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Molecular Characterization of Interferon Regulatory Factor 10 in Red Sea Bream (*Pagrus major*) and Expression Analysis Following RSIV Infection

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참돔(*Pagrus major*)의 인터페론 조절인자 10의 분자적 특성 및 RSIV 감염 후 발현 분석

우원식 · 강경식 · 김경호 · 손하정 · 손민영 · 한현자* · 박찬일†

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Abstract

Interferon regulatory factors (IRFs) exhibit antiviral and antibacterial capabilities. In this research, we isolated the IRF10 genes from red sea bream (*Pagrus major*) and examined their expression following red sea bream iridovirus (RSIV) infection. Analysis of multiple sequences identified preserved components, such as the IRF-association domain, nuclear localization signal, and DNA-binding domain. Phylogenetic examination showed that PmIRF10 grouped with IRF10 equivalents in fish. We utilized quantitative real-time PCR to explore IRF10 expression in normal tissues, revealing increased expression levels in the gill and spleen. Our results indicate that PmIRF10 plays a role in the immune response, as evidenced by the controlled expression levels of IRFs after RSIV infection in red sea bream. This finding aligns with prior research on different fish species. Overall, this investigation characterizes IRF genes in red sea bream and provides a basis for comparative analysis of IRF genes.

Key words : *Pagrus major*, Red sea bream iridovirus, Interferon regulatory factors, IRF10

Ⅰ. Introduction

The development of innate and adaptive immune systems in vertebrates has enabled them to protect themselves against the infiltration of foreign pathogens. These systems encompass endogenous binding sites and the activation of various expression factors, such as interferon regulatory factors (IRFs). Initially identified in humans, IRFs constitute a protein family responsible for regulating the transcriptional and inducible gene expression of interferons (IFNs) (Antonczyk et al., 2019). Since the first discovery of IRF1 in 1988, the scope of IRFs has expanded to comprise IRF1-11. Studies have demonstrated that IRF1-9 can be found in mammals, IRF10 in birds and fish, and IRF11 exclusively in fish (Suzuki et al., 2011; Shu et al., 2015; Guan et al., 2020).

The IRF family is characterized by an N-terminal DNA-binding domain (DBD), a peptide linker (LK),

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and an IRF-association domain (IAD) that includes five tryptophan repeats essential for identifying similar DNA motifs within the highly conserved DBD (Antonczyk et al., 2019). Phylogenetic analysis categorizes the IRF family into four subfamilies: IRF1-G (IRF1, IRF2); IRF3-G (IRF3, IRF7); IRF4-G (IRF4, IRF8, IRF9, IRF10); and IRF5-G (IRF5, IRF6) (Nehyba et al., 2002). The primary function of the IRF family is to regulate the antiviral response in vertebrates' innate immunity by participating in the transcription of IFN and interferon-stimulated genes (ISG) (Paun & Pitha, 2007). In vertebrates, IRFs identify viral infections by recognizing pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) (Medzhitov & Janeway Jr, 2000). Furthermore, IRFs differentiate and regulate dendritic cells (Gabriele & Ozato, 2007), and modulate crucial cytokines and transcription factors involved in T helper cell differentiation (Zhang et al., 2012).

Phylogenetic analysis reveals that IRF10 belongs to the IRF-4G subfamily, exhibiting a strong binding affinity to the interferon-stimulated response element (ISRE) domain, particularly when heterodimerized with IRF1, IRF2, or IRF4 (Alter-Koltunoff et al., 2008). Since its initial identification in birds, IRF10 has been detected in various vertebrates, with the exception of humans and mice (Huang et al., 2010). Structurally, IRF10 shares a compact configuration with IRF4 and is reported to play a role in the late stage of antiviral response in birds (Nehyba et al., 2002). IRF10 has been identified in species such as olive flounder, zebrafish, orange-spotted grouper (*Epinephelus coioides*), Atlantic cod (*Gadus morhua*), rainbow trout, common carp (Cyprinus carpio), and swamp eel (Monopterus albus) (Suzuki et al., 2011; Li et al., 2014; Qiao-Qing et al., 2014; Huang et al., 2015; Inkpen et al., 2015; Xu et al., 2016; Zhu et al., 2020).

