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Molecular characterization and expression analysis of B-cell lymphoma-2 protein in *Amphiprion clarkii* and its role in virus infections

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ABSTRACT

Amphiprion clarkii is increasingly being used as a captive-bred ornamental fish in South Korea. However, its breeding has recently been greatly hindered by destructive diseases due to pathogens. B-cell lymphoma-2 (*Bcl2*), a mitochondrial apoptosis regulatory gene involved in immune responses, has not been investigated in anemonefish, including *A. clarkii*. Herein, we aimed to annotate *Bcl2* in the *A. clarkii* transcriptome and examined its role against virus infections. Sequence analysis indicated that Bcl2 in *A. clarkii* (AcBcl2) contained all four Bcl-2 homology domains. The structure of AcBcl2 closely resembled those of previously analyzed anti-apoptotic Bcl2 proteins in mammals. Expression analysis showed that the highest level of *AcBcl2* was expressed in blood. *AcBcl2* expression in the blood was downregulated within 24 hpi when challenged with immune stimulants poly I:C and lipopolysaccharides. AcBcl2 reduced poly I:C-induced cell death. The propagation of viral hemorrhagic septicemia virus (VHSV) was higher in the presence of AcBcl2. Cell mortality was higher in AcBcl2 when transfected cells were infected with VHSV, and a higher viral transcript was observed compared to their respective controls. In conclusion, AcBcl2 is an anti-apoptotic protein, and its activity may facilitate the propagation of VHSV.

1. Introduction

B-cell lymphoma (Bcl2) family proteins regulate apoptosis during infections and respond to cellular stresses or development [1]. Bcl2 is also involved in non-apoptotic functions, including innate and adaptive immune responses such as lymphocyte activation, homeostasis, cell cycle regulation, cell migration, metabolic modulation, insulin secretion, and embryonic development. Bcl2 family members can be subdivided into three categories based on their protein structures and homology motifs, namely, multi-domain anti-apoptotic proteins (Bcl2 and Bcl-XL), multi-domain pro-apoptotic effector proteins (BAK, BAX, BOK, and BH1-3), and pro-apoptotic BH3 only proteins [2].

Bcl2, a proto-oncogene, is an anti-apoptotic protein in the Bcl2 family that acts as a central gatekeeper in the mitochondrial apoptosis pathway. Bcl2 is predominantly localized to the mitochondria. When overexpressed, Bcl2 inhibits mitochondrial outer membrane permeabilization by inhibiting downstream pro-apoptotic effectors. In addition, Bcl2 inhibits Beclin 1- dependent autophagy. BH domains are important in the apoptotic regulatory role of Bcl2 proteins. In particular, the BH3 domain is important for inhibiting apoptotic factors such as pore-forming factors or BH3-containing pro-apoptotic proteins on the mitochondrial membrane [3].

To date, several studies have been conducted on Bcl2 in vertebrates and invertebrates because of their crucial roles in modulating the process of apoptosis and immunity [4]. Teleost Bcl2 expression has been established to be induced by poly I:C or lipopolysaccharide (LPS) in various species such as Atlantic cod (*Gadus morhua*) [5], grass carp (*Ctenopharyngodon idella*) [6], orange-spotted grouper (*Epinephelus coioides*) [7], pufferfish (*Takifugu obscurus*) [8], and channel catfish (*Ictalurus punctatus*) [9].

The yellowtail clownfish *Amphiprion clarkii*, which belongs to the family Pomacanthidae, is a dominant and high-value anemonefish in the ornamental fish aquarium trade due to its rare color morphs and adaptability. These subtropical fish species inhabit the Pacific and

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Indian oceans as well as the coasts of Japan [10]. Owing to its economic importance as well as the characteristics that differ from other model species such as the ability to change sex and their symbiotic relationship with the sea anemone, fundamental research regarding their genes and genomes is considered to be vital [11]. Despite the recent rise in popularity of *A. clarkii* farming, the spread of destructive diseases has threatened *A. clarkii* culture, causing enormous economic losses in the aquaculture industry. Therefore, studying the functions of immune regulatory proteins in *A. clarkii* is vital to expanding the aquaculture industry.

To the best of our knowledge, no studies have identified the contribution of Bcl2 in *A. clarkii* against RNA virus infections. To this end, in this study, we examined the structure, expression, and role of *AcBcl2* in *A. clarkii*. Furthermore, we investigated the anti-apoptotic nature of AcBcl2 in the presence of poly I:C and viral hemorrhagic septicemia virus (VHSV) to elucidate the function of AcBcl2 during RNA virus infections in anemonefish. The results of this study may contribute towards the understanding of the innate immunity of anemonefish and aid in developing specific and efficient drugs and vaccines against pathogens in fish.

2. Materials and methods

2.1. Identification and in silico analysis of A. clarkii Bcl2 (AcBcl2)

The *AcBcl2* cDNA sequence was retrieved from a previously established transcriptomic database for *A. clarkii* [12] and the retrieved sequence was verified using the NCBI Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence-specific primers were designed based on the retrieved *AcBcl2* cDNA and cloned into a pcDNA3.1+ vector (Table 1). The open reading frame and deduced amino acid sequences were obtained using the ORF finder tool (https://www.ncbi.nlm.nih.gov/orffinder/). The amino acid sequence was translated from the nucleotide sequence using the Expert Protein Analysis System (ExPASy) translation tool (https://web.expasy.org/translate/). The predicted protein size and theoretical isoelectric point were analyzed using the ProtParam tool (https://web.expasy.org/protparam/).

Protein homologs and sequence similarities were identified and analyzed using the BLAST search program at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment was performed using the Clustal X2.0 program (http://www.clustal.org), and a phylogenetic tree was constructed with the neighbor-joining algorithm using MEGA-X software with 1000 bootstrap trials. 3D structures were predicted using the SWISS-MODEL online software (https://swissmodel.expasy.org/).

Table 1
Primers used for this study.

