



Full length article

Lysine 39 of IKK ϵ of black carp is crucial for its regulation on IRF7-mediated antiviral signalingJun Li^{a,1}, Yu Tian^{a,b,1}, Ji Liu^a, Chanyuan Wang^a, Chaoliang Feng^a, Hui Wu^a, Hao Feng^{a,*}^a State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha, 410081, China^b The Second Xiangya Hospital, Central South University, Changsha, 410011, China

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ABSTRACT

Interferon regulatory factor 7 (IRF7) plays a crucial role in the interferon (IFN) signaling in mammals, in which it is activated by the TBK1/IKK ϵ complex during host antiviral innate immune response. There are few reports about the relation between IRF7 and IKK ϵ in teleost fishes. In this study, the IRF7 homologue (bcIRF7) of black carp (*Mylopharyngodon piceus*) has been cloned and characterized. The transcription of *bcIRF7* gene increased in host cells in response to the stimulation of LPS, poly (I:C) and viral infection. bcIRF7 migrated around 56 kDa in immunoblot assay and was identified as a predominantly cytosolic protein by immunofluorescent staining. bcIRF7 showed IFN-inducing ability in reporter assay and EPC cells expressing bcIRF7 showed enhanced antiviral ability against both grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV). IKK ϵ of black carp (bcIKK ϵ) was found to be recruited into host innate immune response initiated by SVCV and GCRV in the previous work; in this paper, the kinase dead mutant of bcIKK ϵ , bcIKK ϵ -K39A was constructed and showed no IFN-inducing activity. The data of reporter assay and plaque assay demonstrated that bcIKK ϵ but not bcIKK ϵ -K39A obviously enhanced bcIRF7-mediated IFN production and antiviral activity. Our data support the conclusion that bcIKK ϵ upregulates bcIRF7-mediated antiviral signaling, which most likely depends on its kinase activity.

1. Introduction

Vertebrates depend on their immunity to protect themselves from the invasion of the pathogenic microbes, such as bacteria and viruses, which is classified into innate immunity and adaptive immunity [1]. The innate immune system of vertebrate is activated after the infection of virus, which depends on the recognition of the invaded viral components by the pathogen recognition receptors (PRRs) and the consequent production of interferons (IFNs) [2]. IFN is the key component of innate immunity of vertebrates, which also functions importantly in the adaptive immunity [3]. The interferon regulatory factors (IRFs) were originally known as transcriptional regulators of type I IFN and IFN-stimulated genes (ISGs), which form the first line of antiviral defense in vertebrates [4].

To date, 11 IRF members (IRF1~11) have been reported in vertebrates, in which IRF11 is exclusively found in fish [5]. All IRF proteins share a conserved amino N terminus DNA-binding domain (DBD), containing several conserved tryptophan residues and forming a helix-loop-helix motif [6]. The DBD of IRF recognizes the consensus sequence A/GNGAAANNGAAACT, which is found in the promoters of type I IFNs

and many ISGs [7]. Additionally, the carboxyl (C) terminus of all IRFs except IRF1 and IRF2 contains an IRF association domain (IAD), which interacts with other proteins to form transcriptional complexes [8].

IRF7 is one of the most intensively studied IRF members due to its crucial role in innate antiviral immunity, which was first described as a binding factor to the Qp promoter region of the Epstein-Barr virus (EBV) - encoded gene EBNA1 [9]. In mammals, IRF7 is generally present in the cytoplasm of uninfected cells in an inactive form. After virus-mediated phosphorylation and dimerization, it translocates to the nucleus and bind to the type I IFN promoters, activating transcription either alone or in coordinate with other transcription factors such as IRF3 and NF- κ B [10]. When IFNs are produced, they are secreted to neighboring cells and trigger the transcription of a set of antiviral ISGs through the Jak-STAT pathway, leading to the establishment of an antiviral state in uninfected cells [11–13].

NF- κ B translocates into the nucleus to initiate the expression of pro-inflammatory cytokines after virus infection, which play the critical role in immune and inflammatory responses in mammals and teleost fish [14]. The degradation of inhibitor of NF- κ B (I κ B) exposes the NF- κ B complex and activates NF- κ B, in which I κ B is phosphorylated by I κ B

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kinase (IKK) and followed by polyubiquitination [15]. IκB kinase ε (IKKε, also known as IKKi) was firstly identified as the serine/threonine kinase induced by lipopolysaccharide (LPS) [16]. IKKε and the other non-canonical IκB kinase, TANK-binding kinase 1 (TBK1), are characterized as activators of NF-κB although they are not essential for NF-κB activation [17]. Moreover, they play critical roles in antiviral response via phosphorylation and activation of transcription factors IRF3, IRF7 and STAT1 [18]. However, the function of IKKε orthologue in teleost fish remains largely unknown.

In our previous study, IKKε of black carp (bcIKKε) have been cloned and characterized, which was implied to function in the RIG-I like receptor (RLR) signaling in host antiviral innate immune response against GCRV and SVCV [19]. To further explore the function of bcIKKε in the IFN production process in host cells after viral infection, bcIRF7 was cloned and characterized in this study, which was considered as an important activator of the transcription of IFNs in black carp like its mammalian counterpart. Our data demonstrated clearly that bcIRF7 mediated IFN production and antiviral activity were obviously elevated by bcIKKε, which was reported in teleost fish for the first time.

2. Materials and methods

2.1. Cells and plasmids

Epithelioma Papulosum Cyprinid (EPC) cells, *C. idella* kidney (CIK) cells and *Mylopharyngodon piceus* kidney (MPK) cells were kept in the lab [20]. All the cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 u/ml penicillin and 100 μg/ml streptomycin. The fish cells was cultured at 26 °C. Lipomax (SUDGEN) was used for EPC transfection (100 ng DNA with 0.3ul lipomax; efficiency around 80%) and MPK transfection (100 ng DNA with 0.2ul lipomax; efficiency around 30%) as previously described [21].

pcDNA5/FRT/TO-HA, pcDNA5/FRT/TO-Flag, pcDNA5/FRT/TO-Flag-bcIKKε and pRL-TK were kept in the lab; zIFN1-Luci was a kind gift from Dr. Yong'an Zhang (Institute of Hydrobiology, CAS), in which the 5'-flanking regulatory sequences of zebrafish IFN1 were cloned and inserted into pGL3-Basic luciferase reporter vector (Promega) to analyze the promoter activity [22]. pcDNA5/FRT/TO-HA-bcIRF7 was constructed by inserting the open reading frame (ORF) of black carp IRF7 (bcIRF7) into pcDNA5/FRT/TO-HA between Kpn I and Not I restriction sites with a HA tag at its N-terminus. bcIRF7-pEGFP-N1 was constructed by inserting the ORF of bcIRF7 into the upstream of enhanced green fluorescent protein in the pEGFP-N1 vector between Kpn I and Hind III restriction sites. bcIKKε-K39A construct was generated by site mutagenesis using overlap PCR as previously [22], in which lysine (K) of residue 39 was altered to alanine (A).