Initially discovered in Japan in 1990 in red sea bream, red sea bream iridovirus (RSIV) is a linear double-stranded DNA virus that belongs to the Iridoviridae family and the Megalocytivirus genus (Inouye et al., 1992). RSIV inflicts significant damage on various fish species annually, as susceptible populations consistently exhibit the presence of the virus (Kim et al., 2018; Lopez-Porras et al., 2018; Girisha et al., 2020; Puneeth et al., 2021).

Red sea bream is a rapidly growing fish known for its exceptional meat quality, making it highly sought after. As a result, it has become a significant aquaculture species in Korea, Japan, and China. Numerous studies have been conducted on red sea bream, focusing on growth and stocking density (Murashita et al., 2018; Ido et al., 2019; Seo et al., 2020; Mzengereza et al., 2021). However, among the existing research on immunity, only a few reports have specifically addressed IRF (Haque et al., 2021; Joo et al., 2022; Kim et al., 2023).

Since IRFs have been shown to play a role in viral pathogen infections, gaining insight into the features and expression of the IRF gene could substantially contribute to research on innate immunity and viral infections. Consequently, this study aimed to explore the molecular properties of IRF10 (belonging to the IRF-4G subfamily) in red sea bream and to characterize their expression during RSIV infection. This report provides the first set of data on IRF10 in relation to innate immunity and viral infection in sea bream.

Ⅱ. Materials and methods

1. Fish and virus

In this study, red sea bream were sourced from a net cage culture in Tongyeong, Gyeongsangnam-do, South Korea. The fish had an average weight of 52.1 ± 4.6 g and a total length of 12.5 ± 1.6 cm. Upon arrival at the laboratory, the fish were acclimatized with aeration in a flowing water tank at 22 ± 1 °C. To ensure the absence of pathogen infection, internal and external clinical symptoms, as well as molecular diagnostics, were employed on five randomly chosen fish. RSIV was isolated from rock bream and cultured in Tongyeong. Subsequent sequencing research confirmed that the virus belonged to genotype II, which is prevalent in Korea.

2. Molecular characteristics and phylogenetic analysis

To verify the integrity of the gene sequence obtained through genome sequencing, subcloning was conducted using *Escherichia coli* (*E. coli*) JM109 and the pGEM T-easy vector (Promega, USA). Sanger sequencing validated the sequence, which was then translated into amino acid sequences using GENETYX software version 8.0 (SDC Software Development, Japan). The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) search method was employed to identify relevant sequences, while ClustalX 2.1 and MEGA 6.0 were utilized for multiple sequence alignment and phylogenetic analysis, respectively. The Expert Protein Analysis System PROSITE Scan program (http://prosite.expasy.org) was used to detect specific domains and motifs.

3. Tissue collection and cDNA synthesis

Tissue samples from five healthy red sea breams were aseptically collected and preserved at -80 °C to investigate the mRNA distribution of PmIRF10. The samples included head kidney, trunk kidney, skin, stomach, gill, heart, liver, spleen, eye, brain, and intestine. Fish were experimentally infected intraperitoneally with 1×10^7 copies/fish of RSIV suspended in PBS. These RSIV-infected red sea breams were employed to assess the temporal expression changes of PmIRF10 mRNA. Gill, spleen, liver, and trunk kidney samples were aseptically collected from five randomly selected red sea breams at 0 (control), 1, 3, 6, 12, 24, and 36 hours, and 3, 5, and 7 days post-RSIV infection. The easy-spin™ Total RNA Extraction Kit (iNtRON Biotechnology, South Korea) was used to extract total RNA from each tissue, while the PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Japan) was employed for cDNA synthesis.