Primer No.	Purpose	Sequence (5′ to 3′)	Restriction sites
1	AcBcl2 qPCR forward	GCCAACTCAGCTTCCAGTAA	NA
2	AcBcl2 qPCR reverse	CCTGCGTGAAGAGAAGTTCA	
3	AcBcl2 cloning into pcDNA3.1 (+) forward	GAGAGAggtacc ATGgCGTACAGTAACAGAGAGCTGG	*Kpn*I
4	AcBcl2 cloning into pcDNA3.1 (+) Reverse	GAGAGActcgag TCACTGTTTCTTAGCAATGAGCACAC	*Xho*I
5	VHSV-GP	TACAACATCACCCTGCCCAACC	NA
6	qPCR	GACCACCCTGTGATCATGTGTCC	
7	Interferon	TGTTCGTTCCCTGGCTGTACCAAA	
8	regulatory factor-3 (irf3 gene) GenBank: HE856621.1,	TGGTCTGAGCCCATGCCTTGAATA	
9	FHM ubquitin-	AATGGAGATGTGAAGCGGCTGGA	
10	like protein 1 (ISG15), GenBank: KM099174,	AACCCGTAACTGCTGAGGCTTCT	
11	FHM viperin	AAGACTTCCTGGACCGCCATAAGA	
12	(vig1), GenBank: KM099177,	GCCTCTTCCACACCAACATCCA	
13	FHM mx,	GGATTGCACGAGTGGCTGTCAAA	
14	GenBank: KM099175,	CAATGCTTCTGTGGTCGCGATGT	
15	EF1-a,	TCGCTTTGCTGTGCGTGACAT	
16	AY643400, qPCR,	GGCAGCCTTCTGTGCAGACTTT	

2.2. Experimental fish and sampling

Healthy *A. clarkii* (length 10 cm ± 0.3 cm, weight 20 g ± 0.5 g) were purchased from the Choryang Aquarium (Busan, Republic of Korea) and acclimatized for one week in separate tanks in a laboratory aquarium (300 L, at 28 °C, salinity 32 PSU).

For sampling, five healthy *A. clarkii* fish were captured, anesthetized using tricaine (MS-222, 200 mg/L), and carefully dissected. The head kidney, spleen, liver, gill, intestine, muscle, skin, heart, and stomach were sampled (approximately 50 mg per tissue). Peripheral blood cells were separated from the plasma by immediate centrifugation at 3000×*g* for 10 min at 4 °C. All samples were snap-frozen in liquid nitrogen and stored at −80 °C until used for RNA extraction. The animal study was reviewed and approved by the Jeju National University Animal Ethics Committee.

2.3. Immune challenge experiment

The acclimatized *A. clarkii* were separated into four groups, and five fish in each group were injected intraperitoneally with lipopolysaccharides (LPS; 2.5 μg/g), poly I:C (2.5 μg/g), *or Vibrio harveyi* (1×10^3 CFU/μL) [13] suspended in 100 μL of phosphate-buffered saline (PBS). Five *A. clarkii* injected with 100 μL PBS was used as a control group. Following this challenge, peripheral blood cells and spleens (approximately 50 mg from each tissue) were collected from all *A. clarkii* at six time points: 0, 6, 12, 24, 48, and 72-h post-injection (hpi). Collected tissues were immediately frozen in liquid nitrogen (−80 °C) until RNA extraction.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from each collected tissue sample using the TRIzol reagent (Invitrogen, USA). First-strand cDNA was synthesized from total RNA using a PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Japan). The synthesized cDNA was diluted and stored in a freezer at −20 °C for further studies.

2.5. Quantitative reverse transcription PCR (RT-qPCR)

RT-qPCR was used to detect transcript levels of *AcBcl2*. The PCR reactions were performed in the final volume of 10 μL (3 μL cDNA, 0.4 μL of each forward and reverse primer (10 pmol/μL) (Table 1), 5 μL 2 × TaKaRa Ex Taq™ SYBR premix, and 1.2 μL dH_2O). Initial denaturation was conducted at 95 °C for 10 s, followed by 45 cycles of a denaturation step at 95 °C for 5 s, annealing at 58 °C for 10 s, extension at 72 °C for 20 s, and a final extension step at 95 °C for 15 s in a PCR Thermal Cycler Dice ™ TP 950 (Takara, Japan). Amplifications were performed in triplicate for each sample and all C_t values were normalized using the $2^{-\Delta\Delta Ct}$ method [14].

2.6. Cloning and recombinant plasmid construction

According to the reference sequence of *AcBcl2*, two pairs of primers were designed with the corresponding restriction enzyme sites (*Kpn1* and *XhoI*) to clone *AcBcl2* into pcDNA3.1+. The coding region of *AcBcl2* was PCR-amplified using cDNA from *A. clarkii*. The PCR mixture consisted of 1x Ex Taq buffer (TaKaRa, Japan), 5 U Ex Taq polymerase, 2.5 mM dNTPs (8 μL), 50 pmol of each primer, 50 ng of cDNA, and nuclease-free water in a total reaction volume of 50 μL. The cycling conditions were as follows: initial denaturation at 94 °C for 1 min, followed by 25 cycles at 94 °C for 30 s and 60 °C for 30s and 72 °C for 50 s and a final extension at 72 °C for 7 min. The PCR products were separated on a 1% agarose gel and respective bands were excised and purified using AccuPrep® PCR purification kit (Bioneer, Korea). The purified PCR products and the expression vectors pcDNA3.1+ and pEGFPN1 were digested with their respective enzymes and ligated using the DNA Ligation Kit, Mighty Mix (TaKaRa, Japan). The lined products were then transformed into competent *Escherichia coli* DH5α cells. The correct constructs were confirmed by DNA sequencing (Macrogen, Seoul, Korea). Positive colonies were grown in LB medium broth with ampicillin and incubated overnight at 37 °C in a shaker at 200 rpm. Finally, a MIDI kit (Qiagen, USA) was used to purify the plasmids, which were then used for cell transfection.

2.7. Cell culture and transfection

Fathead minnow (FHM) cells were grown at 26 °C under a normal atmosphere in L-15 medium (Sigma, USA) containing 10% (v/v) fetal bovine serum (FBS), and antibiotics (1% penicillin and streptomycin). The cells were passaged once every five days. For transfection, 60–90% confluent cells were seeded into respective wells in plates. One microgram of pcDNA3.1+ plasmids was transfected with 3 μL of Xtreme-GENE™ 9 transfection reagent (Roche, USA) following the manufacturer's protocol (DNA: transfection reagent, 3:1). The transfection mixture was incubated at room temperature for 20 min prior to the transfection. Transfection efficiency was calculated using RT-qPCR. The transfection efficiency between 80-90% was used in subsequent assays.

2.8. VHSV cell culture

VHSV [15] was propagated in a monolayer of FHM cells grown in 75 cm^2 screw cap culture plates at 20 °C in L-15 medium supplemented with 2% FBS, 1% penicillin, and 100 μg/mL streptomycin (Invitrogen, USA). Cells were inoculated with the virus at a multiplicity of infection (MOI) of 0.01. Cytopathic effect was assessed daily, and cells were harvested with the medium, vortexed, and centrifuged at 5000×*g* for 30 min at 4 °C to eliminate cell debris. The suspension was filtered through a 0.2 μm pore size filter (DISMIC, ADVANTEC, Japan). The viral titer was quantified using the $TCID_{50}$ method. The filtrate was stored at −80 °C until required.