2.2. Cloning of bcIRF7 cDNA

Total RNA was isolated from the spleen of black carp and the first-strand cDNA were synthesized by using the Revert Aid First Strand cDNA Synthesis Kit (Thermo). Degenerate Primers were designed for amplifying bcIRF7 cDNA based on the IRF7 sequences of *C. carpio* (HQ850444.1), *C. auratus* (AY177629.1) and *D. rerio* (BC058298.1). The coding sequence (CDS) was cloned at the first attempt by using the degenerate primers. Rapid amplification of cDNA ends (RACE) was performed to obtain 5'UTR and 3'UTR of bcIRF7 cDNA by using 5'Full RACE kit and 3'Full RACE kit separately (TaKaRa). The amplified fragments were cloned into pMD18-T vector and sequenced by Invitrogen.

2.3. Virus produce and titer

SVCV and GCRV were kept in the lab [23]. SVCV and GCRV were propagated in EPC and CIK separately at 26 °C in the presence of 2% fetal bovine serum. Virus titers were determined by plaque forming

assay on EPC cells separately as previously described [22]. Briefly, the 10-fold serially diluted virus supernatants were added onto EPC cells and the cells were incubated for 2 h at 26 °C. The supernatant was replaced with fresh DMEM containing 2% FBS and 0.75% methylcellulose (Sigma) after incubation. Plaques were counted at day 3 post-infection.

2.4. Quantitative real-time PCR

The relative bcIRF7 mRNA level in the selected tissues of black carp or MPK cells was examined by quantitative real-time PCR (q-PCR). The primers for bcIRF7 and β-actin (as internal control) were listed in [Supplementary Table 1](#). The q-PCR program was: 1 cycle of 50 °C/2min, 1 cycle of 95 °C/10min, 40 cycles of 95 °C/15s, 60 °C/1min, followed by dissociation curve analysis (60 °C-95 °C) to verify the amplification of a single product. The threshold cycle (CT) value was determined by using the manual setting on the 7500 Real-Time PCR System and exported into a Microsoft Excel Sheet for subsequent data analysis where the relative expression ratio of target gene of treated group versus that of control group were calculated by $2^{-\Delta\Delta CT}$ method [40].

2.5. Immunoblotting

EPC cells in 6-well plate (2×10^6) or MPK cells in 60 mm dish (1×10^6) were transfected with pcDNA5/FRT/TO-HA-bcIRF7 or the empty vector separately. The transfected cells were harvested at 48 h post transfection and the whole cell lysates were used for immunoblot assay as previously [21]. In brief, the proteins were isolated by 10% SDS-PAGE and the transferred membrane was probed with mouse-anti-HA monoclonal antibody (1:4000; Sigma), which was followed by the incubation with goat-anti-mouse IgG (1:30000; Sigma). The target protein was visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma).

2.6. Immunofluorescence microscopy

MPK cells in 24-well plate were transfected with bcIRF7-pEGFP-N1 and infected with SVCV or GCRV (MOI = 10) at 36 h post transfection separately. The cells were fixed with 4% (v/v) paraformaldehyde at 8 h post infection. EPC cells in 24-well plate were transfected with pcDNA5/FRT/TO-Flag-bcIKKε or pcDNA5/FRT/TO-Flag-bcIKKε-K39A separately and the transfected cells were fixed at 24 h post transfection. The fixed cells were permeabilized with Triton X-100 (0.2% in PBS) and applied to immune-fluorescent staining as previously described [23]. Mouse-anti-Flag antibody (Sigma) was probed at the ratio of 1:300 and Alexa 488-conjugated secondary antibody (Invitrogen) was probed at the ratio of 1:1000; and DAPI (4, 6-diamidino-2-phenylindole) were used for the nucleus staining.

2.7. Luciferase reporter assay

EPC cells in 24-well plate (3×10^5) were co-transfected with expression plasmids as required, pRL-TK and zIFN1-Luci. For each transfection, the total amount of DNA was balanced with the empty vector. The cells were harvested and lysed at 24 h post transfection. The centrifuged supernatant was used to measure the activities of firefly luciferase and renilla luciferase according to the instruction of the manufacturer (Promega) as previously [22].

2.8. Statistics analysis

For the statistics analysis of the data of q-PCR, luciferase reporter assay and viral titer measurement, all data were obtained from three independent experiments with each performed in triplicate. Error bar represents the standard error of the mean (+SEM) of three independent experiments. Asterisk (*) stands for $p < 0.05$. The data were analyzed by two-tailed Student's t-test.

