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Interferon Regulatory Factors Functioned as Activators of the Interferon Pathway in the Scallop *Chlamys farreri*

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Interferon regulatory factors (*IRF*s) are a family of transcription factors that control many facets during innate and adaptive immune responses. Vertebrate *IRF*s play important roles in regulating the expression of interferons (IFNs) and IFN-stimulated genes, while only limited studies were conducted on invertebrate *IRF*s. In the present study, four *IRF* family genes (*CfIRF1, CfIRF1-like, CfIRF2*, and *CfIRF8*) were identified from Zhikong scallop (*Chlamys farreri*) through whole-genome scanning. *CfIRFs* contain a highly conserved N-terminal DNA-binding domain and a variable C-terminal regulatory domain. *CfIRFs* were constitutively expressed during development as well as in adult tissues, especially in hepatopancreas, hemolymph, gill, and mantle. In hemolymph, qRT-PCR analysis revealed that *CfIRF1, CfIRF1-like*, and *CfIRF2* were significantly upregulated in response to *Vibrio anguillarum* infection, and their encoding proteins could translocate into nucleus. Dual-luciferase reporter assay on *Cf*IRF1, *Cf*IRF1-like, and *Cf*IRF2 showed that these three proteins were capable to induce a strong activation of ISRE promoters. Notably, in comparison with *CfIRF1* and *CfIRF1-like*, *CfIRF2* showed the most sensitive responses in coping with *V. anguillarum*, and consistently, *Cf*IRF2 exhibited the most significant activation on ISRE. This study would provide valuable information for the innate immune roles of the *IRF* gene family in bivalve molluscs.

Keywords: *Chlamys farreri*, *IRF*, transcriptional activation, interferon-stimulated response element, immune response

1 INTRODUCTION

Interferon regulatory factors (*IRF*s) are a family of transcription factors that were first identified as regulators of IFN (Type I interferon) and IFN-inducible genes (Miyamoto et al., 1988; Harada et al., 1989), which have been extensively studied in vertebrates, showing diverse functions in regulating immune responses, stress responses, reproduction, development, and carcinogenesis (Tamura et al., 2008; Nehyba et al., 2009; Savitsky et al., 2010). So far, a total of 11 *IRF* family members (from *IRF-1*

to *IRF-11*) have been reported in vertebrates (Inkpen et al., 2019), with nine *IRFs* identified in mammals and another two members found in several avian and fish species (Nehyba et al., 2002; Huang et al., 2010). All IRF family members possess a highly conserved N-terminal helix-turn-helix DNA-binding domain (DBD). This domain consists of about 120 amino acids, binding to the core IFN-stimulated response element (ISRE) recognition sequence, GAAANNGAAAG/CT/C (Escalante et al., 1998; Marchler-Bauer et al., 2011). As for the C-terminus, most of the IRFs share an IRF-associated domain 1 (IAD1) or a similar IAD2, which mainly mediates the homomeric or heteromeric formation of IRFs as well as the interaction of IRF with non-IRF members, and the resulting protein complex acts as a transcriptional activator or repressor (Ikushima et al., 2013). It was reported that *IRF1*, *IRF3*, *IRF5*, *IRF7*, and *IRF9* are usually functioned as positive mediators of the host's IFN response, whereas *IRF4* usually acts as a repressor. Furthermore, *IRF2* and *IRF8* can participate in either activating or repressing the target gene transcription, depending on the nature of the pathogen or the signaling pathways that is involved (Taniguchi, 2006; Weiqi et al., 2008; Ikushima et al., 2013). Besides the IAD, the C-terminus is not well conserved, which may confer versatile functions to *IRF* members (Yanai et al., 2012). For example, besides IFN mediator, *IRF* activation through the TLRs or other inflammatory cytokines could interfere with NF-κB signaling, which were necessary to maintain the immunity balance (Anda et al., 2012; Cavlar et al., 2012; Xuan et al., 2020).

Previous studies have revealed that *IRF* genes are present in all principal metazoan groups, and *IRF-like* genes have been detected in genomic and expressed sequence tag (EST) databases (Davidson et al., 2006; Azumi et al., 2007; Huang et al., 2008). Based on the evolutionary molecular relationships, the IRF proteins could be classified into four subfamilies, namely, IRF-1 group (IRF1, 2, and 11), IRF-3 group (IRF3 and 7), IRF-4 group (IRF4, 8, 9, and 10), and IRF-5 group (IRF5 and 6) (Zhan et al., 2016). For the IRF-1 subfamily, IRF1 and IRF2 were first identified as transcriptional regulators of ISGs and type I IFN, which mainly play important roles in antiviral immunity (Harada et al., 1989). IRF11 has only been identified in teleost fish, and its function study is still in the infancy stage (Huang et al., 2010). For the IRF-3 family, researchers found that phosphorylated IRF7 and IRF3 could jointly regulate the rapid production of IFN initially, and ultimately induce the production of IFN in large quantities through a positive feedback regulatory loop (Marié et al., 1998; Sato et al., 1999). For the IRF-4 subfamily, they showed diverse IFN or NF-κB regulating functions depending on the nature of the binding molecules or the cellular differentiation status, and higher homology was found between IRF4 and IRF8 (Meraro et al., 2002; Lehtonen et al., 2005; Lu, 2008). As for the IRF-5 subfamily, IRF5 is mainly involved in the natural inflammatory response, while IRF6 is mainly involved in the embryonic early development (Hatada et al., 1997; Barnes et al., 2001; Barnes et al., 2004; Green et al., 2015).

Compared with the extensive knowledge of *IRFs* in vertebrates, studies on *IRFs* in invertebrates are quite limited. Previously, the interferon response has been thought to be a vertebrate innovation because the genomes of model invertebrates (i.e., *Drosophila*) do not encode interferon or its major effectors (Green et al., 2015). With the abundance and further analysis of invertebrate genome data, several key molecules in the IFN system have been identified, including IRF, interferon-like protein (IFNLP), interferon receptor (IFNR), and interferon-induced protein (Lelong et al., 2015; Zhang et al., 2015; Huang et al., 2017). More recently, studies have revealed that *IRF* genes are present in a lot of invertebrate groups, including sea sponges, placozoans, comb jellies, cnidarians, and bivalves, but are not detected in *Nematoda* and *Hexapoda* (including insects) (Nehyba et al., 2009; Huang et al., 2010). Although *IRFs* have been found in various invertebrates, they are different in number and genomic characteristics from the vertebrate *IRF* family, and there are only few preliminary functional studies on invertebrate *IRFs* through gene cloning. For example, *PfIRF-2* in pearl oyster *Pinctada fucata*; *CgIRF-1*, *-2*, and *-8* in pacific oyster *Crassostrea gigas*; and *LvIRF* in pacific white shrimp *Litopenaeus vannamei* were found to participate in the immune response against Gram-negative bacteria (Huang et al., 2013; Li et al., 2015; Huang et al., 2017; Lu et al., 2018). As invertebrates or vertebrates might have experienced a different pressure during evolution, it may in turn lead to the functional differentiation of *IRF* genes (Huang et al., 2010). Therefore, we need more research into the function of invertebrate *IRFs*, providing valuable information for the origin of the *IRF* family as well as the evolution of innate immunity.