2.9. Antiviral assay for VHSV

FHM cells were transiently transfected with pcDNA3.1(+)/AcBcl2 or pcDNA3.1(+) empty vectors. Cells were infected with VHSV (0.01 MOI) or poly I:C (10 mg/mL to 100 ng/mL) in L-15 growth medium (Sigma, USA) 24 h after transfection and incubated for 48 h at 20 °C. Cell viability was determined in triplicate using the crystal violet method as previously described [16].

rVHSV-ΔNV-EGFP (rVHSV) is a recombinant VHSV which produces EGFP instead of NV protein. Therefore, this virus can be used to study VHSV protein expression in real-time. This provides an indication of VHSV propagation in cells. rVHSV was kindly donated by the Department of Aquatic Life Medicine and the Department of Marine Biomaterials & Aquaculture, Pukyong National University, Busan, South Korea [17]. rVHSV-ΔNV-EGFP was propagated according to the conditions previously used for VHSV maintenance (Section 2.8). FHM cells were grown and transiently transfected with pcDNA3.1(+)/AcBcl2 or pcDNA3.1(+). After 24 h post transfection (hpt), the cells were infected with 10^4 $TCID_{50}$/mL rVHSV. The images were taken after 50 hpi. Fluorescent microscopy (40 × 10 power, Leica Microsystems, Germany) was used for imaging, and images were modified using the Leica Application Suite-X software (Version 3.3).

2.10. Gene expression analysis

FHM cells (1×10^6 cell/well) were transiently transfected with AcBcl2-pcDNA3.1+ constructs at approximately 90% confluency. After 24 h of transfection, cells were harvested at 24 h, 48 h, and 72 h. RNA was extracted using a RNeasy Mini Kit (Qiagen, USA), and cDNA was synthesized as described previously. PCR primers used for gene expression analysis are listed in Table 1.

2.11. Statistical analysis

All experiments were conducted in triplicates. Student's t-test was used ($p < 0.05$) for the data analysis in the immune challenge experiment. The data from RT-qPCR were analyzed using one–way ANOVA using Tukey's comparison and were presented as means, and the significance level was set at $p < 0.05$.

3. Results and discussion

3.1. Bio-informatics analysis of AcBcl2

The cDNA sequence transcribing the mature AcBcl2 protein was submitted to NCBI under accession number MK836052. AcBcl2 encodes a 23 kDa protein consisting of 208 amino acids. The theoretical isoelectric point (pI) was 5.04. Structural modeling of AcBcl2 revealed eight alpha helices (α1–α8) in the structure (Fig. 1A). Helices 1,4, and 5 were parallel to each other. However, helix 2,3,7, and 8 were perpendicular to the above-mentioned cluster. The presence of eight alpha helices is a classical feature of Bcl-xL proteins; however, the first human Bcl2-xL protein contained only seven alpha helices, where two C-terminal alpha helix (helix 7 and 8) were taken together to form the 7th alpha helix [18]. In this study, we separately labeled the last alpha helix as number eight.

The previously described apoptosis-and pro-apoptosis-related proteins indicate the availability of Bcl-2 homology (BH) domains in the protein. For homology modeling, human Bcl2-xL (hBcl2-xL) was used as the template and observed similar positions of BH domains in AcBcl2. The BH1 domain was observed in α4 and α5. The BH2 domain was observed in α7 and 8. The BH3 domain was observed in the α2 helix, and the BH4 domain was observed in the α1 helix (Fig. 1B).

An unrooted phylogenetic tree was created based on the amino acid sequences of known *Bcl2* family protein sequences in vertebrates and invertebrates (Fig. 1C). In comparison with other teleost Bcl2, AcBcl2 was observed to be coupled with damselfish, which belongs to the family Pomacentridae, and was the most similar to Bcl2 of *Stegastes partitus*.

Multiple sequence alignments were performed to identify the sequence alignments of the structural motifs (Fig. 1D). A total of eight alpha helices were observed with highly conserved sequences. In particular, sequences were conserved in BH regions. We identified four BH domains in *AcBcl2*. BH domains are indispensable at multiple levels of function for AcBcl2, as they stabilize the tertiary structure of the protein. Previous studies have suggested that mutations in these domains affect the apoptosis rate. Usually, the BH3 domain participates in apoptosis; however, anti-apoptotic proteins also contain this domain. The N-terminal BH domain, denoted BH4, is conserved only among anti-apoptotic Bcl-2 family members and forms an amphipathic helix on the opposite face of the molecule. This domain also interacts with Bax and