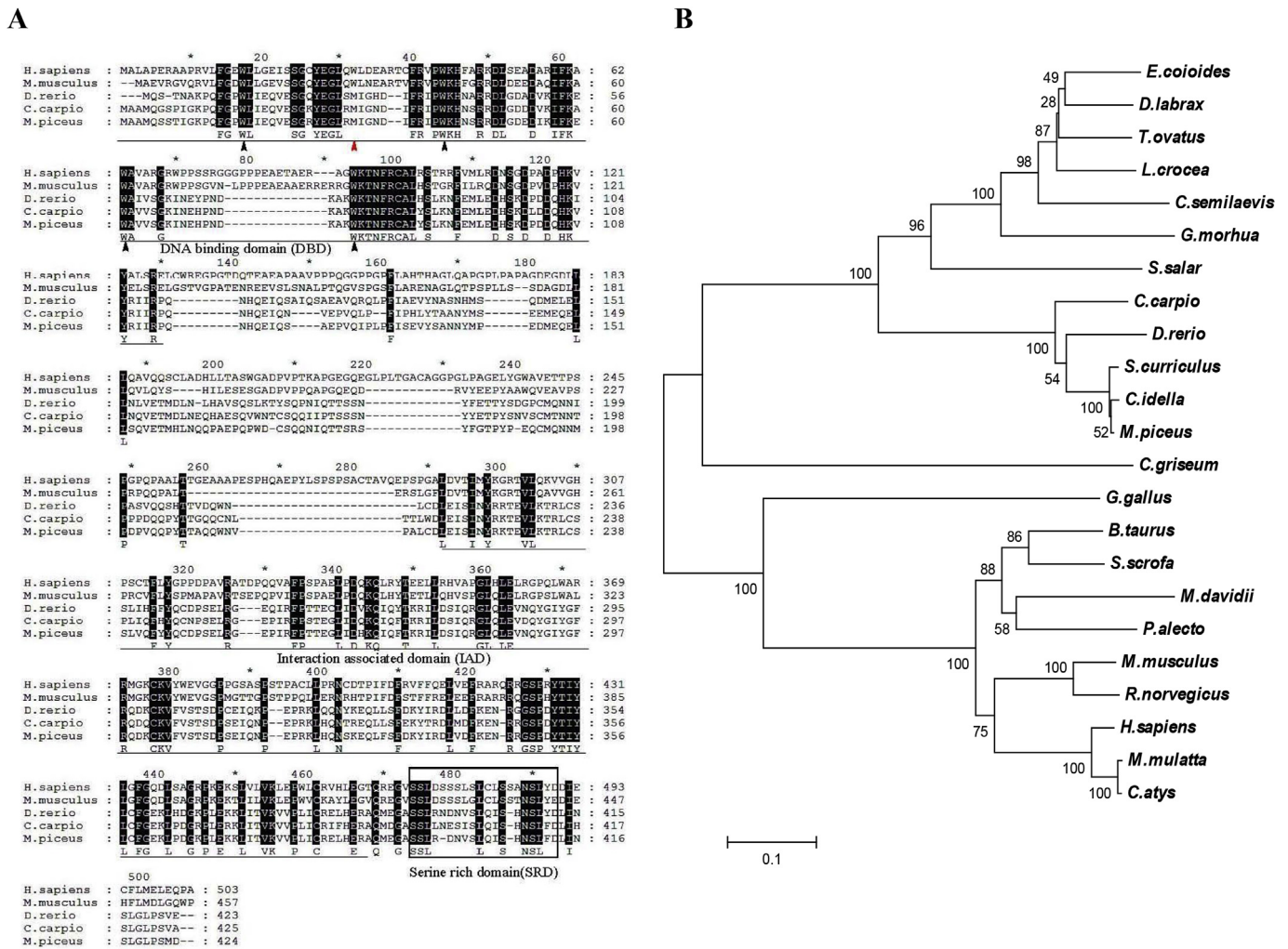


Fig. 1. Evolution study of bcIRF7.

(A): Comparisons of bcIRF7 with other vertebrate IRF7 by using MEGA 6.0 program and GeneDoc program. The conserved four tryptophan residues are marked with black triangle and the red triangle indicates the different conservative tryptophan site between fishes and mammals.

(B): Phylogenetic tree of vertebrate IRF7. The amino acid sequence of bcIRF7 was aligned with IRF7 from different species by using MEGA 6.0 software, which included (GenBank accession number): *M. mulattan* (NP_001129572.1), *H. sapiens* (AAB17190.1), *M. musculus* (NP_058546.1), *R. norvegicus* (NP_00102886 3.1), *M davidii* (AOQ26356.1), *B. Taurus* (NP_001098510.1), *S. scrofa* (NP_001090897.1), *G. gallus* (AJS11515.1), *S. salar* (NP_001130020.1), *E coioides* (ADA57613.1), *C. carpio* (AD255457.1), *D. rerio* (AAH58298.1), *S. curriculus* (AMP81961.1), *C. idella* (ACS34986.1), *M. piceus* (MG210943). The bar stands for the scale length and the numbers on different nodes stand for bootstrap value.

3. Results

3.1. Molecular cloning and sequence analysis of bcIRF7

To study the role of bcIRF7 in the innate immunity of black carp, bcIRF7 was cloned from the spleen of black carp. The full-length cDNA of bcIRF7 consists of 1867 nucleotides containing a 5-UTR of 44 bp, a 3-UTR of 548 bp and an open reading frame (ORF) of 1275 bp (NCBI accession number: MG210943). The predicted bcIRF7 protein contains 425 amino acids, including a DNA binding domain (DBD), an Interaction associated domain (IAD) and a Serine rich domain (SRD). In the DBD of bcIRF7, four tryptophan residues are found in the tryptophan cluster, which is unique among all fish IRF7 members (Fig. 1A and Supplementary Fig. 1). The calculated molecular weight of bcIRF7 is 49.3kDa and its theoretical isoelectric point is 5.68 (Calculated by EXPASy Compute PI/Mw).

To gain insight into bcIRF7 evolution, amino acid (aa) sequence of bcIRF7 was subjected to multiple alignments with those of IRF7 proteins from different species, including mammals and birds. The comparison of amino acid sequences of IRF7 proteins of these species

showed that bcIRF7 shared high amino acid sequence identity with grass carp (98.6%) and Barble chub (98.4%), which correlated with the close genetic relationship among these cyprinoid fishes (Table 1). Phylogenetic analysis of IRF7 of the above selected species demonstrated that these homologue proteins could be divided into two groups, consisting of human/mammalian/bird and piscine branches, in which bcIRF7 was clustered together with other Cyprinidae IRF7 including grass carp and Barble chub (Fig. 1B).

3.2. Tissue-specific expression of bcIRF7

To investigate the mRNA level of bcIRF7 *in vivo*, total RNA was isolated from intestine, heart, liver, spleen, kidney or gill of the black carp separately, which was injected with GCRV, SVCV or PBS. bcIRF7 transcription was detected in all the selected tissues and bcIRF7 mRNA level in heart and gill was obviously higher than those of other tissues. In GCRV and SVCV injected group, bcIRF7 mRNA level in kidney, intestine and liver increased significantly post virus injection. Additionally, in SVCV injected group, bcIRF7 mRNA level in spleen increased after viral infection that is different from the GCRV injected

Table 1
Comparison of bcIRF7 with other vertebrate IRF7 (%).

Species	Full-length sequence	
	Similarity	Identity
<i>Mylopharyngodon piceus</i>	100	100
<i>Bos. Taurus</i>	32.8	47.5
<i>Macaca. mulatta</i>	30.3	44.1
<i>Sus. scrofa</i>	31.0	45.8
<i>Homo. sapiens</i>	30.5	43.8
<i>rattus norvegicus</i>	32.8	47.6
<i>Myotis davidii</i>	29.3	42.2
<i>Gallus. gallus</i>	33.7	45.7
<i>Cercocebus atys</i>	43.8	30.4
<i>Pteropus alecto</i>	43.3	31.1
<i>Mus musculus</i>	48.1	33.1
<i>Dicentrarchus labrax</i>	64.5	49.9
<i>Chiloscyllium griseum</i>	47.1	32.6
<i>Cynoglossus semilaevis</i>	61.4	49.1
<i>Trachinotus ovatus</i>	62.9	50.2
<i>Gadus morhua</i>	63.5	49.3
<i>Larimichthys crocea</i>	63.3	50.3
<i>Salmo salar</i>	48.7	61.1
<i>Epinephelus coioides</i>	50.2	63.4
<i>Danio rerio</i>	80.1	86.1
<i>Cyprinus carpio</i>	82.7	90.2
<i>Squaliobarbus curriculus</i>	97.2	98.4
<i>Ctenopharyngodon idella</i>	97.9	98.6

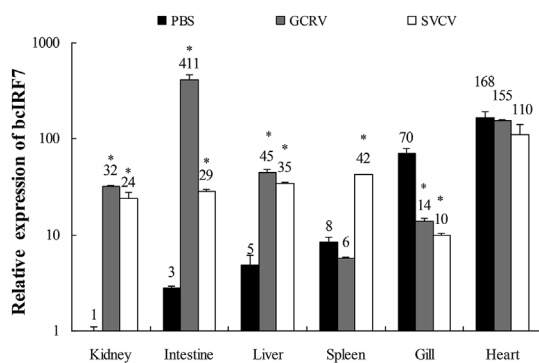


Fig. 2. Tissue-specific expression of bcIRF7.