4. Reverse transcription-quantitative real-time PCR (RT-qPCR) analysis

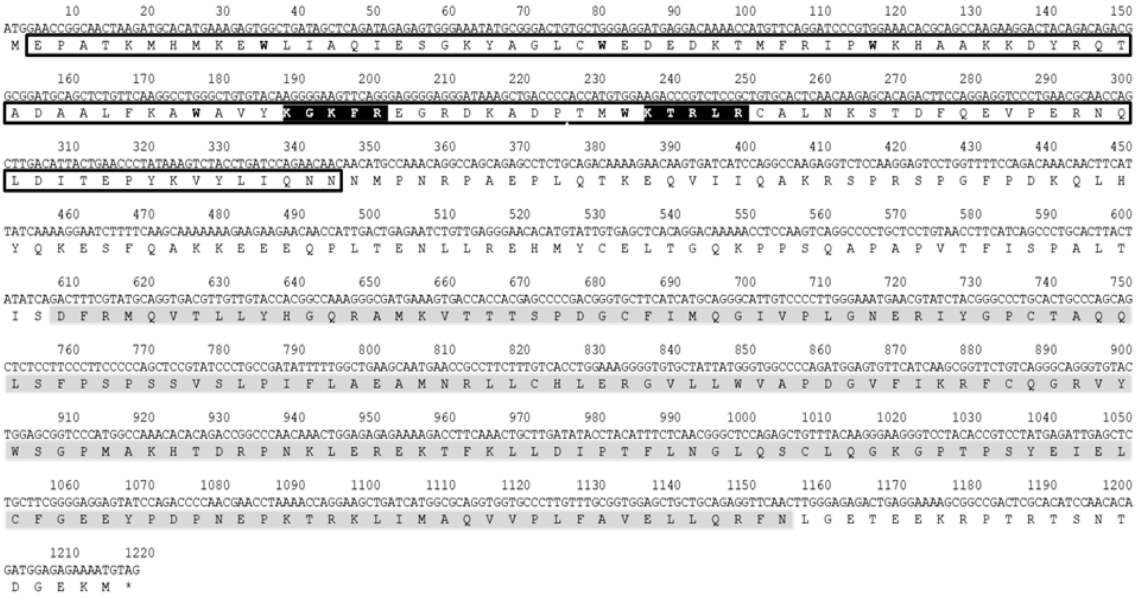
To determine the mRNA expression levels for each gene, RT-qPCR was performed using TB Green™ Premix Ex Taq™ (Takara). The sequences and details of the primer sets employed can be found in <Table 1>. The PCR reactions were carried out using a Thermal Cycler Dice® Real-Time System III (Takara). The $2^{-\Delta\Delta Ct}$ method was applied to quantify the relative mRNA expression, utilizing EF-1 α (AY190693.1) as the reference gene (Livak and Schmittgen, 2001). These experiments were conducted in triplicate.

5. Statistical analysis

The expression of PmIRF10 in RSIV-infected tissues was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test, employing IBM SPSS software version 19 (IBM, USA). The standard deviation (SD) of the experimental results is denoted, and all samples were examined in triplicate.

<Table 1> Primer sequences and condition were used in this study

Primer name	Primer sequence (5′ -3′)	Usage	Condition
qPCR-PmEF-1 α -F	ACGTGTCCGTCAAGGAAATC	RT-qPCR amplification	95℃/5 minutes 95℃/30 seconds 60℃/30 seconds 72℃/30 seconds 72℃/5 minutes 4℃/∞ Number of cysles: 35
qPCR-PmEF-1 α -R	TGATGACCTGAGCGTTGAAG		
qPCR-PmIRF10-F	CTGTCAGGGCAGGGTGTACT		
qPCR-PmIRF10-R	GGAGCCCGTTGAGAAATGTA		