Bivalve molluscs belong to the most speciose phylum of marine invertebrates, which could well adapt to the highly diverse and hostile environment with various stressors (bacteria, pollution, etc.). Scallops are highly prized as a food source, while in recent years, their aquaculture industry suffers huge economic loss due to the etiological diversity of pathogens that cause repeated appearance of disease outbreaks (Liu et al., 2004; Teng et al., 2012). Scallops generally lack the adaptive immune system and rely solely on innate immunity mediated by both cellular and humoral components (Loker et al., 2010). Functional studies of *IRFs* on scallops would be helpful for revealing their immune defense mechanisms and understanding the origin and evolution of bivalve innate immunity. In the present study, we take *Chlamys farreri* (Zhikong scallop), one of the most important maricultural scallop species in China, as research subject to systematically identify the *IRF* gene family. Their expression profiles during development and in different healthy adult tissues were analyzed. Meantime, their responses after *Vibrio anguillarum* challenge in hemocytes were investigated. We further explored their subcellular localization as well as the transcriptional activity using pISRE-Luc reporter plasmids in HEK293T cells, thereby providing insights into the immune function of *IRF* genes in bivalves.

2 MATERIALS AND METHODS

2.1 Database Mining, Gene Identification, and Sequence Analysis

To identify *IRF* genes, the transcriptome and whole genome sequence databases of the *C. farreri* were searched using the available IRF protein sequences from representative invertebrates and vertebrates, including *Homo sapiens, Mus*

musculus, Gallus gallus, Xenopus tropicalis, Danio rerio, C. gigas, P. fucata, Mytilus galloprovincialis, Hyriopsis cumingii, Pecten maximus, Lottia gigantea, Biomphalaria glabrata, and *Elysia chlorotica*. These IRF proteins from representative species were retrieved from NCBI (http://www.ncbi.nlm.nih.gov), Ensembl (http://useast.ensembl.org), MolluscDB (http://mgbase.qnlm.ac/), and OysterBase (http://www.oysterdb.com/) databases. TBLASTN was used to obtain the initial pool of *IRFs* transcriptome sequences from the Zhikong scallop, and then, BLASTN was performed to verify the cDNA sequences by comparing the transcriptome sequences with the whole genome sequences. The candidate *CfIRFs* sequences were submitted to the ORF Finder program (https://www.ncbi.nlm.nih.gov/orffinder/) to predict the open reading frame (ORF), and the ORFs were translated into amino acid sequences. The translated sequences were submitted to the SMART program (http://smart.embl-heidelberg.de/) for identification of the signal peptide and other conserved domains. The putative isoelectric point (*pI*) and molecular weight were computed using the Compute *pI*/Mw (http://web.expasy.org/compute_pi/). The subcellular localization and nuclear localization signals (NLSs) were predicted through the online prediction website (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/, https://www.genscript.com/wolf-psort.html/, https://sunflower.kuicr.kyoto-u.ac.jp/~smatsuda/slplocal.html, http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). The protein structures of all the identified IRF proteins were drawn with IBS1.0.3 software. Multiple alignment analysis of CfIRFs were performed with the ClustalW multiple alignment programs (http://www.ebi.ac.uk/clustalw/).

2.2 Phylogenetic Analysis

The IRF proteins from other vertebrates and invertebrates listed in the Section 2.1 were used for phylogenetic analysis together with the Zhikong scallop IRFs. The amino acid sequences of IRF proteins from these species were retrieved from the NCBI and Ensembl Genome Browser. Protein sequences were aligned using the ClustalW method in the MEGA-X software (Sudhir et al., 2018). Phylogenetic relationships of IRF amino acid sequences were estimated using maximum-likelihood (ML) analyses with FastTree 2.0.0. FastTree accounts for variable rates of evolution across sites by assigning each site to one of 20 categories, with the rates geometrically spaced from 0.05 to 20. FastTree sets each site to its most likely category by using a Bayesian approach with a gamma prior. Branch supports evaluated 10,000 pseudo-replicates of the ultrafast bootstrap procedure (Thi et al., 2017). Whole amino acid sequences were used in the phylogenetic analyses. This analysis involved a total of 59 amino acids across 13 species. The accession numbers of 59 IRFs are listed in **Supplementary Table 1**.

2.3 Expression Analysis

For expressional analysis, the RPKM (reads per kilo per million reads) value of each *IRF* gene was retrieved from the published RNASeq datasets of various developmental stages and adult tissues of Zhikong scallop (Li et al., 2017). To visualize the expression patterns of *IRF* genes in Zhikong scallop, the expressional heatmaps were shown *via* heatmap package under the R environment and the statistical analysis of the data was performed with edgeR package under R environment using the *F*-test. Differences were considered significant at $p < 0.05$. For examining the correlation relationship of *IRF* genes, a regression analysis was performed.

2.4 Sample Collection and Bacteria Treatment

Two-year-old healthy Zhikong scallops were collected from artificial scallop-rearing substrates installed in Xunshan Fishery Group Co., Rongcheng (Shandong Province, China). All the procedures involved in the handling and the treatment of scallops during this study were approved by the Ocean University of China Institutional Animal Care and Use Committee (OUC-IACUC) prior to the initiation of the study. The scallops were acclimated in the laboratory at ambient seawater temperature for 1 week prior to the experiments, which is within the optimum temperature range for their survival.

Gram-negative (*V. anguillarum*) bacteria were used to challenge scallops in our study (Zhi et al., 2011). *V. anguillarum* was cultured in liquid 2216E broth (5 g/L of Tryptone, 1 g/L of yeast extract, and 0.1 g/L of C6H5Fe·5H2O, pH = 7.6) at 28°C and harvested by centrifugation at 2000×*g* for 5 min, as described by Kong et al. The pellet was suspended in filtered seawater and was adjusted to 1×10^7 CFU/ml in seawater, respectively (Zhi et al., 2011; Ragab et al., 2014).