Black carp of six months (weight of 120 g) were injected intraperitoneally with GCRV (2.52×10^6 pfu/fish), SVCV (2.43×10^6 pfu/fish) or sterile PBS (as healthy control) separately and cultured at 25 °C. Three fish were collected for each injected group. The injected black carp were sacrificed at 33 h post injection and total RNA was isolated from the selected tissues independently. Total RNA samples of each tissue were combined in each group and used for cDNA synthesis. Relative mRNA level of bcIRF7 after virus challenge or in healthy condition was detected by q-PCR separately. The numbers above the error bars stand for the average bcIRF7 mRNA level, Error bars represent the standard error of the mean (\pm SEM) of three independent experiments. Asterisks (*) indicates a significant difference between the experimental data and the control data ($p < 0.05$).

group. However, bcIRF7 transcription in gill obviously decreased after both GCRV and SVCV infection (Fig. 2). The data suggested that bcIRF7 was involved into host innate immune response initiated by GCRV and SVCV.

3.3. bcIRF7 expression in response to different stimuli

To learn bcIRF7 mRNA profile during host innate immune activation, MPK cells were subjected to different stimuli and applied to qPCR analysis. In LPS treated MPK cells, bcIRF7 mRNA level increased post treatment and reached the peak at 24 h for all doses except an extreme increase at 2 h point of 10ug/ml treatment (Fig. 3A). In the poly (I:C) treated MPK cells, the transcription of bcIRF7 gene increased right after

stimulation (2 h). It was interesting that bcIRF7mRNA level at 48 h post treatment was obviously lower than that of control (0h). It was speculated that a negative feedback regulation was caused when the cells stimulated with high concentration of poly (I:C) for long time (Fig. 3B). In SVCV infected MPK cells, bcIRF7 mRNA level of all groups increased post infection and reached the peak at 48 h post infection (hpi) except a fierce increase at 24hpi of 1MOI group (Fig. 3C). In the GCRV infected MPK cells, bcIRF7 mRNA level increased right after viral infection and reached the peak at 48 hpi except a fierce increase at 12hpi in 1MOI group (Fig. 3D). These data indicated that bcIRF7 had been recruited into host innate immune response initiated by virus and bacteria.

3.4. bcIRF7-mediated antiviral signaling

A specific band of ~56 KDa was detected in the whole cell lysate of EPC cells and MPK cells transfected with plasmids expressing bcIRF7 (Fig. 4A). To further examine the subcellular distribution of bcIRF7, MPK cells were transfected with bcIRF7-pEGFP-N1 and the subcellular location of bcIRF7 was examined by immunofluorescence staining (IF) assay. The IF data showed clearly that bcIRF-7 was localized predominantly in the cytoplasm, and it translocated to the nucleus (30%–50% of the cells expressing bcIRF7) within 8 h post viral infection (Fig. 4B). To determine the IFN-inducing activity of bcIRF7, EPC cells were transfected with pcDNA5/FRT/TO-HA-bcIRF7 and used for dual luciferase reporter assay. For the induced promoter activity of zebrafish IFN1, the zIFN1 fold induction increased as the bcIRF7 input increased and the highest value was 416.7 times that of control (Fig. 4C). These data demonstrated clearly that bcIRF7 possessed the ability to induce the production of IFN.

To investigate the antiviral activity of bcIRF7 during host innate immune response, EPC cells were transfected with pcDNA5/FRT/TO-HA-bcIRF7 and infected with virus infection at 24 h post transfection. In GCRV infected group, both the CPE ratio and the viral titer in the supernatant media of the EPC cells expression bcIRF7 were obviously lower than those of empty vector transfected cells (Fig. 5A&B). The viral titer in the media of bcIRF7-expressing cells showed the biggest reduction (> 1000 times) when the cells were infected with GCRV at the dose of 0.01 MOI. The data of SVCV infected group was similar to that of GCRV group, both CPE ratio and viral titer in the media of the EPC cells expressing bcIRF7 obviously lower than those of control cells except 1MOI group. It could be explained by that the antiviral ability of bcIRF7 might be covered by the high dose SVCV infection (Fig. 5C and D). Our data showed clearly that exogenous bcIRF7 enhanced the antiviral ability of EPC cells against both GCRV and SVCV, which demonstrated that this fish IRF7 orthologue functioned importantly during host antiviral innate immune response.

3.5. The antiviral activity of bcIKKε and bcIKKε-K39A

In the previous study, bcIKKε was cloned and characterized. To further explore the role of bcIKKε in host IFN signaling, the kinase dead mutant K39A (short for bcIKKε-K39A) was generated, which was used as the kinase inactive form of bcIKKε just like its human counterpart IKKε-K38A (Fig. 6 A). In the immunoblot assay of EPC cells, K39A showed the identical migration size and similar expression level to those of bcIKKε (Fig. 6B). In the immunofluorescence staining (IF) assay, K39A mutant was proved to be a cytosolic protein just like the wild type bcIKKε (Fig. 6C). The reporter assay demonstrated that bcIKKε slightly activated the promoter activity of zebrafish IFN1; however, K39A inhibited the activity of zebrafish IFN1 promoter in a dose dependent manner (Fig. 6D).