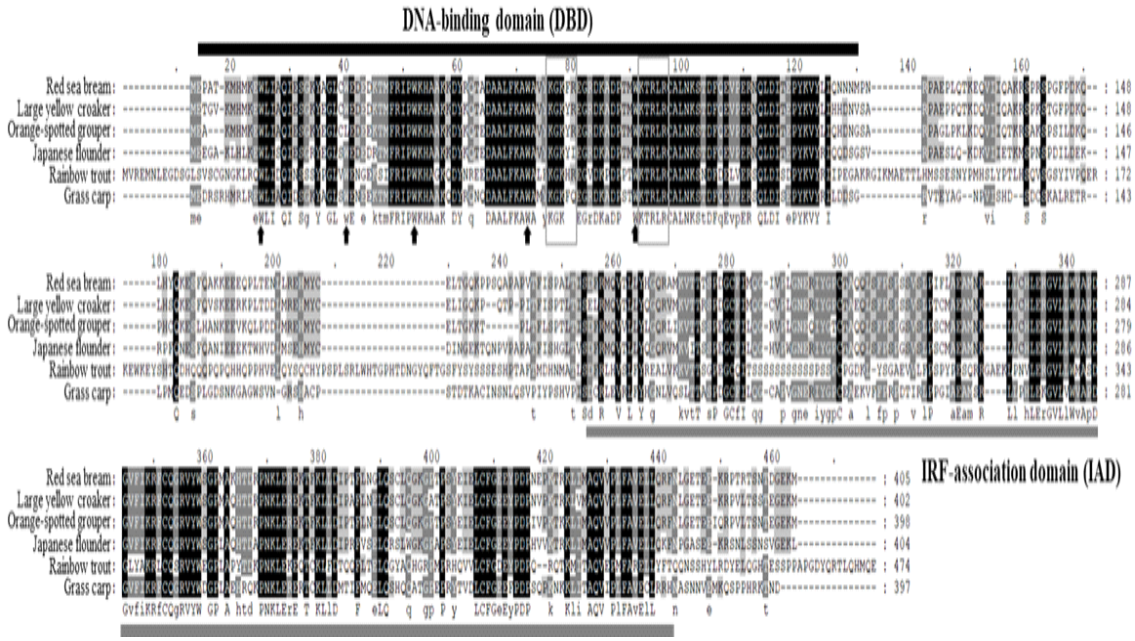


[Fig. 1] IRF10 transcript sequences from red sea bream. The nucleotide sequence cDNA of PmIRF10 (GenBank accession: OK340063.1). The nucleotide sequence number is indicated above the sequence. An asterisk (*) indicates the stop codon. Black empty boxes indicate DNA binding domain (DBD), and bold letters indicate five tryptophans. White text in black boxes represents nuclear localization signals (NLS), and gray boxes represent the IRF-association domain (IAD).

Ⅲ. Results

1. PmIRF10 Gene Structure and Amino Acid Sequence Characterization

Genome sequencing of PmIRF10 revealed molecular properties, such as mRNA structure and amino acid (aa) sequences ([Fig. 1]). The predicted molecular weight of PmIRF10 (OK340063.1) was 46 kDa, with a theoretical pI of 8.79. The PmIRF10 sequence extended across 1218 bp (406 aa) in total, encompassing a DNA-binding domain (2-115 aa) and an IRF-association domain (203-385 aa). Moreover, PmIRF10 both possessed five tryptophan residues, and the nuclear localization signal (NLS) was highly conserved.



[Fig. 2] Multiple sequence alignments are analyzed in red sea bream IRF10 with homologs of other vertebrates. Black lines indicate DNA binding domains and arrows indicate five tryptophans. Gray boxes represent nuclear localization signals, and dark gray lines represent IRF-association domains.

2. Multiple alignments and phylogenetic analysis of PmIRF10

Multiple alignment analyses of PmIRF10 revealed that the five tryptophan residues and the DBD and IAD domains were highly conserved across all spec ies examined ([Fig. 2]). PmIRF10 exhibited the hig hest homology with large yellow croaker (86.67%) and orange-spotted grouper (82.34%) ([Fig. 2]). Phy logenetic analysis confirmed that PmIRF10 formed distinct clusters ([Fig. 3]).

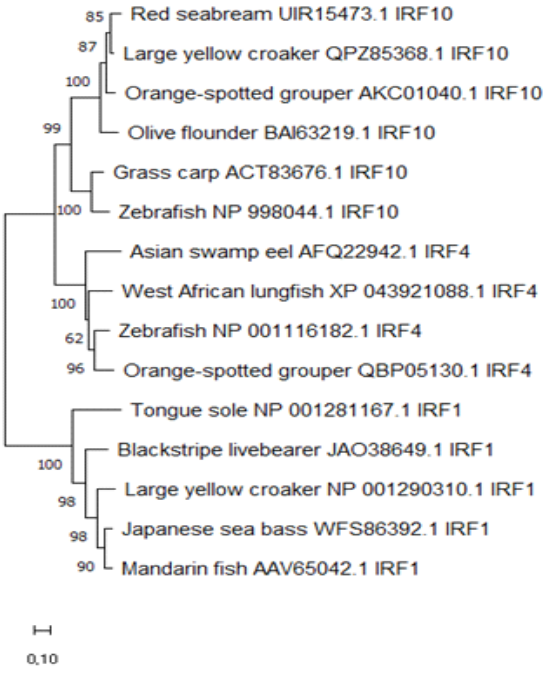
3. Analysis of PmIRF10 mRNA expression in healthy red sea bream

Expression analysis of PmIRF10 in healthy red s ea bream revealed that PmIRF10 demonstrated the highest expression in the gills as well (19.7-fold), f ollowed by the spleen (10.2-fold), liver (5.2-fold), h eart (4.7-fold), and skin (4.3-fold) ([Fig. 4]).