A total of 75 individuals were randomly and equally divided into five groups. At 0 h, 5 h, 24 h, 48 h, and 72 h post-infection, 5 individuals were randomly collected from each group. The 0 h group was employed as the control group, and other groups were used as experimental groups. The hemolymph samples were collected from adductor muscles using a syringe and were immediately centrifuged at 800×*g*, 4°C for 10 min to harvest the hemocytes (Gao et al., 2007). The extracted hemocyte sample was immediately frozen in liquid nitrogen and then subsequently frozen at −80°C before processing.

2.5 RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was isolated following the method described by Hu et al. (2010), and then was digested with DNase I (TaKaRa, Shiga, Japan). A Nanovue Plus spectrophotometer (GE Healthcare, NJ, USA) was used to assess the concentration and purity of RNA; RNA integrity was determined by agarose gel electrophoresis. First-strand cDNA was synthesized using Moloney murine leukemia virus (MMLV) reverse transcriptase (Thermo, USA) following the manufacturer's protocol. All of the cDNA products were diluted to 5 ng/ml for use as the template in real-time PCR.

Real-time PCR was conducted using the SsoFast™ EvaGreen® Supermix on a Light Cycler 480 Real-time PCR System (Roche Di-agnostics, Mannheim, Germany). The running program was as follows: 50°C for 2 min, 94°C for 10 min, and 40 cycles at 94°C for 15 s and at 62°C for 1 min. Cytochrome B (CB), DEAD-βox RNA helicase (HELI), and EF1-

A gene were designated as internal reference genes for the normalization of gene expression in healthy adults and test subjects during the real-time PCR experiment, respectively (Li et al., 2010; Feng et al., 2013). All the primers used in the real-time PCR were designed using Primer Premier 5.0 and are listed in **Table 1**.

Data from the real-time PCR were analyzed using the Relative Expression Software Tool (REST) version 2009 (Pfaffl et al., 2002); gene expression is shown as the fold change. For the experimental groups, the control group (0 h) was used for normalization. The statistical analysis of the data was performed with SPSS (version 16.0) software using the independent *t*-test. Differences were considered to be significant at $p < 0.05$.

2.6 Subcellular Localizations

The full length of ORFs of three *CfIRF* genes was amplified from *C. farreri* cDNA, using primers listed in **Table 1**. For *CfIRF1* and *CfIRF2*, the PCR products were ligated and subcloned into pEGFP-N1 (Clontech, USA) by way of overlap extension PCR to construct recombinant plasmids pEGFP-*CfIRF1* and pEGFP-*CfIRF2*, while for the *CfIRF1-like*, PCR products were digested with *Kpn*I and *Sma*I, and ligated and subcloned into pEGFP-N1 vector digested by the corresponding restriction enzymes to construct recombinant plasmids pEGFP-*CfIRF1-like*. The constructed recombinant plasmids were subsequently verified by DNA sequencing.

In scallops, as well as in other marine bivalves, there are no mature cell lines. Thus, we choose HEK293T cells to perform our experiment. HEK293 cells were maintained in Modified Eagle Medium (MEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) and antibiotics (100 mg/L streptomycin and 10^5 U/L penicillin, Gibco) at 37°C in a humidified incubator under 5% CO_2. For DNA transfection, cells were seeded and allowed to grow to more than 70% confluence, and then plasmids were transfected by using the Lipofectamine 3000 Reagent (Invitrogen, USA) following the manufacturer's recommendations. HEK293T cells were transiently co-transfected with 0.8 μg of expression plasmid and 1 μl of Lipofectamine 3000 in each well in a 24-well plate. All assays were performed with three independent transfections.

At 48 h post-transfection, HEK293 cells were washed with PBS twice and fixed with paraformaldehyde for 15 min. Then, the cells were washed three times with PBS and were stained with DAPI (Sigma, USA) to mark the nucleus followed by washing three times. Immunofluorescence was visualized and captured with confocal microscopy (Nikon, Japan).

2.7 Dual-Luciferase Reporter Assays

The full length of ORFs of three *CfIRF* genes was amplified from *C. farreri* cDNA, using primers listed in **Table 1**. For *CfIRF1* and *CfIRF2*, the PCR products were ligated and subcloned into

TABLE 1 | List of primers used in this study.

Name	Sequence (5'-3')
For RT-PCR	TGACGATGATGAGAGCAATG
IRF1-F	
IRF1-R	GGACGGATATTTGAAGGGATG
IRF1-like-F	CAGGTGACAATAGACCTGAAG
IRF1-like-R	ACACCACAGACACGAATATG
IRF2-F	CAGACTACCACATTGAGATCG
IRF2-R	CGACTTCTTCGTCTGTTAGG
IRF8-F	CTTATCTTACGGCCAGGAAC
IRF8-R	GGTTCTTCAGCATCGTATCA
For construction of plasmids[a]	
IRF1-GFP-F	CGTCAGATCCATGGCAATTTCCGAGATTGAAC
IRF1-GFP-R	CGGGACACACATCCCAGTCAACCATGGTGA
IRF1-GFP-N1-F	ATCCCAGTCAACCATGGTGAGCAAGGGCGAG
IRF1-GFP-N1-R	GGTTTAGTGAACCGTCAGATCCATGGCAATTT
IRF1-like-GFP-F	TCC**CCCGGG**ATGAGCAAAGTGAAGAAAAAGATGG
IRF1-like-GFP-R	GGG**ACCGGT**AACTGAATTTTTCGAATCTGGTTCG
IRF2-GFP-F	CGTCAGATCCATGGTTGTGTCAAAGAAAATGC
IRF2-GFP-R	ATACACAAGTATTTTGGGCCTGACCATGGTGA
IRF2-GFP-N1-F	TTTGGGCCTGACCATGGTGAGCAAGGGCGAG
IRF2-GFP-N1-R	GGTTTAGTGAACCGTCAGATCCATGGTTGTGT
IRF1-gene-F	ACCCAAGCTGATGGCAATTTCCGAGATTGAAC
IRF1-gene-R	GGACACACATCCCAGTCATAACCGCTGATCA
IRF1-pcDNA3.1-F	CCAGTCATAACCGCTGATCAGCCTCGACT
IRF1-pcDNA3.1-R	CTATAGGGAGACCCAAGCTGATGGCAATTT
IRF1-like-pcDNA3.1-F	ATAAGAAT**GCGGCCGC**ATGAGCAAAGTGAAGAAAAAGATGG
IRF1-like-pcDNA3.1-R	CGG**GGTACC**TCAAACTGAATTTTTCGAATCTGGTT
IRF2-gene-F	ACCCAAGCTGATGGTTGTGTCAAAGAAAATGC
IRF2-gene-R	CACAAGTATTTTGGGCCTGTAACCGCTGATCA
IRF2-pcDNA3.1-F	GGGCCTGTAACCGCTGATCAGCCTCGACT
IRF2-pcDNA3.1-R	CTATAGGGAGACCCAAGCTGATGGTTGTGT