To explore the antiviral ability of bcIKKε and K39A, EPC cells were transfected with pcDNA5/FRT/TO-Flag-bcIKKε or pcDNA5/FRT/TO-Flag-bcIKKε-K39A and infected with GCRV (Fig. 7A&B) or SVCV (Fig. 7C&D) at 24 h post transfection. In both groups, both CPE ratio

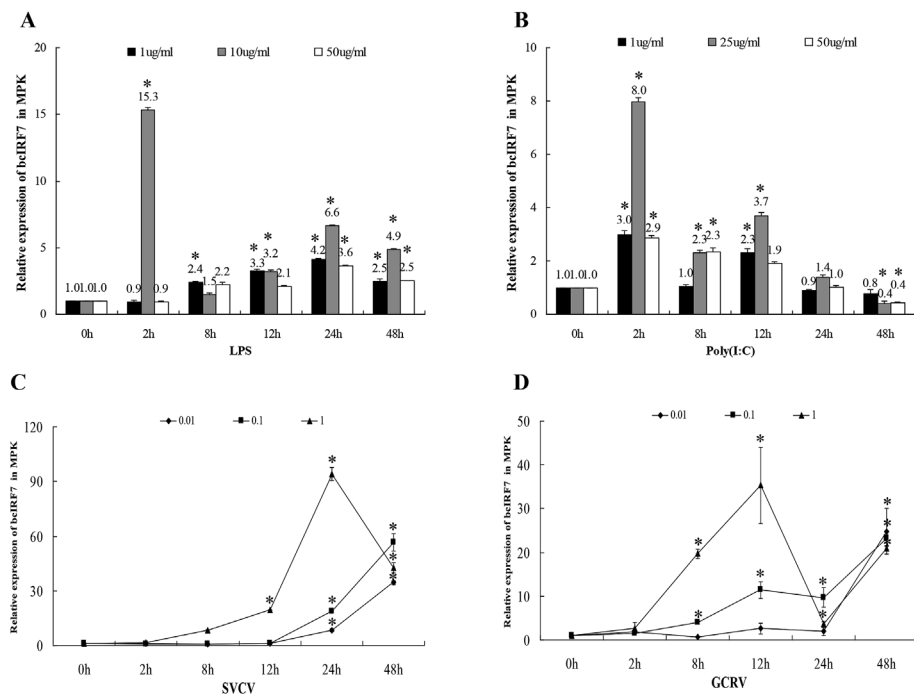


Fig. 3. Expression of bcIRF7 in response to different stimuli.

MPK cells were seeded in 6-well plate (2×10^6 cells/well) at 16 h before stimulation. The cells were treated with poly (I:C) or LPS at the indicated dose (A&B) or infected with GCRV or SVCV at indicated MOI separately (C&D) and harvested for qPCR independently at the indicated time points post stimulation. The number above the error bar represents average bcIRF7 mRNA level. Error bars represent the standard error of the mean (\pm SEM) of three independent experiments. Asterisks (*) indicates a significant difference between experimental data and control data ($p < 0.05$).

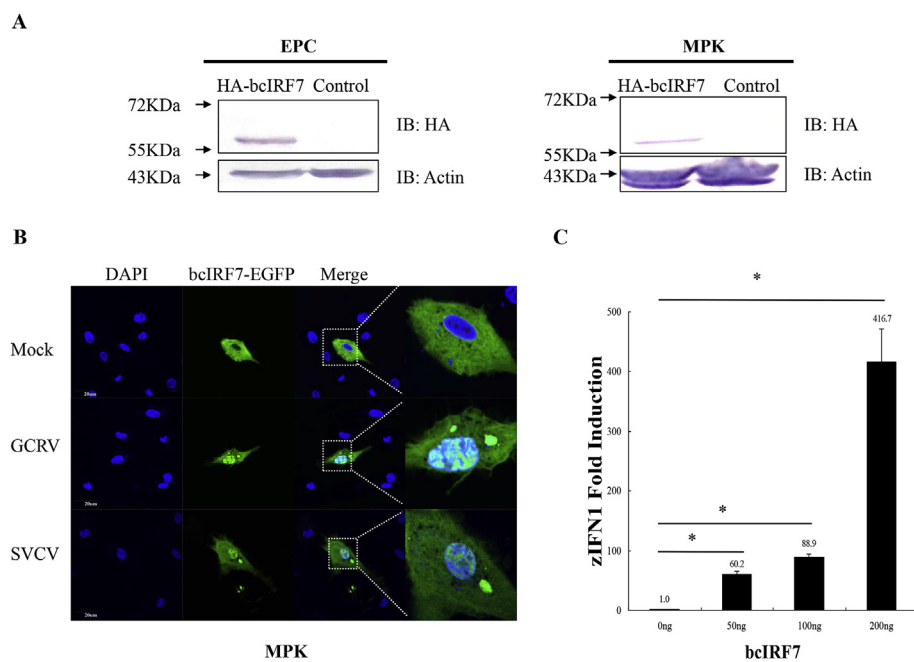


Fig. 4. Protein expression, subcellular distribution and IFN-inducing ability of bcIRF7.

(A): Immunoblot assay of bcIRF7 in EPC and MPK cells. Control: cells transfected with empty vector, HA-bcIRF7: pcDNA5/FRT/TO-HA-bcIRF7, IB: immunoblot. (B): Immunofluorescence staining of bcIRF7 in MPK cells. bcIRF7-EGFP: bcIRF7-pEGFP-N1; the bar stands for the scale of 20μm. (C): zebrafish IFN1 promoter activity (zIFN1) induced by bcIRF7. bcIRF7: pcDNA5/FRT/TO-HA-bcIRF7. The numbers above the error bars stand for the average IFN fold induction. Error bar represents the standard error of the mean (\pm SEM) of three independent experiments. Asterisks (*) indicates a significant difference between the experimental data and the control data ($p < 0.05$).

and viral titer in the supernatant media of the EPC cells expressing bcIKKε but not K39A were slightly lower than those of empty vector transfected cells except that in 1MOI-infection group, which might be explained by that the “gentle” antiviral ability of bcIKKε was covered by GCRV/SVCV infection with high dose.

3.6. bcIKKε but not bcIKKε-K39A upregulated bcIRF7-mediated IFN production

In mammals, the RLRs recognize the viral components and transduce the signaling downstream to IKKε after viral invasion [24]. IKKε phosphorylates IRF3/7 together with TBK1 and the activated IRF3/7 translocate into nuclear to trigger the transcription of IFNs, which finally initiate the host antiviral innate immune response [25]. To

explore the relationship between bcIRF7 and bcIKKε, EPC cells were co-transfected with pcDNA5/FRT/TO-HA-bcIRF7 and pcDNA5/FRT/TO-Flag-bcIKKε (pcDNA5/FRT/TO-Flag-bcIKKε-K39A); then used for reporter assay. When co-expressed with bcIKKε, bcIRF7-induced zebrafish IFN1 transcription were obviously increased, which was in a dose dependent manner (50 ng, 100 ng, 200 ng) of the bcIKKε input. On the contrary, K39A did not enhance but suppressed the bcIRF7-induced zebrafish IFN1 production (Fig. 8). These data demonstrated that bcIKKε functioned positively in bcIRF7-mediated signaling, which was related with its kinase activity. As to the decreased zebrafish IFN1 production in EPC cells co-transfected with pcDNA5/FRT/TO-HA-bcIRF7 and pcDNA5/FRT/TO-Flag-bcIKKε-K39A, it was speculated that over-expressed K39A in EPC cells competitively inhibited the endogenous IKKε.