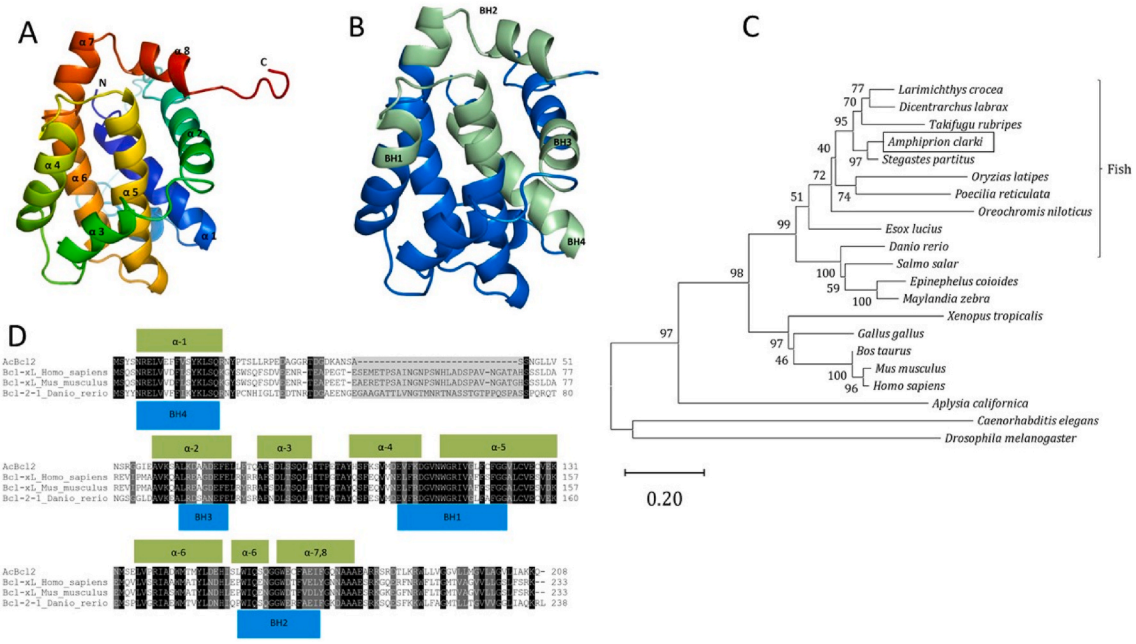

Fig. 1. Predicted 3D structures of AcBcl2 (A) AcBcl2 was predicted to contain seven alpha helixes in the structure. (B) In the predicted 3D structure four BH domains (BH1, BH2, BH3, and BH4) were identified. Relative positions of the BH domains in the protein are indicated in green color. (C) Phylogenetic analysis of Bcl-2 from different species. Clustal Omega was used to align amino acid sequences. The tree was constructed with neighbor joining method in MEGA 7.0, and bootstrap analysis was performed using 1000 replicates. NCBI gene bank accession numbers used to construct the tree are listed in Supplementary Table 1. According to the phylogenetic tree, AcBcl2 was observed to be closely related to Bcl2 from *Stegastes partitus*, which is another damselfish. (D) Homologs of AcBcl2 were identified using the NCBI BLAST. Here, three sequence homologs were identified from humans (*Homo sapiens*), mice (*Mus musculus*), and zebrafish (*Danio rerio*). Sequences were aligned using multiple sequence alignment suite in Clustal Omega. After alignment, conserved regions were visualized with Color Align Conservation online tool. Seven α-helixes observed in the protein structure were marked with green boxes and the BH domain regions were denoted by blue boxes. Higher sequence conservation was observed among alpha helix regions, especially the BH domain regions. This may indicate the functional conservation of the Bcl2 proteins among different animals.

its antagonists to inhibit cytochrome-c release from the mitochondria and is thus negatively associated with the apoptosis process [19].

3.2. Tissue distribution of AcBcl2 expression

The expression pattern of *AcBcl2* was examined using RT-qPCR with gene-specific primers in non-immune-challenged fish. *AcBcl2* was detectable in selected tissue samples, and the highest expression of *AcBcl2* was observed in blood, followed by the heart and spleen. The lowest expression levels were observed in the liver and kidneys (Fig. 2). These results were similar to those of a study conducted on Atlantic cod Bcl-XL [5].

The ubiquitous expression pattern of AcBcl2 in all tested tissues implies that AcBcl2 has a regulatory function in apoptosis in all tissues studied. Initially, Bcl2 proteins were believed to play a role in the survival of cancer cells [20]. However, recent findings suggest that Bcl2 is crucial to the survival of healthy cells as well [21]. The balance between apoptotic and anti-apoptotic proteins may confirm the fate of a given cell. Therefore, the constant expression of anti-apoptotic proteins, such as Bcl2, may be vital for cell survival. The additional role of Bcl2 in mitochondrial Ca^{2+} homeostasis has been described, and Bcl2 has been shown to regulate the uptake of extra Ca^{2+} to the mitochondria and protect them from excessive Ca^{2+} accumulation [22]. These factors may explain the natural, ubiquitous expression of *AcBcl2* in the studied tissues.

Cytotoxic lymphocytes such as natural killer cells and cytotoxic T cells can promote apoptosis in infected, physiologically stressed, or worn out blood cells, and these cells are continuously being removed from the circulatory system [19]. We can speculate that in healthy fish, without immune challenge, the elevated expression of anti-apoptotic proteins such as AcBcl2 can be vital for the survival of blood or cardiac cells, which can otherwise be vulnerable to apoptosis-induction via the above-mentioned apoptotic cells or factors.

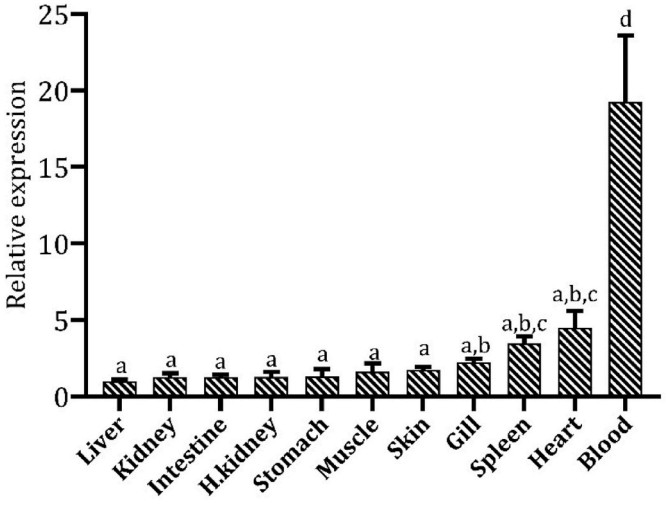

Fig. 2. Expression pattern for *AcBcl2 in vivo*. *AcBcl2* was ubiquitously expressed in all the tissues with the highest expression found in the blood. Expression was quantified by RT-qPCR and normalized using $2^{-\Delta\Delta Ct}$ method. The mean value of three replicates was mentioned along with the respective positive standard deviation (SD). *A. clarkii* tissue *elongation factor-1β* (*EF-1-β*) was used as the internal control for the experiment. One-way ANOVA with Tukey's comparison ($p < 0.05$) was used for statistical analysis. Different lowercase letters represent the significant differences between the mean expression of *AcBcl2* in different tissues.

Tissue-specific expression of Bcl2 has been previously reported in several invertebrates and vertebrates. In invertebrates, the expression of

Bcl2 mRNA in tissues suggests an organism-specific tissue expression profile. In the Zhikong scallop (*Chlamys farreri*), relatively high expression levels were detected in the adductor muscle [23]. Studies in other fish, including pufferfish, found the highest expression of anti-apoptotic Bcl-2 in the blood, liver, and gills; and the lowest in the muscle, head kidney, heart, spleen, and brain [8]. In Atlantic cod, strong expression was found in tissues such as the blood and brain [5]. These studies further corroborate our data.

3.3. Immune challenge experiment

To determine the expression pattern of *AcBcl2* under different immune stimulants, an immune challenge experiment was conducted. According to the results, in the blood (Fig. 3A), *AcBcl2* expression was significantly downregulated at the initial phase of the immune challenge experiment (0–12 hpi). At 24 hpi, *AcBcl2* expression significantly increased in the presence of both poly I:C and LPS. Interestingly, *AcBcl2* expression was significantly downregulated at 24 hpi in the presence of *V. harveyi*. At the end of the immune challenge experiment (72 hpi), all immune stimulants caused significant downregulation of *AcBcl2* expression in the blood. When analyzing *AcBcl2* expression in the spleen (Fig. 3B), the expression was either unchanged or significantly

