRNA level of AaIRF3 and AaIRF7 was significantly up-regulated in response to PolyI:C stimulation in all examined tissues/organs except in muscle, with a lower level of increase observed in response to lipopolysaccharide (LPS) challenge and *Edwardsiella tarda* infection, indicating that AaIRF3 and AaIRF7 may be more likely involved in antiviral immune response. In addition, some pattern recognition receptors genes related with the production of type I IFNs and those genes in response to type I IFNs were identified in the European eel genome database, indicating a relatively conserved system in the production of type I IFN and its signalling in the European eel.



## 1. Introduction

Interferon regulatory factor (IRF) family represents one of the most important transcription factor families, with multiple biological roles such as in the regulation of immune response, and in apoptosis and proliferation [1,2]. Since the identification of the first member in this family, IRF1, originally as a regulator of IFN-β gene expression, 11 members in the family have been found in vertebrates [3,4]. Each IRF member contains a well conserved N terminal 'tryptophan cluster' DNA-binding motif which possesses five conserved tryptophan repeats and resembles closely to the DNA binding domain (DBD) of Myb transcription factors [2,5]. In addition, all IRFs, with the exception of IRF1 and IRF2, contain an IRF associated domain (IAD) at the C terminus, which is responsible for the formation of homodimers or heterodimers with other IRFs or other transcription factors [6,7]. Among the IRF family, two members, IRF3 and IRF7, share a high degree of amino acid sequence identity in the N-terminal DBD [8], and these two IRFs have been analysed extensively in the regulation of type I IFN gene expression [1,9–12]. IRF3 is constitutively expressed in a variety of tissues and is present in a latent inactive form in cytoplasm [13]. After viral infection, IRF3 is phosphorylated in its C-terminal serine and threonine residues by IκB kinase (IKK) ε or TANK-binding kinase 1 (TBK1), resulting in the formation of either IRF3 homodimer or an IRF3/IRF7 heterodimer to form a transcriptional complex for binding its target DNA sequence in nucleus [14–16]. Unlike IRF3, IRF7 is expressed at low level in lymphoid cells [17], and can also be strongly induced by viral infection or by type I IFN mediated signalling. Upon viral infection, the dimers of IRF-3 translocate to nucleus, resulting in IFN-β induction. Initially produced IFN-β then induces IRF-7 expression in target cells, which in turns acts on both IFN-α and IFN-β, generating a positive feedback loop for high IFN-α and IFN-β production [18]. Similar to IRF3, IRF7 resides primarily in the cytoplasm and undergoes phosphorylation at Ser425/Ser426 in

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C-terminal region (corresponding to Ser385/Ser386 in human IRF3) following activation, which allows its dimerization, then translocation into nucleus for inducing target gene expression [19].

At present, IRF3 and IRF7 have been cloned from several species of fish or been predicted through searching genome databases of some fish species [4,20–26]. Constitutive expression of IRF3 has been found ubiquitously in tissues of fish, and its transcription can be triggered by polyinosinic:polycytidylic acid (PolyI:C), type I IFN and virus infection [8,22]. IRF3 is localized exclusively in cytoplasm and can translocate to nucleus following stimulation with PolyI:C [23]. In addition, over-expression of IRF3 induces significant increase in the production of type I IFN [8]. IRF7 displays an expression profile similar to that of IRF3. It is expressed constitutively in all analysed tissues of healthy fish individuals with higher levels in immune related tissues [22,24,27]. Furthermore, IRF7 in fish has similar intracellular distribution as its mammalian and avian counterparts, and can translocate into nucleus following virus infection [24]. Using a luciferase reporter assay, IRF7 was shown to be capable of activating type I IFN promoter in orange spotted grouper (*Epinephelus coioides*) and Japanese flounder (*Paralichthys olivaceus*) [24,27]. Interestingly, two different IRF7 genes, named as IRF7A and IRF7B with an overall identity of 77%, have been cloned in Atlantic salmon (*Salmo salar*). *In vivo* analysis showed that the two IRF7 genes were expressed at low level in most organs, but IRF7A had quite high expression level in ovaries, while IRF7B, on the other hand, had a higher expression in gills and intestine. Furthermore, the IRF7A seemed to have a lesser role in inducing the expression of one IFN, the IFNa1, which implied that the IRF7A may have a larger role in activating other IFN genes in Atlantic salmon [8].

The European eel, *Anguilla anguilla*, an important species being cultivated in some European and Asian countries, is a high-priced fish in market. In recent years, however, European eel stock has shown a dramatic decline caused by changes in oceanic circulation, habitat disruption, chemical contamination and overfishing at different development stages, as well as by the impact of diseases [28]. In this case, studies on its immune system may enable the understanding of its immune responses, which may then lead to the development of much more targeted immune prophylactic approaches, for example, the designing of eel-appropriate vaccines, in order to prevent diseases in aquaculture. In the present study, IRF3 and IRF7 genes, named as AaIRF3 and AaIRF7 respectively, were cloned and characterized in the European eel. The expression profile of these two genes was examined in different tissues/organs following PolyI:C and lipopolysaccharide (LPS) challenge and *Edwardsiella tarda* infection.

