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# Molecular characterization and expression analysis of eleven interferon regulatory factors in half-smooth tongue sole, *Cynoglossus semilaevis*

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ABSTRACT

Interferon regulatory factors (IRFs) act as transcription mediators in virus-, bacteria-, and interferon (IFN)-induced signaling pathways and play diverse functions in antimicrobial defense, immune modulation, hematopoietic differentiation, and cell apoptosis. In this study, we described for the first time eleven IRFs (IRF1, IRF1L, IRF2X1, IRF3, IRF4a, IRF4b, IRF5, IRF6, IRF7, IRF8, and IRF9) from half-smooth tongue sole (*Cynoglossus semilaevis*) and examined their tissue distributions and expression patterns under different conditions. The deduced protein sequences of these IRFs (except IRF1) share high identities (71.8–86.6%) with other corresponding IRFs in other teleosts, whereas the sequence identity of IRF1 with the corresponding IRF1 in other teleosts is only 58.1%. A conserved N-terminal DNA binding domain (DBD), which is characterized by a winged type helix-loop-helix motif with four to six tryptophan repeats, is present in all IRFs. Another conserved IRF associated domain (IAD), which mediates the interactions in the C-terminal part of the protein, is present in all IRFs except IRF1 and IRF2X1, which instead contain the IAD2 domain. Several special domains also were found, including a serine-rich domain (SRD) in IRF3, IRF4a, IRF4b, and IRF7; a proline-rich domain (PRD) in IRF9; nuclear localization signals (NLSs) in IRF5, IRF8, and IRF9; and a virus activated domain (VAD) in IRF5. Quantitative real time RT-PCR (qRT-PCR) analysis showed that expression of all IRFs occurred in multiple tissues. IRF1, IRF2X1, IRF4a, IRF5, IRF7, and IRF8 exhibited relatively high levels of expression in immune organs, whereas the other five IRFs displayed high levels of expression in non-immune organs. Infection with extracellular and intracellular bacterial pathogens and virus upregulated the expression of IRFs in a manner that depended on tissue type, pathogen, and infection stage. Specifically, IRF1 and IRF2X1 were highly induced by bacterial and viral pathogens; IRF1L and IRF6 responded mainly to extracellular and intracellular bacterial pathogens; IRF3, IRF5, IRF7, IRF8, and IRF9 were markedly induced by intracellular bacterial pathogen and virus; IRF4a and IRF4b were mainly induced by virus and intracellular bacterial pathogen respectively. These results indicate that the IRFs of *C. semilaevis* can be categorized into several groups which exhibit different expression patterns in response to the infection of different microbial pathogens. These results provide new insights into the roles of teleost IRFs in antimicrobial immunity.


## 1. Introduction

Interferon regulatory factors (IRFs) were originally identified as transcription factors participating in the regulation of interferon (IFN) expression [1]. In recent decades, IRFs have become the focus of numerous immunological and medical studies [2,3]. A growing body of research suggests that IRFs participate in a variety of functions, such as antiviral defense, immune regulation, hematopoietic cell development, and maturation of the immune system [4,5]. To date, a total of 11 IRFs (IRF1–11) have been identified in vertebrates, and some virus-encoded analogs of cellular IRF have also been found [6]. IRFs are classified into the following four subfamilies: IRF1 (IRF1, 2, and 11), IRF3 (IRF3 and 7), IRF4 (IRF4, 8, 9, and 10), and IRF5 (IRF5 and 6). In certain species, some of these genes are absent, for example, humans and mice lack IRF10 [7] and chickens lack IRF3 and IRF9 [8].

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In terms of structure, all IRFs share a highly conserved N-terminus consisting of 115 amino acids, which contain the DNA-binding domain (DBD) that is characterized by five tryptophan repeats. In addition to the DBD, all IRFs except IRF1 and IRF2 possess an IRF associated domain (IAD) in the C-terminus, whereas IRF1 and IRF2 (and possibly IRF11) possess an IAD2. Both IAD and IAD2 mediate the formation of homo- or hetero-dimers or the recruitment of other IRFs and transcription factors to target promoters [9,10]. Functionally, the conserved DBD in the IRF family recognize a similar DNA sequence, and the less conserved C-terminal region acts as a regulatory domain and is used to classify IRFs into three groups: activators (e.g., IRF1, IRF3, IRF5, IRF9, and IRF10), repressors (e.g., IRF8), and bifunctional factors that both activate and repress the gene transcription depending on the target gene (e.g., IRF2, IRF4, IRF7) [1,7,11].