[a]*Nucleotides in bold indicate restriction enzyme sites.*

pcDNA3.1 V5/H vector (Invitrogen, USA) by way of overlap extension PCR to construct recombinant plasmids pcDNA3.1-IRF1 and pcDNA3.1-IRF2, while for the *CfIRF1-like*, the pcDNA3.1-IRF1-like was constructed using the same method as mentioned above but digested with *Not*I and *Kpn*I. The constructed recombinant plasmids were subsequently verified by DNA sequencing. For reporter plasmids, pISRE-Luc (ClonTech, USA) was used, and pRL-TK renilla luciferase plasmid (Progema, USA) and pGL3-basic vector (Progema, USA) were used as an internal control and blank group, respectively. EndoFree Plasmid MiKit (OMEGA, USA) was used for the transfection of the plasmids according to the manufacturer's instruction.

The cell culture assays were performed according to Section 2.6. For dual-luciferase reporter assays, HEK293T cells were transiently co-transfected with 0.2 mg of expression plasmid, 0.5 mg of reporter gene plasmid, 0.01 mg of pRL-TK renilla luciferase plasmid, and 0.1 μl of Lipofectamine 3000 in each well in a 24-well plate. The luciferase reporter vector pGL3-basic was used as a blank group. All assays were performed with three independent transfections. At 48 h post-transfection, HEK293 cells were washed with PBS twice and lysed. Firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instruction. Cell lysate (20 μl) was transferred to a 1.5-ml EP tube and 100 μl of luciferase assay reagent II and 100 μl of Stop & Glo® Reagent were added in sequence, then firefly and renilla luciferase activities were measured, respectively.

3 RESULTS

3.1 Sequence Identification and Analysis

Four *IRF* genes, *CfIRF1, CfIRF1-like*, *CfIRF2*, and *CfIRF8*, were identified from the genome of Zhikong scallop. The basic information (total length, ORF length, number of exons, amino acids length, theoretical *pI*, and weight of protein) of these *IRF* members were summarized in **Table 2**. The ORFs of *CfIRF1, CfIRF1-like, CfIRF2*, and *CfIRF8* were respectively 1,107, 5,913, 1,053, and 1,311 bp, encoding 368, 1,970, 350, and 436 amino acids. The predicted molecular weights of these four genes ranged from 40.05 to 212.51 KD, with the predicted *pI* values from 4.97 to 6.96 (**Table 2**). The genomic structural analysis showed that the length as well as the exon–intron pattern of these four *CfIRF* genes varied greatly. The longest *CfIRF1* gene was 28,913 bp with 9 exons, and the shortest *CfIRF2* gene was 6,565 bp with 10 exons. Moreover, *CfIRF1-like* and *CfIRF8* had 9 and 8 exons, respectively (**Figure 1**).

All four *Cf*IRF proteins had a single well-conserved DBD domain (113 aa in length), which was found to be helix-turn-helix at the N-terminal (**Figure 2**). Furthermore, *Cf*IRF8 was predicted to contain an *IRF*-associated domain (IAD) at the C-terminus, and *Cf*IRF1-like possessed seven C2H2-type (classical) zinc fingers (ZnF_C2H2) at the C-terminal (**Figure 2**). Multiple sequence alignments of *Cf*IRFs showed that they share high similarity within the DBD domain (**Figure 3**). Moreover, five conserved tryptophan (W) residues were revealed, in which the W^{71} residue was found mutated as Y^{71} in *Cf*IRF1 protein.

TABLE 2 | Summary of sequence features of *CfIRF* genes.

Name	Total length (bp)	ORF length (bp)	Exon number	Protein length(aa)	*pI*	Molecular weight (Da)
IRF1	28913	1107	9	368	6.04	41469.13
IRF1-like	22192	5913	9	1970	6.96	212509.5
IRF2	6565	1053	10	350	4.97	40046.71
IRF8	10031	1311	8	436	6.21	49815.55

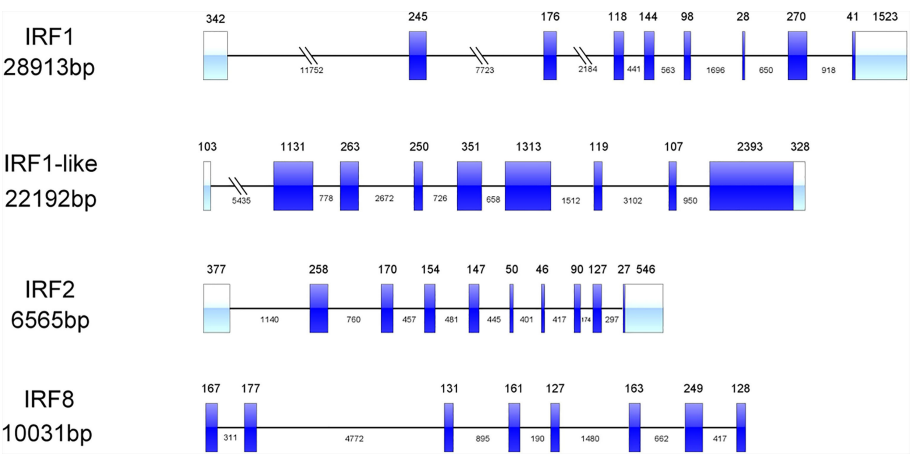

FIGURE 1 | Genetic structure of the *CfIRF* genes. The light blue boxes indicate the 3' UTRs and the 5' UTRs. The dark blue boxes indicate the exons. The horizontal line with sporadic double slash indicates the introns.