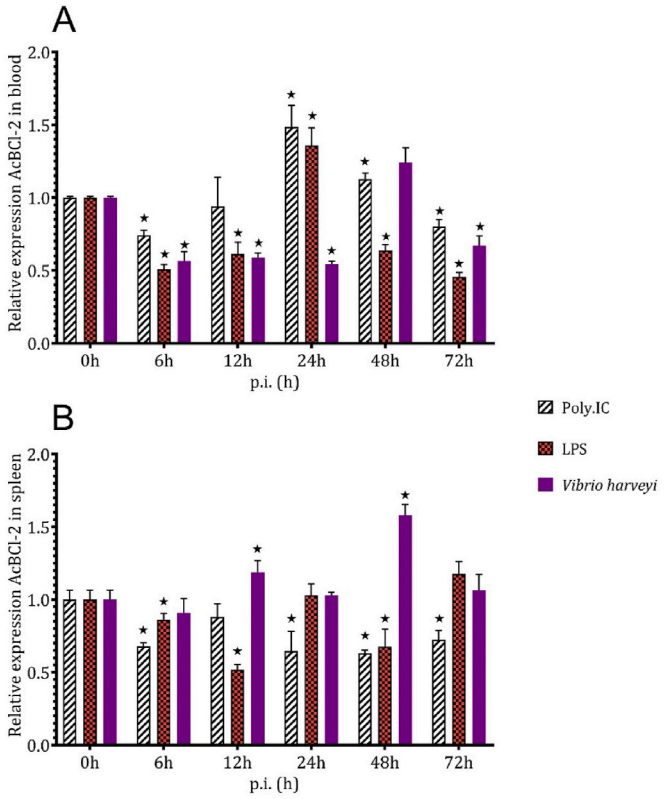

Fig. 3. Expression pattern of the *AcBcl2* in the blood and spleen with the presence of immune stimulants. Three different immune stimulants, poly I:C, LPS, and *V. harveyi* were intraperitoneally administered. PBS was used as the control. Expression of *AcBcl2* was then analyzed by RT-qPCR and normalized to that of *A. clarkii EF-1-β* gene. Data were analyzed with $2^{-\Delta\Delta Ct}$ method, and Student's t-tests were used to analyze significant differences. Statistical difference was compared to the 0 hpi samples. In the blood **(A)**, *AcBcl2* exhibited a significant downregulation at the initial phase of the challenge experiment for all immune stimulants. However, *AcBcl2* expression was significantly upregulated at and after 24 hpi. In the spleen **(B)**, expression of *AcBcl2* in the presence of poly I:C and LPS was significantly reduced throughout the immune challenge experiment. However, expression of *AcBcl2* was significantly higher when treated with *V. harveyi*. Each bar in the graph is a mean value ± SD of five (n = 5 fish) indicating a significant difference between PBS and the challenge group.

downregulated throughout the challenge experiment with poly I:C and LPS. Contrary to that in the blood, the expression of *AcBcl2* was significantly upregulated in the spleen throughout the immune challenge experiment for *V. harveyi*.

The three immune stimulants used in this study represent a wide range of immune stimulations. Poly I:C is a potent stimulant of double-stranded RNA viruses, and lipopolysaccharide (LPS) is used to mimic bacterial endotoxins. *Vibrio harveyi*, a pathogen for both fish and humans, has been used as a live bacterial pathogen [24]. A similar expression pattern of *AcBcl2* in the presence of poly I:C, LPS, and *V. harveyi* in the blood during the initial phase of the immune challenge suggests that AcBcl2 is a common intermediate in immune-related signal transduction pathways. Furthermore, blood is a common pool of immune cells that interconnects immune organs throughout the body. These factors may have resulted in the observed expression pattern of *AcBcl2* in blood. In the initial phase of the challenge experiment, the concentration of LPS and poly I:C was expected to be the highest, and in the presence of apoptotic stimulants such as LPS and poly I:C, significant downregulation of anti-apoptotic proteins was expected in the blood. However, we speculated that at around 24 hpi the concentration of both LPS and poly I:C would decrease in the blood. This would be associated with the expression of anti-apoptotic proteins such as *AcBcl2* to counterbalance the effect of prior apoptosis stimulation. Thus, we believe that the observed elevated expression of *AcBcl2* at 24 hpi was due to this reason; however, this pattern could be disrupted in the presence of live pathogens. Injected live pathogens tend to accumulate in the blood over time; therefore, the expression of anti-apoptotic proteins should be downregulated, and we believe that this phenomenon can explain the downregulation of *AcBcl2* in the blood for all hpi (with the exception at 48 hpi).

The spleen readily interacts with blood and helps to filter blood; therefore, antigens present in the bloodstream tend to accumulate in the spleen. When compared to that of the blood, continuous downregulation of *AcBcl2* in the spleen may indicate the accumulation of antigens. The spleen has a distinct expression pattern owing to its characteristic cellular composition. A previous study conducted in Atlantic cod indicated similar downregulation of *Bcl-X1* and *Bcl-X2* as that observed in our study [5]. Interestingly, during *V. harveyi* challenge, a significant upregulation of *AcBcl2* was observed. Previous studies have demonstrated the ability of several bacteria to negatively regulate apoptosis. During bacterial infections, apoptosis is one of the primary defense mechanisms. This is particularly true for intracellular pathogens. If bacteria can inhibit apoptosis, they can propagate rapidly [25].

V. harveyi produces LPS, which causes pathogenicity by triggering apoptosis and inhibiting anti-apoptotic protein synthesis [24]. In the blood, we observed similar downregulation of *AcBcl2* expression in the presence of both LPS and *V. harveyi*. This might be caused by *V. harveyi*-induced LPS production.

Several studies have examined the expression pattern of *Bcl2* with immune stimulants, a study conducted in channel catfish indicated that bacterial infection-induced expression of anti-apoptotic proteins similar to that of Bcl2 [9]. Further, *Bcl2* is highly expressed in tumors [26]. In fact, Bcl2 might play an important role in helping cancer cells evade apoptosis under seemingly harsh conditions for normal cells, such as high levels of reactive oxygen species and different ratios of metabolites. Similar harsh conditions may arise during pathogenic infections in cells, especially during viral and intracellular bacterial pathogens, which can alter the microenvironment of the cells to support pathogen survival. Therefore, the alteration of apoptosis regulatory proteins is crucial for balancing cell survival or death.

3.4. In vitro gene expression analysis in the presence of AcBcl2

As our goal in this study was to understand the role of AcBcl2 during VHSV infections, we analyzed the expression of key antiviral genes in the presence of AcBcl2. IFN regulatory factor-3 (Irf3) is a key

transcription factor for interferon gene regulation. Mx proteins have a GTPase activity, which is important for RNA virus defense. Mx proteins have been identified as key proteins in the antiviral defense. Viperin is a classic example of an antiviral protein, and its mechanism has validated. Among the interferon-stimulated genes (ISG), the Isg15 is one of the most abundantly expressed genes. Isg15 has shown its role in inhibiting virus infections. Therefore, by analyzing the expression of these genes we may be able to understand the role of AcBcl2 in antiviral defense mechanisms [27].