4. PmIRF10 mRNA expression in red sea bream following RSIV infection

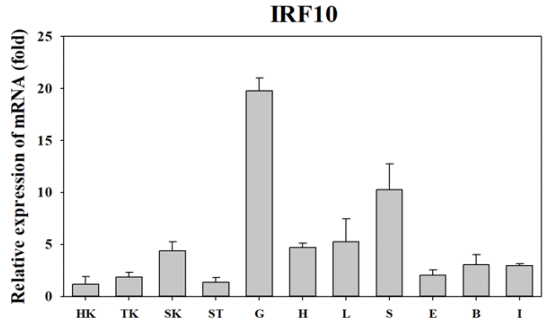
Hourly expression analysis of PmIRF10 following RSIV infection revealed that PmIRF10 expression w as observed to be downregulated in the gills until 3 hours post-infection (hpi), after initial expression, and then strongly upregulated at 6 hpi ([Fig. 5]).

Beyond this time point, expression persisted but decreased and exhibited low regulation at 7 days p ost-infection (dpi). In the spleen, upregulation was observed up to 6 hpi after initial expression, follow ed by downregulation until 36 hpi. The highest upr egulation was observed at 5 dpi, succeeded by low er regulation at 7 dpi. In the liver, initial expressio n was followed by rapid upregulation at 3 hpi and 6 hpi, and then downregulation at 12 hpi. Up to 3 6 hpi, low regulation was observed, followed by up regulation at 3 dpi, and finally, low regulation at 7

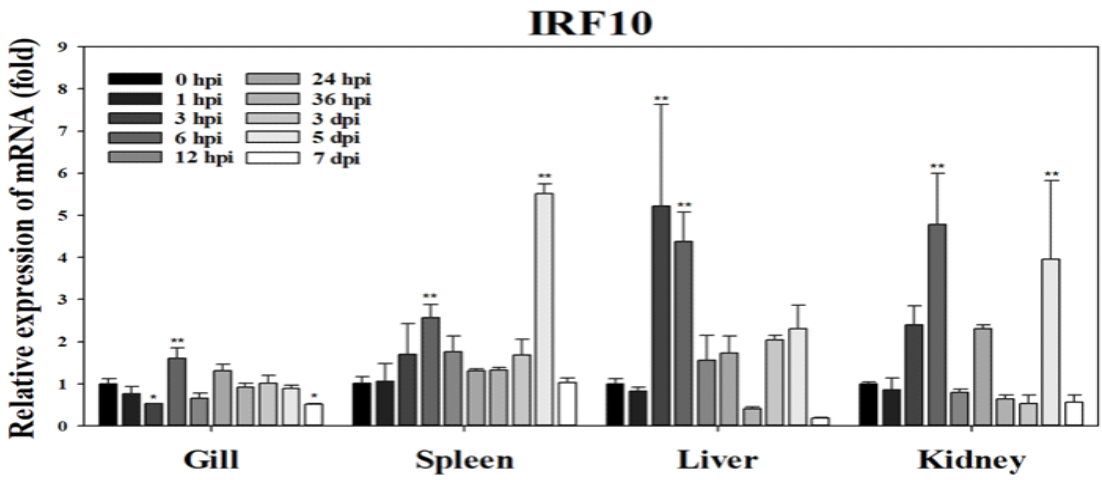


[Fig. 3] Phylogenetic analysis of Interferon regulatory factor family protein. The phylogenetic tree was constructed using the Neighbor-Joining method.

dpi. After initial expression in the kidney, the stron gest regulation occurred at 6 hpi, followed by dow nregulation, high upregulation at 5 dpi, and low reg ulation at 7 dpi.



[Fig. 4] Expression analysis of PmIRF10 in normal tissue. The relative expression level for each transcript was calculated as fold units after it was first normalized to the expression of a house-keeping gene (EF-1a). HK, TK, SK, ST, G, H, L, S, E, B, and I represent head kidney, trunk kidney, skin, stomach, gill, heart, liver, spleen, eye, brain, intestine. The results represent the mean ± SD of five fish.