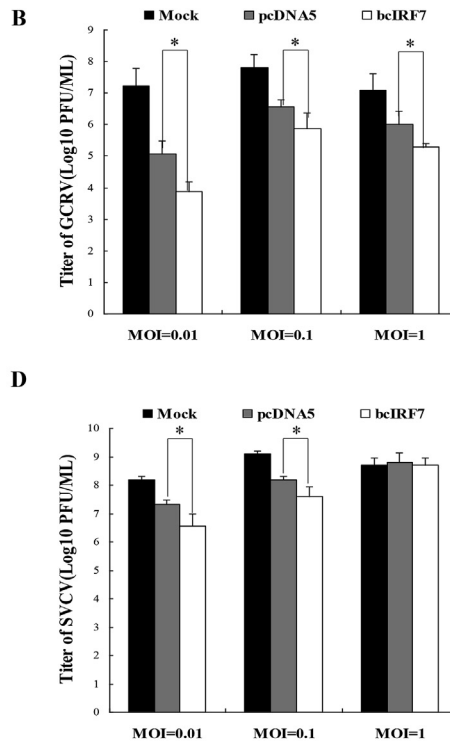
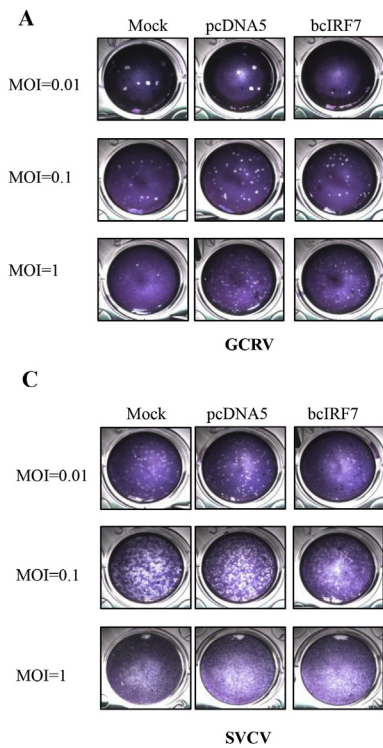


Fig. 5. EPC cells expressing bcIRF7 represented enhanced antiviral ability.

EPC cells in 24-well plate were transfected with 500 ng of bcIRF7 or the empty vector separately and infected with GCRV or SVCV at the indicated MOI at 24 h post transfection. The cell monolayers were stained with crystal violet (A&C) and the virus titers in the supernatant media were determined by plaque assay at 24hpi (B&D). Mock: EPC cells without transfection; pcDNA5: pcDNA5/FRT/TO-HA; bcIRF7: pcDNA5/FRT/TO-HA-bcIRF7. Error bar represents the standard error of the mean (\pm SEM) of three independent experiments. Asterisks (*) indicates a significant difference between experimental data and control data ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.7. bcIKK ϵ but not bcIKK ϵ -K39A upregulated bcIRF7-mediated antiviral activity

IFNs are crucial to the antiviral state of the cells, which are induced after virus invasion. To further explore the function of bcIKK ϵ in bcIRF7-mediated antiviral activity in host cells, EPC cells were transfected with pcDNA5/FRT/TO-HA-bcIRF7 or co-transfected with pcDNA5/FRT/TO-HA-bcIRF7 and pcDNA5/FRT/TO-Flag-bcIKK ϵ (or

pcDNA5/FRT/TO-Flag-K39A); then infected with GCRV or SVCV at 24 h post transfection. Crystal violet staining (Fig. 9 A&C) and plaque assay (Fig. 9 B&D) demonstrated that both CPE rate and viral titer in the supernatant media of the EPC cells co-transfected with pcDNA5/FRT/TO-HA-bcIRF7 and pcDNA5/FRT/TO-Flag-bcIKK ϵ were obviously lower than the cells expressing bcIRF7 alone. However, EPC cells co-expressing bcIRF7 and K39A showed no-enhanced antiviral activity than the cells expressing bcIRF7, which was correlated with the

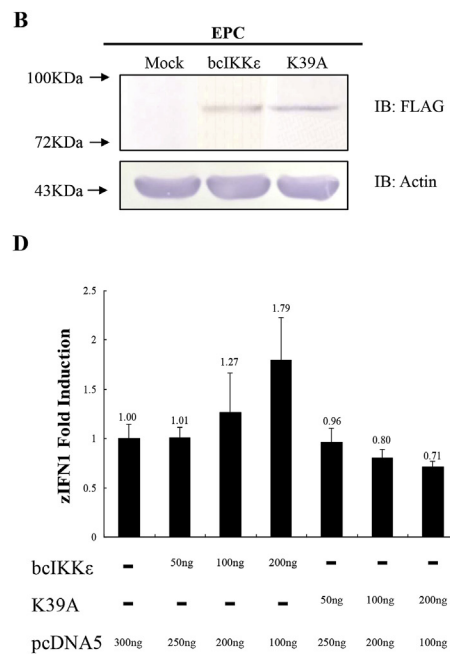
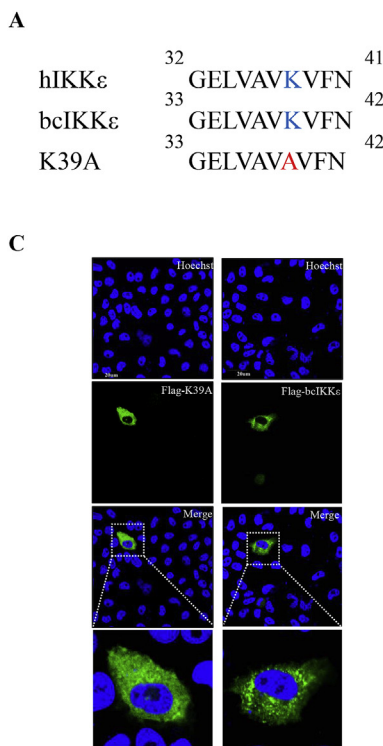


Fig. 6. Protein expression, subcellular distribution and IFN-inducing ability of bcIKK ϵ and K39A.