This experiment was performed under two conditions: in the first condition, AcBcl2 was overexpressed alone, and the antiviral gene expression was analyzed. In the second condition, AcBcl2 was overexpressed, and the gene expression was analyzed following infection by VHSV (Fig. 4).

Certain groups of viruses can stimulate the expression of apoptosis inhibitors, such as Bcl2 [28]. Apoptosis is among the primary defense mechanisms against viruses. If virus-infected cells cannot undergo apoptosis, the virus has a high chance of infecting adjacent cells. However, the precise mechanism whereby certain viruses inhibit apoptosis is unknown.

When AcBcl2 was overexpressed alone, analyzed genes were significantly downregulated at all time points, except for Mx, at 72 hpt. This result suggests that overexpression of AcBcl2 alone can reduce cellular antiviral gene expression.

Under VHSV infection, three patterns were observed. First, in the early stages of infection (approximately 24 hpi), the expression of *viperin* and *isg15* were downregulated in the AcBcl2 overexpressed cells. *Mx* expression was unchanged and the expression of *irf3* was increased slightly compared to the respective controls. However, this pattern of expression changed at 48 hpi, where all the antiviral genes were significantly upregulated compared to controls. The upregulation in antiviral gene expression observed at 48 hpi in the presence of AcBcl2 can be explained as follows: in the AcBcl2 overexpressed and VHSV infected samples, VHSV has a better chance to replicate and produce high copy numbers, induce high cellular damage, and release higher titers of the virus, eventually leading to the high expression of antiviral genes in a dose-dependent manner. The third pattern was observed at the latter stages of the infection, where the antiviral genes analyzed were again significantly downregulated in the AcBcl2 overexpressed samples compared to the controls. The higher cell mortality of the AcBcl2 overexpressed cells might have caused this reduction of the antiviral gene expression.

In summary, we demonstrated the ability of *AcBcl2* to modulate gene expression patterns in fish cells. In particular, the downregulation of apoptosis-associated genes observed in the presence of AcBcl2 further confirmed its anti-apoptotic activity. Even with VHSV infection, the expressions of genes were downregulated or were not significantly different at 24 hpi, which may indicate anti-apoptotic activity.

3.5. *VHSV proliferation and cell damage was higher in the presence of AcBcl2*

This experiment was performed to investigate the propagation and release of VHSV in the presence of AcBcl2. In the previous section, we observed the effect of AcBcl2 on antiviral gene transcription. Especially, at 48 hpi, we observed a strong expression of antiviral genes. Here we sought reasons for this upregulation.

We speculate that AcBcl2 supports the propagation and release of VHSV *in vitro*. To study the propagation and spread of VHSV, we used EGFP-tagged recombinant VHSV (rVHSV) [17]. Thus, the propagation of VHSV can be determined in real-time by using fluorescence microscopy. We observed a relatively higher fluorescence signal in cells expressing AcBcl2 compared to that seen in control samples (Fig. 5A and B).

Monolayer damage in cell culture may resemble virus assembly and

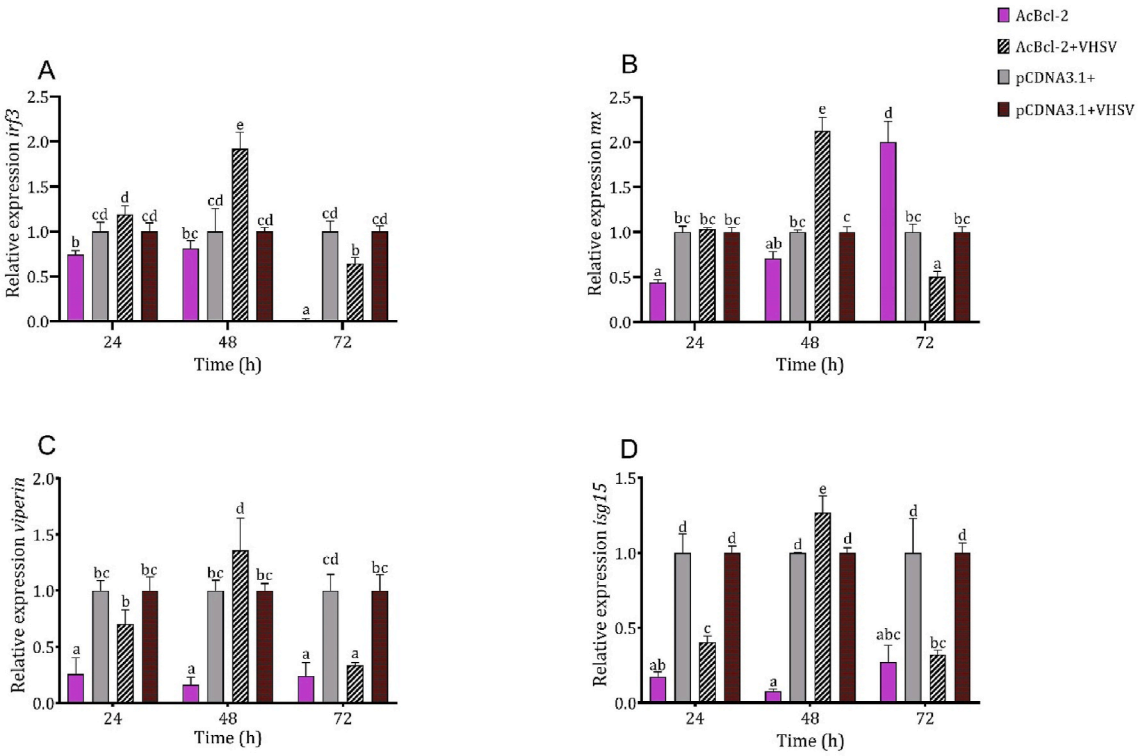

Fig. 4. Antiviral system related gene expression with the presence of AcBcl2 *in vitro*. FHM cells were transfected with either pcDNA3.1/AcBcl2 constructs. One set was infected with VHSV (0.01 MOI). Samples were taken at 24 hpi, 48 hpi, and 72 hpi, and the gene expression was analyzed by RT-qPCR. **(A),** *irf3* **(B),** *mx* **(C),** *viperin* **(D),** *isg15*. When VHSV was infected, the expression of the antiviral genes was downregulated at 24 hpi. However, at 48 hpi, expression of antiviral genes was upregulated. One-way ANOVA with Tukey's comparison ($p < 0.05$) was used for statistical analysis. Different lower-case letters represent the significant differences between the mean expression of AcBcl2 in different tissues.