IRF1 and IRF2, which are expressed in most cells, show high sequence homology in the N-terminal region and very low homology in the C-terminal region, which suggests the possibility of opposite functions. IRF1 was originally identified as an activator of IFN-β [12], and subsequent studies revealed that IRF1 also plays a critical role in antiviral defense, immune-related cell maturation and activation, major histocompatibility complex (MHC) I expression, and cellular apoptosis [13,14]. IRF2 can bind to the same recognition site of IRF1 and suppress the transcriptional activity of IRF1 [12]. Accumulated data indicate that IRF2 also acts as an activator, activating the transcription of the histone 4 gene [15], promoting the maturation of natural killer cells [16], and stimulating the expression of MHC I antigenic peptide transporter [17]. IRF3, 5, and 7 are involved in modulating transcription of type I IFN and IFN-stimulated genes (ISGs) [18–20]. Moreover, IRF3 contributes to the enhancement of the host antiviral response [21]. IRF5 exhibits functions in apoptosis, tumorigenicity, and the immune response to pathogens [22,23]. IRF7 plays an important role in differentiation of monocytes to macrophages [24] and increases the tumoricidal activity of macrophage cells [25]. Similar to IRF7, IRF6 also participates in the switching from proliferation to differentiation of keratinocyte [26]. IRF4 and IRF8 exhibit a high level of sequence identity. In contrast to IRF1, IRF2, and IRF3, which are universally expressed, IRF4 and IRF8 are expressed predominantly in immune-related cells (i.e., myeloid and lymphoid cells) [27] and act as critical determinants for the development of B and T cells as well as differentiation of myeloid progenitor cells [28,29]. As a bifunctional factor, IRF8 can also repress the IRF1-mediated responses, transcription of Fas-associated phosphatase 1, toll-like receptor (TLR3) gene expression, and DNA binding activity of IRF9 [30,31]. IRF9 is a component of the tertiary complex interferon–-stimulated gene factor (ISGF3), which is formed in IFN-treated cells to stimulate transcription [32], and it participates in the antiviral effect of Type I IFN. Moreover, IRF9 can form a DNA binding complex with the signal transducer and activator of transcription 1 (STAT1) homodimer or with STAT2 alone, which binds to special DNA sequences such as ISGF3 [33]. So far few studies about IRF10 and IRF11 have been reported.

To date, several IRF family members have been studied in teleosts, including *Danio rerio* [34,35], *Ctenopharyngodon idella* [36], *Scophthalmus maximus* [37,38], *Paralichthys olivaceus* [39,40], *Siniperca chuatsi* [41,42], *Epinephelus coioides* [43,44], *Tetraodon nigroviridis* [45], *Salmo salar* [46,47], *Oncorhynchus mykiss* [48,49], and *Larimichthys crocea* [50,51]. In half-smooth tongue sole (*Cynoglossus semilaevis*), only IRF1 was reported [52]. In this study, we characterized 11 putative IRFs (IRF1, IRF1L, IRF2X1, IRF3, IRF4a, IRF4b, IRF5, IRF6, IRF7, IRF8, and IRF9) in half-smooth tongue sole and examined their conserved features and expression profiles under different conditions. Our results indicate that the tongue sole IRFs are involved in antiviral and antibacterial immunity.

## 2. Materials and methods

### 2.1. Pathogen strains and culture conditions

The fish pathogens *Edwardsiella tarda* TX1, *Vibro harveyi*, and megalocytivirus RBIV-C1 have been reported previously [53–55]. Viral proliferation and bacterial culturing in Luria–Bertani broth (LB) medium were reported previously [53–55].

### 2.2. Fish

Half-smooth tongue soles (*C. semilaevis*) were purchased from a commercial fish farm in Shandong Province, China and maintained at 24 °C in aerated seawater and changed daily. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Before experiment, fish were randomly sampled for the examination of bacterial recovery or megalocytivirus DNA from blood, liver, kidney, and spleen as described previously [56]. No bacteria or virus were detected from the examined fish. Fish were euthanized with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA) before tissue collection.

### 2.3. Sequence analysis

All of the IRF sequences were obtained from GenBank, and the accession numbers of IRF1, IRF1L, IRF2X1, IRF3, IRF4a, IRF4b, IRF5, IRF6, IRF7, IRF8, and IRF9 are listed in Table S1. Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple sequence alignment was carried out with ClustalX program. Sequence percentage similarities were calculated using the Megalign program of DNAStar software package (DNASTAR Inc. Madison, WI, USA). Phylogenetic analysis was performed with ClustalX and the Neighbor-joining algorithm of MEGA 4.0.

### 2.4. IRFs expression in fish tissues under normal physiological conditions

Blood, brain, gill, heart, intestine, head kidney (HK), liver, muscle, and spleen were taken aseptically from five tongue soles (average 15.3 g) and used for total RNA extraction and cDNA synthesis as reported previously [57]. Real-time quantitative polymerase chain reaction (qRT-PCR) was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR Premix Ex Taq (Takara, Dalian, China) as described previously [58]. The expression level of IRFs was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with β-actin (ACTB) as an internal control as reported previously [59]. The PCR primers for IRFs are listed in Table 1. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The experiment was performed three times.

### 2.5. IRFs expression upon bacterial and viral infection

Bacterial infection was performed as reported previously [58]. Briefly, *E. tarda* TX and *V. harveyi* were cultured in LB medium at 28 °C to an $OD_{600}$ of 0.8. The cells were washed with PBS and resuspended in PBS to $2 \times 10^6$ CFU (colony forming unit)/ml and $2 \times 10^7$ CFU/ml for *E. tarda* TX1 and *V. harveyi* respectively. Tongue soles were divided randomly into three groups (20 individuals/group) and injected intraperitoneally (i.p.) with 50 μl *E. tarda*, *V. harveyi*, or PBS per fish, and maintained at 24 °C. Fish were euthanized at 6 h, 12 h, 24 h, and 48 h post-infection, and spleen,