(A): Amino acid sequence of human IKK ϵ (hIKK ϵ) (32-41aa), bcIKK ϵ (33-42aa) and K39A (32-42aa). (B): Immunoblot assay of bcIKK ϵ and K39A in EPC cells. Mock: cells transfected with the empty vector; bcIKK ϵ :pcDNA5/FRT/TO-Flag-bcIKK ϵ K39A: pcDNA5/FRT/TO-Flag-bcIKK ϵ -K39A; IB: immunoblot. (C): Immunofluorescence staining of bcIKK ϵ and K39A in EPC cells. Flag-bcIKK ϵ : pcDNA5/FRT/TO-Flag-bcIKK ϵ ; Flag-K39A:pcDNA5/FRT/TO-Flag-bcIKK ϵ -K39A; the bar stands for the scale of 20 μ m. (D): zebrafish IFN1 promoter activity (zIFN1) induced by bcIKK ϵ and K39A. pcDNA5: pcDNA5/FRT/TO-Flag bcIKK ϵ ; pcDNA5/FRT/TO-Flag-bcIKK ϵ K39A: pcDNA5/FRT/TO-Flag-bcIKK ϵ -K39A. The number above the error bar stands for the average IFN fold induction.

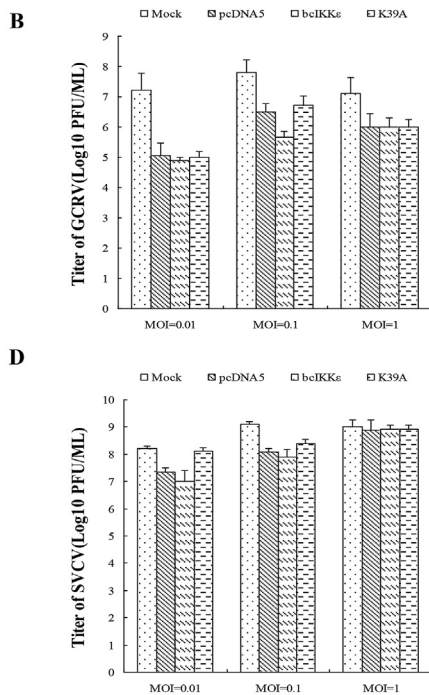
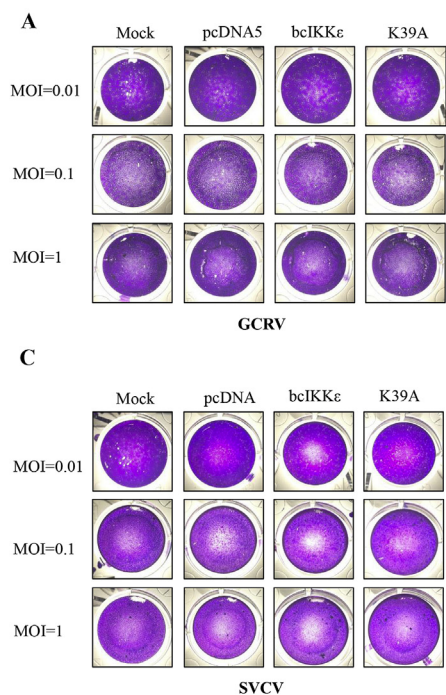


Fig. 7. Antiviral ability of bcIKKε and bcIKKε-K39A against SVCV and GCRV
 cells in 24 well-plate were transfected with 250 ng of bcIKKε, K39A or the empty vector separately and infected with GCRV or SVCV at the indicated MOI at 24 h post transfection. The cell monolayers were stained with crystal violet (A&C) and the virus titers in the supernatant media were determined by plaque assay at 24 hpi (B&D) Mock: EPC cells without transfection; pcDNA5: pcDNA5/FRT/TO-Flag; bcIKKε: pcDNA5/FRT/TO-Flag-bcIKKε; K39A: pcDNA5/FRT/TO-Flag-bcIKKε-K39A. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reporter assay result. Thus, our data demonstrated clearly that the antiviral ability of EPC cells mediated by bcIRF7 was upregulated by bcIKKε but not its kinase dead mutant, K39A.

4. Discussion

In the present study, the full-length cDNA of IRF7 homologue has been cloned from black carp and the predicted bcIRF7 possesses three conserved functional domains like other fish and mammalian IRF7, including an N-terminal DBD domain, a C-terminal IAD domain and a C-terminal SRD (Fig. 1). Mammalian DBD is characterized by a cluster of five well-spaced tryptophan residues and forms a helix-turn-helix motif that binds to the interferon stimulating response element (ISRE) and IRF regulatory element (IRF-E) consensus in target promoters [26]. Studies revealed that three of the five tryptophan residues 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 [27]. Different to their IRF counterparts, bcIRF7 and other fish IRF7s lack the second residue of the tryptophan cluster [28,29]. The IAD is responsible for the formation of IRF homo/hetero dimer and the association with other transcription factors [30]. The SRD is important for virus-induced phosphorylation and the following dimerization and exhibits a higher conservation in IRF-7 in mammal [31].

Phylogenetic tree analysis indicated that bcIRF7 was well clustered with fish IRF7 and was closely related to that of grass carp and Barble chub (Table 1). The previous study have demonstrated that grass carp IRF7 and Barble chub IRF7 acted as positive regulators on the transcription of IFN and were involved in host antiviral reaction [30,32]. In addition to these two, IRF7 genes were cloned from other teleost species and their expression were analyzed. In the study of orange-spotted grouper (*Epinephelus coioides*), the transcription of IRF7 was mainly detected in spleen, kidney, intestine and skin, and was differentially up-regulated after the infection with bacteria and virus [28]. Japanese