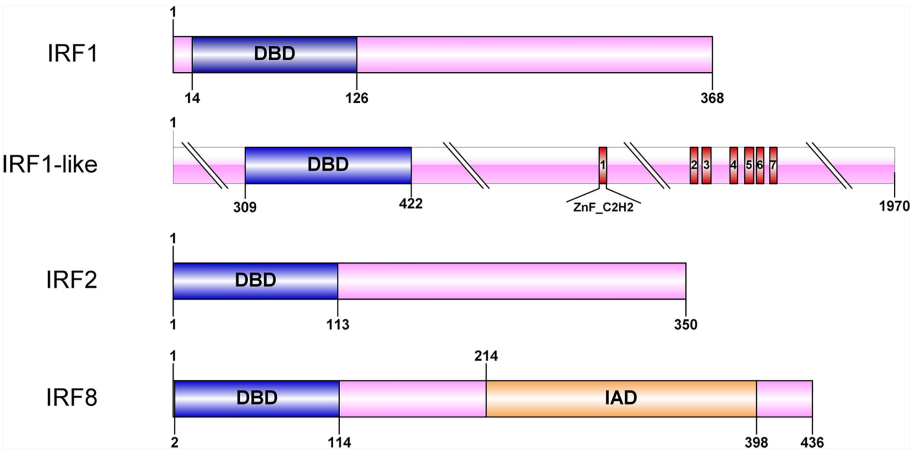

FIGURE 2 | Protein structure of the *CfIRF* genes. The blue boxes indicate the DBD domain. The orange boxes indicate the IAD domain. The red boxes indicate the ZnF_C2H2 domain.

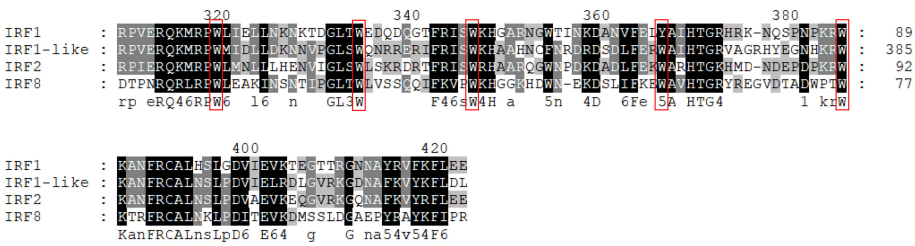

FIGURE 3 | Alignment of DBD domains of the four *Cf*IRF proteins. Alignment was performed using ClustalW2. Identical residues are indicated in black, and similar residues are in light gray. Dashes indicate gaps. Five conserved tryptophan (W) residues are marked with red boxes.

To confirm the identification of the *Cf*IRFs, a phylogenetic tree was constructed through the ML method using 59 IRF proteins across 13 species. Four IRF subfamilies (IRF-1, -3, -4, and -5) were identified (**Supplementary Figure 1**) and all *Cf*IRFs were clustered into its own clade. The result showed that *Cf*IRF1, *Cf*IRF1-like, and *Cf*IRF2 were clustered into the IRF-1 subfamily. *Cf*IRF8 was firstly grouped together with IRF8-like from *Peten maximus* and IRF8 from *C. gigas*, and they were clustered into the IRF-4 subfamily.

3.2 Spatiotemporal Expressions of *CfIRF*s

The RPKM data were used to analyze the expression patterns of four *CfIRF* genes during eleven developmental stages (**Figure 4A**, **Supplementary Figure 2A**). Their expression during early development (from zygote to trochophore larvae) was quite low (<10 RPKM). After D-shaped larvae formation, *CfIRF1-like* and *CfIRF8* remained at a low expression (<5 RPKM) while expression of *CfIRF1* and *CfIRF2* was obviously elevated. Although *CfIRF2* showed higher expression level than *CfIRF1* (>3-fold), their expression pattern was similar, both of which showing the highest expression in creeping larvae and juvenile. In adult tissues, we found that expression of *CfIRF1* and *CfIRF2* was obviously higher than *CfIRF1-like* and *CfIRF8* (**Figure 4C**, **Supplementary Figure 2B**), consistent with their expression tendency during development. In general, *CfIRF1-like* and *CfIRF8* remained low expression in all the tissues (<25 RPKM), while *CfIRF1* was dominantly expressed in hepatopancreas and hemolymph (>160 RPKM), and the highest expression of *CfIRF2* was observed in gill and hepatopancreas (>220 RPKM). To illustrate the role of *CfIRF1* and *CfIRF2* in development stages and adult tissues, we performed correlation analysis between the expression of *CfIRF1* and *CfIRF2* with E2/T ratio. According to the results, *CfIRF1* has a significantly positive correlation with *CfIRF2* expression both in different developmental stages (r = 0.86, $p < 0.001$) (**Figure 4B**) and in adult tissues (r = 0.65, $p <$ 0.05) (**Figure 4D**).

3.3 Temporal Responses of *CfIRF*s in Coping With Bacterial Infection

To examine the immune responses of *CfIRFs* to *V. anguillarum* challenge (Qiu et al., 2007; Costa et al., 2009), their expression level was investigated at four time points (5 h, 24 h, 48 h, and 72

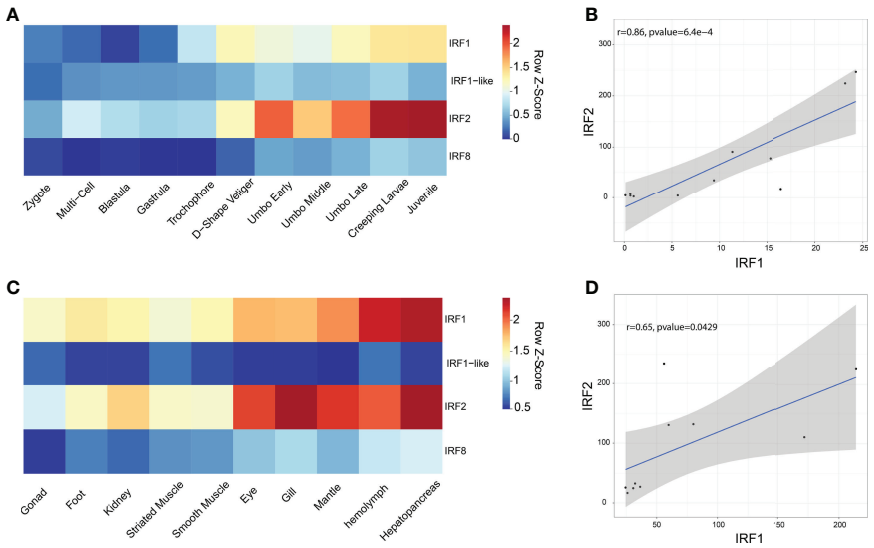

FIGURE 4 | Expression profiles of *CflRFs* in different developmental stages and adult tissues. **(A)** Heatmap of *CflRFs* expression profile in different embryonic and larval stages. **(B)** The positive correlation between *CflRF1* and *CflRF2* in different developmental stages. **(C)** Expression profiles of *CflRFs* in adult tissues. **(D)** The positive correlation between *CflRF1* and *CflRF2* in adult tissues.