[Fig. 5] Expression analysis of PmIRF10 in RSIV infection tissue. The relative expression level for each transcript was calculated as fold units after it was first normalized to the expression of control (0 hpi). The results represent the mean ± SD of five fish. Asterisks (*) above each bar indicate a significant difference from the control group (*, $P \leq 0.05$; **, $P \leq 0.01$).

Ⅳ. Discussion

IRFs are acknowledged for their antiviral and antibacterial properties in animals. These extensively investigated transcription factors play a role in responding to viral infections and regulating apoptosis or the development of immune responses (Savitsky et al., 2010). Via the DBD domain, IRFs bind to the ISRE as homodimers and heterodimers, interacting with the ISRE half-site on the opposite side of the DNA in a proximal direction, thus activating TLR and IFN signaling pathways (Andrilenas et al., 2018). Five tryptophan residues within the DBD domain are associated with the promoters of target genes, such as IFN regulatory elements and ISRE. Although there is an abundance of research on IRFs in mammals, studies in fish are relatively limited, and the variation in gene expression among different fish species is more distinct than in mammals. Consequently, it is crucial to investigate IRFs in individual fish species.

In this study, the nucleotide sequences of IRF10 from red sea bream were identified and displayed considerable similarity to previously reported fish sequences, suggesting that they are part of the IRF family. Furthermore, the DBD domain, an essential domain of IRF, was found to contain five tryptophan residues, and both the NLS sequence and the IAD domain were verified to be highly conserved.

Following the expression analysis of PmIRF10 after RSIV infection, it was observed that all tissues exhibited upregulation at 6 hpi, subsequently downregulation, and then upregulation again at 5 dpi in the spleen, liver, and kidney. In the case of orange-spotted grouper, upon examination of immune tissue expression of IRF10 after poly (I:C) treatment, the highest expression was detected at 6 h post-treatment. Similarly, after poly (I:C) treatment in common carp, spleen, head kidney, foregut, and hindgut displayed notable upregulation at 6 h post-treatment (Huang et al., 2015; Zhu et al., 2020). Moreover, IRF10 of Atlantic cod demonstrated the highest expression at 6 hpi following poly (I:C) treatment. In Japanese flounder, the highest expression was observed 6 h after poly (I:C) administration (Suzuki et al., 2011; Inkpen et al., 2015). The consistent early expression of IRF10 across multiple fish species implies that an antiviral immune response elicits prompt feedback from IRF10. The initial upregulation in PmIRF10, succeeded by a decrease and then an increase in expression pattern, may correlate with the finding that IRF10 in zebrafish acts as a negative regulator to maintain equilibrium in the antiviral immune response (Li et al., 2014). By integrating the outcomes of this study with those from prior research, it can be concluded that PmIRF10 assumes a unique role in the immune response of red sea bream, akin to the role of IRF10 in previously investigated fish, and it has been demonstrated to play a crucial function in antiviral immunity specifically.

In this study, high expression of PmIRF10 was confirmed in the immune tissues of the gills and spleen. In previous studies, normal tissue expression in rainbow trout, Asian swamp eel, and grass carp showed high levels of immune tissue-related expression, and in Japanese flounder IRF10 was strongly expressed in the gills, intestines, head kidney, and trunk kidneys (Suzuki et al., 2011; Xu et al., 2016). However, IRF10 in common carp showed different results, with strong expression in the gonad and the lowest expression in the head kidneys (Zhu et al., 2020). Although there are differences between species in normal tissue expression analysis, it is suggested that IRF10 in red sea bream is related to the immune response, similar to the previously reported

IRF10 of other fish species.

We have verified the molecular characteristics and expression analysis of IRF10 in red sea bream. Although further investigation is required to ascertain their precise functions, PmIRF10 appear to exhibit distinct roles in the antiviral immune response, consistent with observations of IRF10 in previous studies. Consequently, this research provides a foundational dataset for understanding IRF characteristics in red sea bream and expands upon existing reports in teleost fish. Moreover, PmIRF10 are crucial immune genes in red sea bream, and additional immune-related studies are warranted to thoroughly elucidate their gene functions.

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