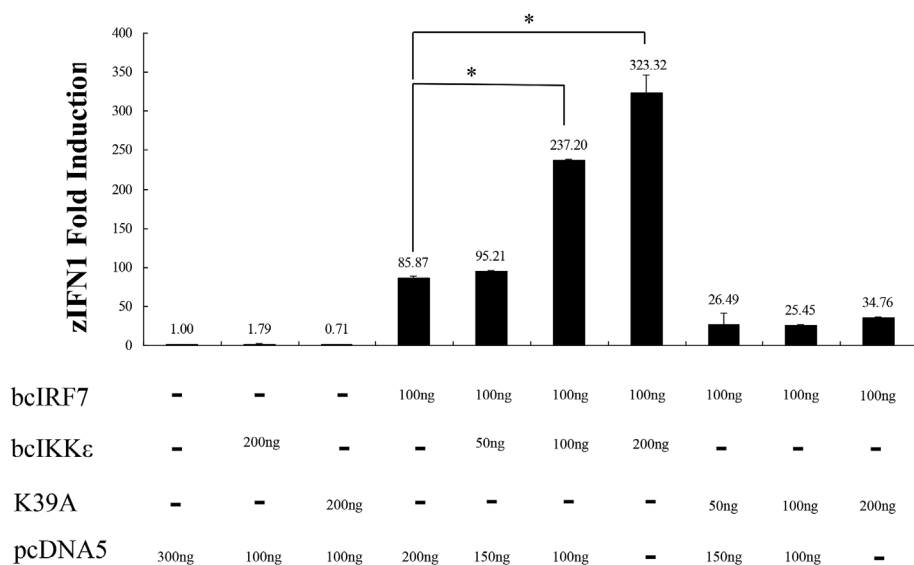


Fig. 8. IFN-inducing activity of bcIRF7 was up-regulated by bcIKKε.
 EPC cells in 24-well plate were co-transfected with bcIRF7 and bcIKKε (K39A) and applied to reporter assay. For each transfection, the total amount of DNA was balanced with the empty vector. pcDNA5: pcDNA5/FRT/TO-Flag; bcIRF7: pcDNA5/FRT/TO-HA-bcIRF7; bcIKKε: pcDNA5/FRT/TO-Flag-bcIKKε, K39A: pcDNA5/FRT/TO-Flag-bcIKKε-K39A; The number above the error bars stands for the average IFN fold induction. Error bar represents the standard error of the mean (± SEM) of three independent experiments. Asterisks (*) indicates a significant difference between experimental data and control data (p < 0.05).

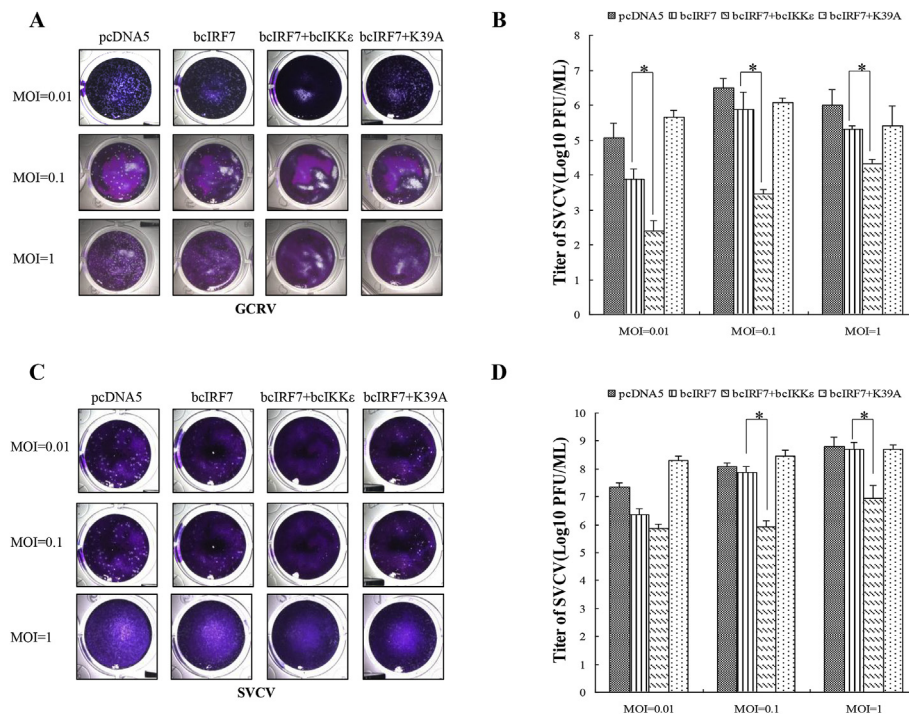


Fig. 9. Up-regulated antiviral ability of bcIRF7 against GCRV and SVCV by bcIKKε. EPC cells in 24-well plate were transfected with 250 ng bcIRF7 alone or co-transfected with 250 ng bcIKKε (K39A); then infected with GCRV or SVCV at 24 h post transfection. The cell monolayers were stained with crystal violet (A&C) and the virus titers in the supernatant media were determined by plaque assay at 24 hpi (B&D). pcDNA5: pcDNA5/FRT/TO; bcIRF7: pcDNA5/FRT/TO-HA-bcIRF7; bcIKKε: pcDNA5/FRT/TO-Flag-bcIKKε; K39A: pcDNA5/FRT/TO-Flag- bcIKKε-K39A. Error bars represent the standard error of the mean (\pm SEM) of three independent experiments. Asterisks (*) indicates a significant difference between experimental data and control data ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

flounder (*Paralichthys olivaceus*) IRF7 was constitutively detected in tested tissues of healthy individuals and the increased mRNA level of IRF7 corresponded to the up-regulated expression of antiviral protein Mx, after lymphocystis disease virus infection [33]. However, the mechanism behind the activation and regulation of teleost IRF7 needs further exploration.

The canonical IKKs, including IKK α and IKK β , form a complex with the adaptor protein NEMO that is required for NF- κ B activation [34,38]. IKK α and IKK β contain an N-terminal kinase domain, a central leucine-zipper and helix-loop-helix domain, and C-terminal NEMO-binding domain (NBD). The non-canonical IKKs, TBK1 and IKK ϵ , share a domain structure similar to IKK α and IKK β . However, TBK1 and IKK ϵ do not have NBD, and presumably do not associate with NEMO [35]. It is known that IKK ϵ is required for antiviral immune response and it was reported that loss of IKK ϵ reduced the induction of a subset of ISGs, including ISG54 (or IFIT2), ADAR1, IFIT3 and Mx1 [36,39]. In mammal, IKK ϵ and TBK1 form a complex to phosphorylate IRF3 and IRF7 in response to viral invasion [18]. The function of IKK ϵ orthologue in teleost fish remains largely unknown, and the relationship between IKK ϵ and IRF7 during fish innate immune activation needs comprehensive exploration.

In this paper, the data of both reporter assay and plaque assay demonstrated that bcIKK ϵ remarkably improved bcIRF7-mediated IFN signaling when co-expressing with bcIRF7 in EPC cells, although this fish IKK member showed weak IFN-inducing activity when expressing alone (Figs. 6 and 7). It was speculated that bcIKK ϵ could phosphorylate bcIRF7 and activate the IRF7-mediated antiviral signaling. These data suggest that bcIKK ϵ is an activator of IRF7 during host innate immune response initiated by viral invasion, which is similar to its mammalian counterpart [37]. In our previous study, bcTBK1, the other non-canonical IKK, showed much stronger IFN-inducing activity than bcIKK ϵ did [21]. It is speculated that bcTBK1 is another important activator of IRF7. However, the mechanism behind the activation of bcIRF7 during host innate immune response still remains largely unknown. bcIKK ϵ -K39A, the kinase-inactive mutant of bcIKK ϵ , did not enhance the bcIRF7-mediated IFN signaling like wild type bcIKK ϵ did (Figs. 8 and 9), which implied that the kinase activity of this fish IKK was crucial for its positive regulation on bcIRF7-mediated IFN signaling.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.fsi.2018.04.012>.

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