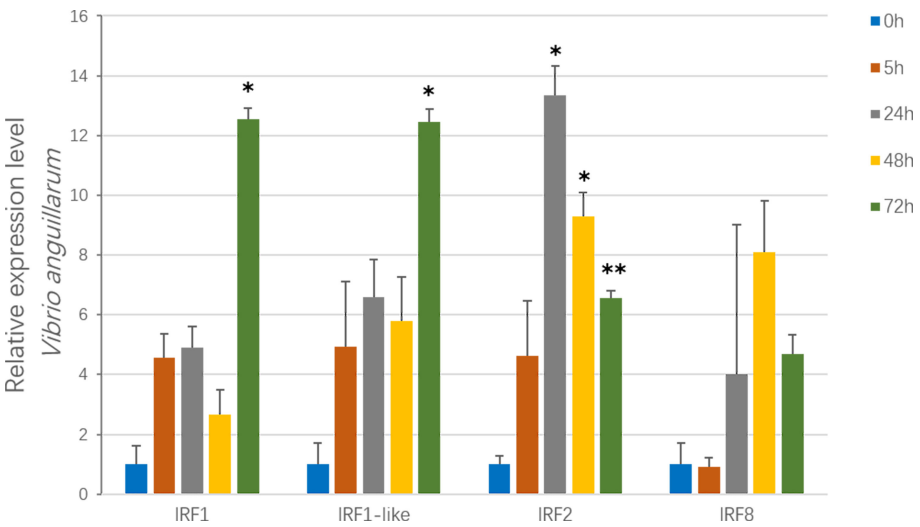

FIGURE 5 | Expression of *CflRF* genes after challenge with the *V. anguillarum*. Vertical bars represent the mean ± S.E. (*N* = 10). The asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$).

h) after infection (**Figure 5**). Overall, three *CflRFs* from the IRF-1 subfamily (*CflRF1*, *CflRF1-like*, and *CflRF2*) were significantly upregulated. Notably, *CflRF2* showed the most sensitive responses, which showed significant upregulation after 24-h infection (>13-fold, $p < 0.05$) and sustained at a significantly higher expression level till 72 h. Moreover, the expressions of *CflRF1* and *CflRF1-like* were significantly upregulated at 72 h post-infection (>12-fold, $p < 0.05$), while *CflRF8* only showed mild upregulated tendency after 24-h infection (4- to 8-fold).

3.4 Subcellular Localizations of *CflRF1*, *CflRF1-Like*, and *CflRF2*

Subcellular localization prediction showed that *Cf*IRF1, *Cf*IRF1-like, and *Cf*IRF2 all possess nucleus localization (**Table 3**). Furthermore, ^{163}RSRRRKKPCVKKE^{175} in *Cf*IRF1 was predicted as a nucleus localization signal with a high score (score: 9.5), further suggesting that *Cf*IRF1 was a nuclear-localized protein. HEK293T cells were transfected with plasmids encoding pEGFP-tagged *CflRF1*, *CflRF1-like*, or

TABLE 3 | Summary of the predicted subcellular localization of *CfIRF1, CfIRF1-like and CfIRF2*.

Name	Euk-mPLoc 2.0	WoLF PSORTⅡ	SLP-Local
IRF1	nucl. cyto.	nucl. nucl-cyto. cyto	nucl. cyto.
IRF1-like	nucl.	nucl.	nucl. cyto.
IRF2	nucl.	nucl.	nucl. cyto.

CfIRF2 to investigate their subcellular localization. The immunofluorescence image analysis showed that recombinant *Cf*IRF1-GFP protein was located in the nucleus, consisting of its subcellular localization prediction and the NLS prediction. Unlike *Cf*IRF1, the fluorescent signal of *Cf*IRF1-like and *Cf*IRF2 recombinant proteins was distributed in both nuclei and cytoplasm (**Figure 6**). Thus, the divergence of *CfIRFs* by subcellular localization tentatively suggested that these three *CfIRFs* may be involved with different cellular functions.

Dual-Luciferase Reporter Assays

To analyze the transcription activities of *Cf*IRF1, *Cf*IRF1-like, and *Cf*IRF2, dual-luciferase reporter assays were performed in HEK293T cells. As control, pcDNA3.1-IRFs were co-transfected with pGL3-basic, and it did not show any effect on the ISRE reporter. By using pISRE-Luc, after co-transfection with pcDNA3.1-IRF1, pcDNA3.1-IRF1-like, and pcDNA3.1-IRF2, the luciferase activity of the ISRE reporter was significantly upregulated (**Figure 7**), suggesting their obvious transcriptional activation on