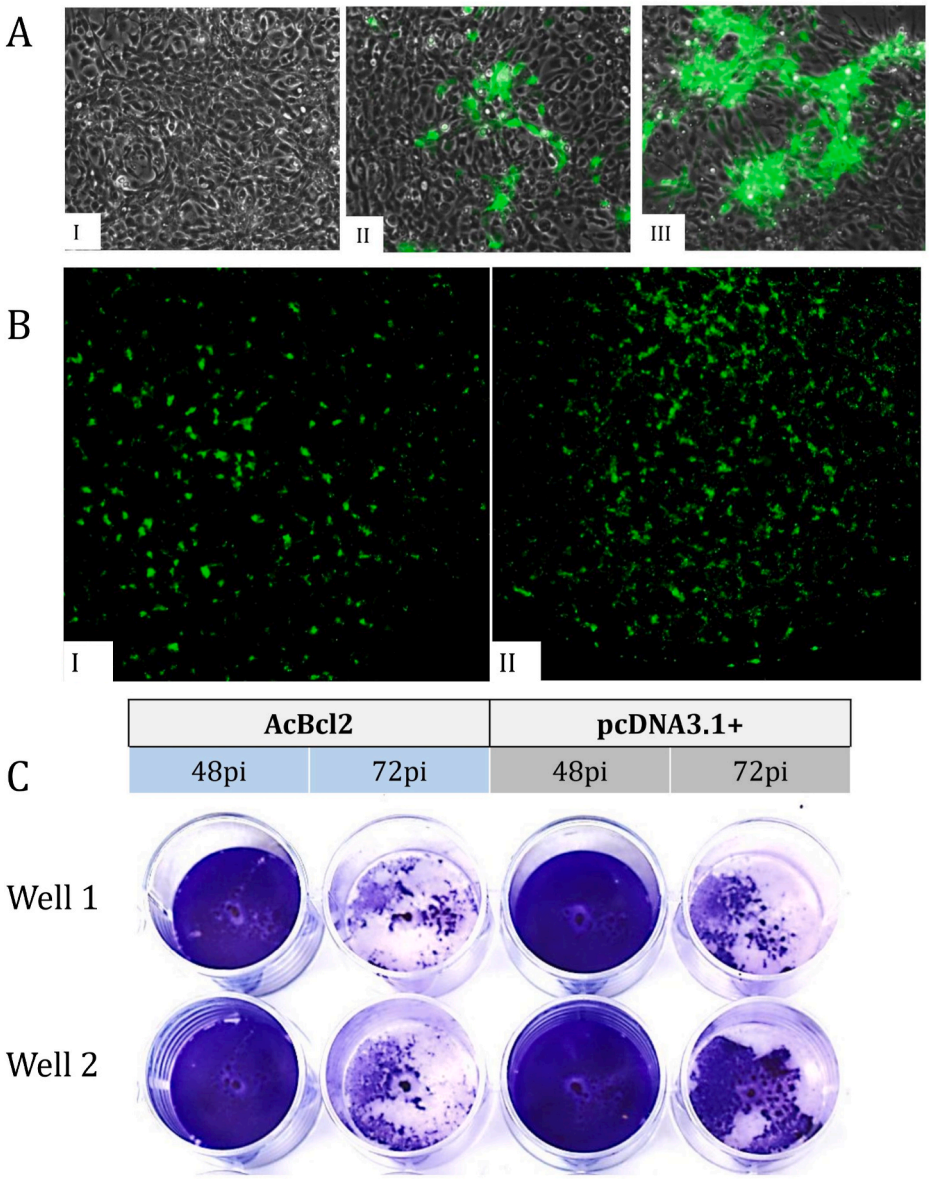

Fig. 5. Effect of AcBcl2 on the VHSV propagation. (A), FHM cells were grown to 100% confluency and transfected with either pcDNA3.1+/AcBcl2. At 24 hpt, cells were treated with rVHSV-ΔNV-EGFP (10^4 $TCID_{50}$/mL). Images were obtained after 50 hpi. (A–I) FHM cell control. (A-II) Cells transfected with pcDNA3.1+ control vectors and then treated with rVHSV-ΔNV-EGFP. (A-III) Cells transfected with AcBcl2 and treated with rVHSV-ΔNV-EGFP. AcBcl2 transfected cells exhibited higher fluorescence signals compared to that of control cells. This may indicate higher VHSV proliferation in the presence of AcBcl2. **(B),** Low-magnification (4 ×) image of the rVHSV-ΔNV-EGFP experiment showing one-quarter of wells. (B–I) Cells transfected with pcDNA3.1+ control vectors and then treated with rVHSV-ΔNV-EGFP. (B-II) Cells transfected with AcBcl2 and treated with rVHSV-ΔNV-EGFP. **(C),** Effects of AcBcl2 on the cell monolayer. FHM cells were grown to 100% confluency and transfected with either pcDNA3.1+ or AcBcl2. At 24 hpt, cells were treated with VHSV and subsequently stained with 0.5% crystal violet at 48 hpi and 72 hpi. Residual stain was washed with water. AcBcl2 transfection was shown to cause higher damage to the cellular monolayer, most probably due to higher viral titer.

release [29]. Crystal violet staining was used to understand the monolayer damage caused by VHSV. This experiment (Fig. 5C) indicated greater cellular damage in the presence of AcBcl2 compared to that in the absence of AcBcl2. Moreover, we believe that the upregulation of the antiviral genes at 48 hpi in section 3.4 most probably occurred due to this increment of the virus titer at the latter stages of the infection.

3.6. AcBcl2 expression enhanced viability of cells treated with RNA virus stimulant poly I:C

In this experiment, poly I:C was used as an apoptotic inducer [12], and the results showed that cell viability was higher in the AcBcl2 transfected cells than in the controls when treated with 100 μg/mL poly I:C (Fig. 6A). This indicated that AcBcl2 has reduced apoptosis. Anti-apoptotic proteins such as Bcl2 are known to be desensitized in the IFN signaling pathway [30]. Virus-induced apoptosis is a key defense mechanism against RNA viruses, which is initiated through the IFN signaling pathway. Studies have indicated the ability of cells over-expressing Bcl2 to neglect viral stimulation and promote viral propagation under such conditions. Similar observations have been made in previous studies involving Bcl2 in other animals [31]. When considering these factors together, we can assume that AcBcl2 plays an inhibitory role in virus-induced apoptosis in fish.

3.7. Cell viability in the presence of VHSV

As cells expressing AcBcl2 exhibited higher cell viability in the presence of poly I:C, we checked cell viability in the presence of VHSV (Fig. 6B). As we expected, VHSV decreased cell viability in the presence of AcBcl2. Gene expression analysis revealed that the antiviral genes required for VHSV defense were significantly downregulated in the presence of AcBcl2. We speculate that this initial downregulation of antiviral genes in the presence of AcBcl2 may have led to a higher viral titer in the AcBcl2 transfected samples. This indicates that AcBcl2 overexpression allows the virus to propagate at higher titers, impairing cell viability. Thus, AcBcl2 overexpressed and VHSV infected samples decreased their viability compared to that of the control.