## 2. Materials and methods

### 2.1. Sample collection

European eels weighing about 100 g each were maintained in aquarium with aerated water (28 ± 2 °C) for 2 weeks before being used in experiments. For examining the expression of AaIRF3 and AaIRF7, two groups of eels were injected intraperitoneally with 0.2 mg PolyI:C (Sigma) (in phosphate buffered saline (PBS), 2 mg/ml, 100 μl per fish) or LPS (Sigma; 2 mg/ml, 100 μl per fish), respectively. Control group was injected with same volume of PBS. At 3, 6 and 12 h post injection (hpi), three fish from each group were anaesthetized in 0.05% 2-phenoxyethanol, and thymus, pronephros, spleen, liver, kidney, intestine, gill, skin and muscle were collected separately for RNA isolation. The sampling time points were chosen since other report showed a similar time pattern following PolyI:C treatment in fish [26].

*E. tarda* was cultured at 25 °C with tryptic soy broth (TSB, BD Biosciences) prepared with distilled water. The bacterial cells were harvested through centrifugation at 3500 × *g* for 10 min, before being suspended in PBS for an appropriate concentration. One group of three fish was injected individually with live microbial suspension in PBS at a concentration of $1 \times 10^6$ cfu/ml, as reported by other authors [29], and equal amount of control fish were injected with PBS. 24 hpi, infected fish, when showing obvious disease symptom, such as skin ulceration and hemorrhagic lesions, were sacrificed with tissues/organs collected as described above for real time PCR analysis.

#### 2.1.1. Isolation of peripheral blood leukocytes (PBLs) and PolyI:C stimulation in vitro

To isolate peripheral blood leukocytes, blood was taken from caudal vessel with a heparinized syringe, and was diluted at 1:10 with pre-cold L-15 medium (Invitrogen Life Technologies) supplemented with 10 U/ml heparin, 10 mM HEPES, 60 mM NaCl, 5% FBS, 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco). The cell suspension was placed on 34/51% Percoll (GE Healthcare UK) gradient and centrifuged at 400 × *g* for 40 min at 4 °C. Cells suspended from the Percoll interface were collected, before being washed three times with pre-cold L-15 medium. Leukocytes were resuspended in the supplemented L-15 medium with cell numbers counted.

For stimulation experiment, 2 ml suspension was added in a 24-well plate at a density of $1 \times 10^6$ cells per well, and incubated with 100 μl PolyI:C solution at final concentrations of 0, 0.5, 5, 50 and 500 μg/ml. Cell cultures were then incubated at 28 °C and 5% $CO_2$ for 12 h. Afterwards, cells were centrifuged at 400 × *g* for 5 min at 4 °C, before being resolved in Trizol for RNA extraction to detect IRF3 and IRF7 at mRNA level by realtime PCR. Each treatment was performed in triplicate.

#### 2.1.2. Cloning and sequencing of AaIRF3 and AaIRF7 genes

Total RNA from spleen was extracted using Trizol (Invitrogen Corp) according to manufacturer's instruction. 2 μg total RNA was reverse-transcribed using the Powerscript II reverse transcriptase [SMART rapid amplification of cDNA end (RACE) cDNA Amplification kit, Clontech] with oligo dT primer. The IRF3 and IRF7 sequences from other teleost fish (Table 1) were used to search against the eel transcriptome database (http://compgen.bio.unipd.it/eeelbase/) with tblastn tool. Primer sets (Table 2) were designed based on the transcriptome sequence. PCR products were purified with EZNAO Gel Extraction Kit (Omega, Bio-Tek) according to manufacturer's instructions. The purified PCR product was inserted into pMD18-T vector and transformed into *Escherichia coli* strain DH5α. Sequencing of positive clones was performed in BGI life Tech Co., Ltd (Beijing, China) using ABI 3730 automatic sequencer (Applied Biosystems, Foster City).

#### 2.1.3. Sequence analysis

Sequences comparison with known IRFs were performed with the Basic Local Alignment Search Tool (BLAST) analysis tool of National Center for Biotechnology information website (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The PROSITE database was searched to detect conserved domain structures within the deduced amino acid sequences (http://us.expasy.org/tools/scanprosite). Polyadenylation signal sites were identified with DNA Functional Site Miner (http://dnafsminer.bic.nus.edu.sg/). Multiple alignments of the amino acid sequences were carried out with CLUSTALW 1.8 program. Sequence percentage similarities were calculated using the Megalign program of DNAStar software package (DNASTAR, Inc). The phylogenetic tree based on multiple protein alignment was constructed using the Neighbor-Joining