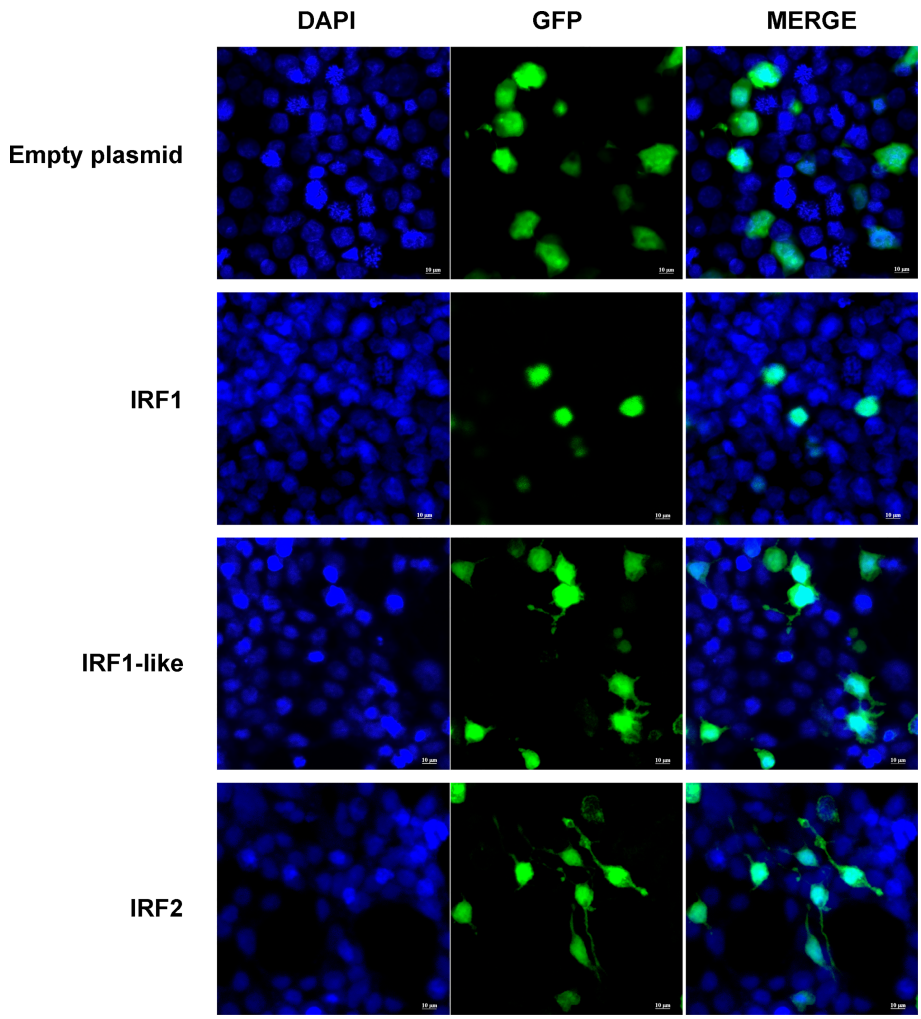

FIGURE 6 | Subcellular localization of *CfIRF* genes in HEK293T cells. Immunohistochemistry was performed to analyze the expression of *Cf*IRF in hemocytes. The left-hand panels depict DAPI staining, the middle panels depict GFP staining, and the right-hand panels depict merged DAPI/GFP staining. The upper panels depict localization of the GFP negative control, and the lower panels depict localization of the *Cf*IRF-GFP proteins.

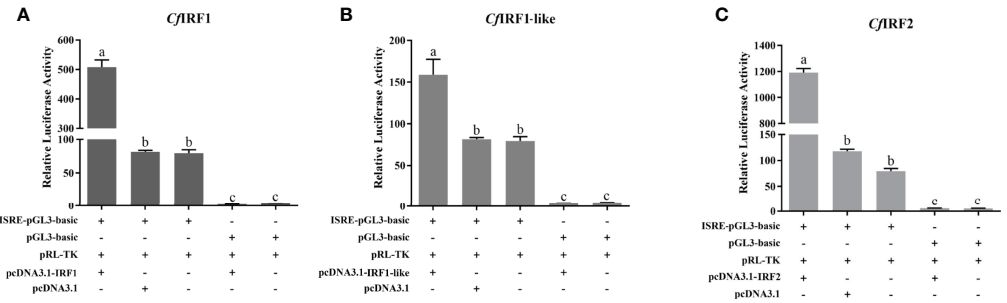

FIGURE 7 | Relative luciferase activity of expression *Cf*IRF plasmids on the luciferase reporter gene pISRE-Luc in HEK293T cells. **(A)** Relative luciferase activity of expression *Cf*IRF1 plasmids. **(B)** Relative luciferase activity of expression *Cf*IRF1-like plasmids. **(C)** Relative luciferase activity of expression *Cf*IRF2 plasmids. All of the groups were co-transfected with pRL-TK as internal reference. Vertical bars represent the mean ± S.E. ($N = 3$). p-values are calculated by one-way analysis of variance (ANOVA) in SPSS software. Different letters indicate significant differences ($p < 0.05$).

interferon-stimulated response element. These results indicated that these *CfIRFs* could activate the expression of ISRE luciferase reporter genes, suggesting that scallop *IRFs* can specifically activate interferon signaling.

4 DISCUSSION

The innate immune system is the first line of defense against the invasion of pathogens. *IRFs* are key transcription factors involved in type I IFN responses, playing a pivotal role in the regulation of interferon activity (Kimura et al., 1994). In the present study, we successfully identified four *IRF* genes in a bivalve mollusc *C. farreri*, namely, *CfIRF1*, *CfIRF1-like*, *CfIRF2*, and *CfIRF8*. All four *Cf*IRF proteins have a single well-conserved N-terminal helix-turn-helix IRF superfamily domain (also named as DBD). Through the motif of Trp repeats, the conserved DBD domain in vertebrate was proved to recognize and bind DNA sequence containing 5′-GAAA-3′ tetranucleotide as a determinant of interferon regulation (Escalante et al., 1998; Mamane et al., 1999). Consistently, we found well-conserved Trp repeats inside the DBD domain of scallop IRFs, suggesting their similar binding activities with vertebrate IRFs. Moreover, *Cf*IRF8 was predicted to contain an IRF-associated domain (IAD) at the C-terminus, which has been reported to mediate the formation of homologous dimers or the coupling with other transcription factors to form heterodimers (Honda and Taniguchi, 2006; Yanai et al., 2012). According to the phylogenetic analysis, *Cf*IRF1, *Cf*IRF1-like, and *Cf*IRF2 were clustered into the IRF-1 subfamily, and *Cf*IRF8 was clustered into the IRF-4 subfamily.

Spatiotemporal expression levels of *CfIRF1* and *CfIRF2* were found to be obviously higher than *CfIRF1-like* and *CfIRF8*, suggesting the initial requirement for them during development as well as in adult tissues. Correlation analysis showed that *CfIRF1* and *CfIRF2* have a significantly positive correlation during development as well as in adult tissues, indicating that they may be functionally synergistic. *CfIRF1* and *CfIRF2* were highly expressed after the D-shape veliger formation, especially in creeping larvae and juvenile, the key stage that multi-organs began to developed. In adult tissues, high expression of both *CfIRF1* and *CfIRF2* was detected in multiple immune-related tissues, such as hemolymph, hepatopancreas, gill, and mantle (Fan et al., 2018; Lu et al., 2018; Gan et al., 2020), indicating the crucial roles of these genes in the host immune response. Hemocytes, one of the major immune tissues in molluscs, has been reported as the main site where the recognition and elimination of bacterial pathogens occurs (Zhou et al., 2015). Hepatopancreas is the main digestive tissue; thus, it needs to cope with the pathogens incoming with the ingested algae. Furthermore, hepatopancreas was also proved as a toxin-rich tissue, acting as major "centers" for toxin accumulation in *C. farreri* (Li et al., 2017), which may also induce immune responses. The gill and mantle are constantly in contact with the external environment *via* water filtering and serve as the front line of the host defense (Lee et al., 2013). The tissue expression patterns of *IRF* genes have been widely characterized in various species, and high expression has been detected in multiple immune-related tissues. For example, high expression levels of *CgIRF-2* were detected in *C. gigas* hemocytes, hepatopancreas, and mantle, and *LcIRF* from *Larimichthys crocea* were highly expressed in hemocytes, gill, and spleen (Lu et al., 2018; Guan et al., 2020). Taken together, the high expression levels of *IRFs* in these immune-related tissues may suggest their conservative roles in scallop innate immune response.