3.8. VHSV gene expression in the presence of AcBcl2

In the previous section, we observed an enhancement in cell viability in the presence of AcBcl2 and VHSV in the latter stages of infection, and

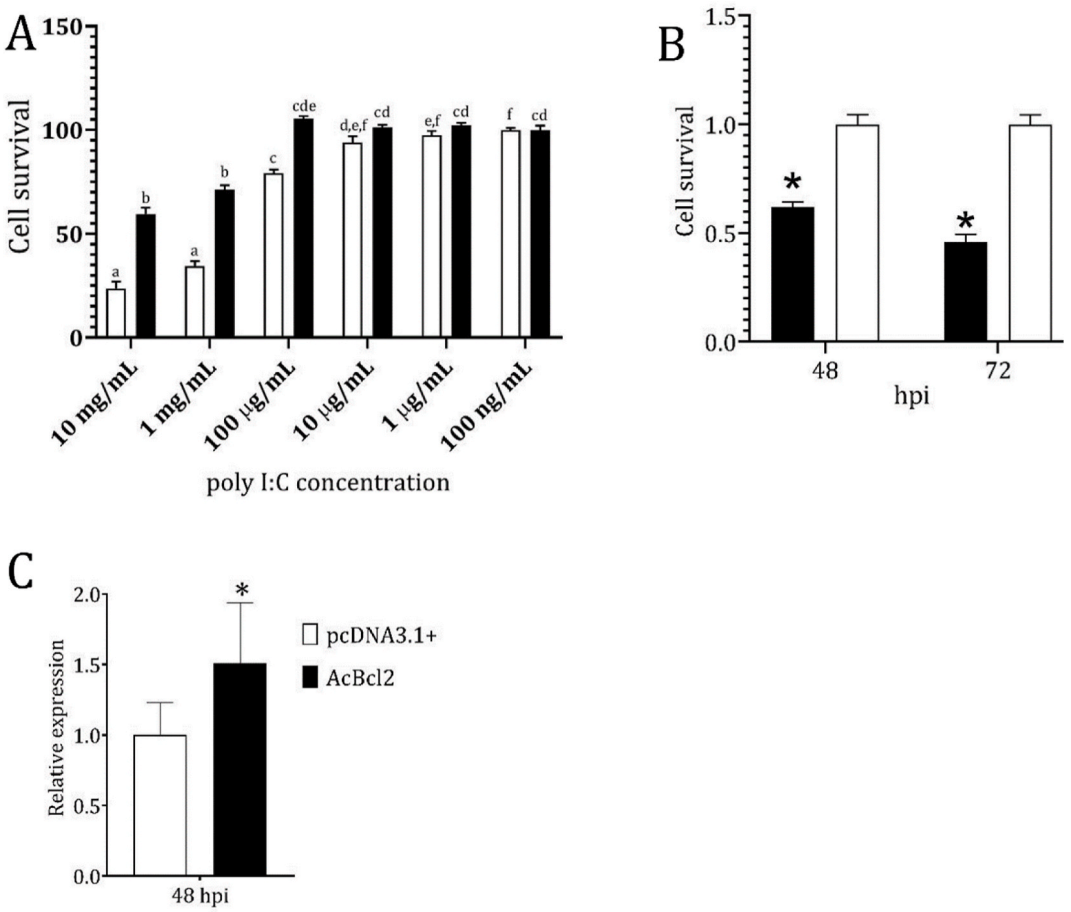

Fig. 6. Cell viability in AcBcl2 transfected cells. **(A),** Cells were grown up to approximately 80% confluency and transiently transfected with AcBcl2. At 24 hpt, cells were re-transfected with six dilutions of poly I:C (10 mg/mL to 100 ng/mL, ten-fold dilutions). After 48 hpi, cell viability was determined by the crystal violet method. Results indicated higher viability of cells treated with 100 µg/mL poly I:C compared to the control cells. This indicates the anti-apoptotic nature of the AcBcl2. **(B),** Cell viability after VHSV transfection. Cells were grown in cell culture plates and transiently transfected with AcBcl2. These were then infected with 0.01 MOI VHSV, and the cell viability was measured by the crystal violet method. According to the results, cells transfected with AcBcl2 exhibited less viability after VHSV infection compared to that of the cells transfected with pcDNA3.1+ control vectors. **(C),** Cells were transiently transfected with AcBcl2 and then infected with VHSV. After 48 hpi, gene expression was analyzed by RT-qPCR. Viral *G-protein* expression was upregulated in the presence of AcBcl2.

further studied viral gene expression in the presence of AcBcl2 (Fig. 6C). The expression of the VHSV *G-protein* (*GP*) was significantly upregulated in the AcBcl2 overexpressed samples compared to that in the control.

Previous evidence suggests that Bcl2 expression promotes virus infections in fish [32,33]. Whereas inhibition of the apoptosis process could enhance virus propagation, on the other hand, the importance of IFN in virus attenuation is a well-established fact. In this study, we observed downregulation of antiviral gene expression in the early stages of VHSV infection. Multiple studies have shown that antiviral genes such as Mx and viperin enervate VHSV gene expression [34]. Therefore, in an environment where the expression of these key antiviral proteins is decreased, VHSV gene expression may be enhanced. Either inhibition of apoptosis or downregulation of antiviral genes may have an influence on the observed higher virus proliferation in this study. However, more studies are required to fully understand the role of Bcl2 during virus infections.

4. Conclusion

In this study, we analyzed the sequence, structure, and expression of *AcBcl2* and its role during VHSV infections. Sequence analysis indicated the presence of all four BH domains in *AcBcl2*. The structure of AcBcl2 was similar to those of previously characterized anti-apoptotic Bcl2 in mammals. Expression analysis indicated downregulation of *AcBcl2* in the presence of immune stimulants. Furthermore, AcBcl2 downregulated the expression of major antiviral genes analyzed. *In vitro,* VHSV propagation and cell damage analyses indicated higher virus titers in the presence of AcBcl2. When infected with VHSV, cell viability was reduced in AcBcl2-transfected samples. Viral *GP* was upregulated when AcBcl2 was expressed. Collectively, these results suggest that AcBcl2 is an anti-apoptotic protein that facilitates VHSV propagation.

CRediT authorship contribution statement

K.P. Madushani: Conceptualization, Methodology, Software, Formal analysis, Writing – original draft. **K.A.S.N. Shanaka:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Formal analysis. **H.M.S.M. Wijerathna:** Writing – review & editing. **Chaehyeon Lim:** Resources, Writing – review & editing, Supervision. **Taehyug Jeong:** Resources, Writing – review & editing, Supervision. **Sumi Jung:** Resources, Writing – review & editing, Supervision. **Jehee Lee:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2022.09.005.

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