To provide insights into the functions of *CfIRFs* during the innate immune response, one of the major bacterial pathogens, *V. anguillarum*, was employed to perform the infection experiment and the responses of *CfIRFs* in hemocytes were investigated. Only *CfIRFs* from the IRF-1 subfamily (*CfIRF1*, *CfIRF1-like*, and *CfIRF2*) were found to be increased significantly post-infection, which confirmed the involvement of these *CfIRFs* in the innate immune response against bacterial invasion. In comparison with *CfIRF1* and *CfIRF1-like*, *CfIRF2* showed the most sensitive response. Subcellular localization prediction showed that *Cf*IRF1 possessed an NLS in its DNA-binding domain, and subcellular localization analysis confirmed that it mainly translocated in the nucleus; meantime, *Cf*IRF1-like and *Cf*IRF2 were expressed in both nucleus and cytoplasm. Similarly,

IRFs have shown the divergence in subcellular localization in some other species. For example, in marine bivalves *C. gigas* and *P. fucata*, researchers found that *Cg*IRF-1 and *Cg*IRF-2 proteins were both primarily expressed in nucleus and cytoplasm (Huang et al., 2017; Lu et al., 2018), and *Pf*IRF-2 was located in the nucleus (Huang et al., 2013). Previous studies showed that most species only have one *IRF1* gene, while in *C. gigas*, there are two *IRF1* members, named *Cg*IRF1a and *Cg*IRF1b (Fan et al., 2018). They found that *Cg*IRF1a significantly activated the ISRE reporter gene, whereas *Cg*IRF1b did not. According to our data, both *CfIRF1* and *CfIRF1-like* showed significant responses against bacterial infection, and they both could activate ISRE significantly; however, they have different functional domains, spatiotemporal expression patterns, and subcellular localizations, suggesting that functional differences may exist for *CfIRF1* and *CfIRF1-like*.

Many studies have shown that *IRFs* are typical interferon-stimulated genes in mammals, birds, and fish (Andrea et al., 2002; Liu et al., 2018; Zhu et al., 2020). Among *IRF*s, *IRF1* and *IRF2* were originally characterized as transcriptional regulators of type I IFNs and IFN-stimulated genes (ISGs), which played an important role in the antiviral immune response (Miyamoto et al., 1988a; Harada et al., 1989b). Our findings from the dual-luciferase reporter gene assays showed that *Cf*IRF1, *Cf*IRF1-like, and *Cf*IRF2 could significantly activate the expression of the IRSE reporter gene, revealing obvious transcriptional activation on interferon-stimulated response element. Similar to their responses against *V. anguillarum*, in comparison with *Cf*IRF1 and *Cf*IRF1-like, *Cf*IRF2 showed the most significant activation effects, which may contribute to its most sensitive responses to bacterial infection. Similar results have been shown in other bivalves; for example, the recombinant *Cg*IRF-1 or *Pf*IRF-2 exhibited the activity to bind ISRE *in vitro*. Previous studies in vertebrates have shown that this conserved binding and activation of ISRE is mainly attributable to the DBD domain in IRFs. It was reported that the DBD in vertebrate IRFs could form a helix-turn-helix domain and bind to the core DNA sequence GAAA in the IFN-stimulated response element (ISRE, A/GNGAAANNGAAACT) (Escalante et al., 1998). For instance, IRF-1 from zebrafish (*Dr*IRF-1) could bind to ISRE/*IRF*-E motifs within the IFN promoters through the DBD helix α3 to induce its transcription (Feng et al., 2015), while compared with the extensive knowledge of *IRF*s in vertebrates, the possible mechanisms of these bivalve *IRF*s binding and activating IFN system need further investigation.

5 CONCLUSION

In conclusion, we identified four *IRF* genes in Zhikong scallop: *CfIRF1*, *CfIRF1-like CfIRF2*, and *CfIRF8*. *Cf*IRFs contained highly conserved N-terminal DNA-binding domain and variable C-terminal regulatory domain, and were constitutively expressed during development as well as in adult tissues, especially in hepatopancreas, hemolymph, gill, and mantle. Furthermore, we determined that *CfIRF1*, *CfIRF1-like*, and *CfIRF2* genes played pivotal roles in the innate immune defense against bacterial infection, and their encoding proteins could translocate into nucleus. Functionally, *Cf*IRF1, *Cf*IRF1-like, and *Cf*IRF2 had been proven to induce a strong activation of ISRE promoters. Moreover, in comparison with *CfIRF1* and *CfIRF1-like*, *CfIRF2* showed the most sensitive responses in coping with *V. anguillarum*, and consistently, *Cf*IRF2 exhibited the most significant activation on ISRE. Our data would provide valuable information for further investigations into the evolution and functional characterization of *IRFs* in bivalve molluscs.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

This animal study was reviewed and approved by the Ocean University of China Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

SL and JH conceived and designed the study. NH and XZ performed the experiments. FS and XC participated in data analysis. NH, SL, JH, LZ, SW, and ZB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIALS

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.865707/full#supplementary-material.

Supplementary Figure 1 | The phylogenetic tree was constructed based on the protein sequences of *Cf*IRFs, in addition to those of other species. FastTree 2.0.0 was used to construct the phylogenetic tree by the maximum-likelihood (ML) analyses. Solid circle: vertebrate IRFs, Hollow circle: invertebrate IRFs, Red hollow circle: *Cf*IRFs.

Supplementary Figure 2 | The expression profiles in different developmental stages and adult tissues. **(A)**. Expression levels of *CfIRFs* in different embryonic and larval stages. **(B)**. Expression levels of *CfIRFs* in adult tissues. Vertical bars represent the mean ± S.E. (N=3). *P* values are calculated by one-way analysis of variance (ANOVA) in SPSS software. Different letters indicate significant differences ($P < 0.05$).

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