Fish and Shellfish Immunology 86 (2019) 794–804

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Molecular cloning and expression analysis of *coagulation factor VIII* and *plasminogen* involved in immune response to GCRV, and immunity activity comparison of grass carp *Ctenopharyngodon idella* with different viral resistance

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ARTICLE INFO

Keywords:
Ctenopharyngodon idella
cDNA cloning
Viral infection
Antioxidant factor
Serum immune factor

ABSTRACT

The grass carp reovirus (GCRV) has been shown to cause lethal infections in the grass carp *Ctenopharyngodon idella* (*C. idella*). In order to investigate the immune response to GCRV infection, the full-length cDNA sequences of *coagulation factor VIII* (*CiFVIII*) and *plasminogen* (*CiPLG*) from *C. idella* were cloned and their involvement in the immune response was studied. The immunity factor levels in *C. idella* with different GCRV resistances were also analyzed. The full-length 2478 bp cDNA of *CiFVIII* contained an open reading frame of 1965 bp and encoded a putative polypeptide of 654 amino acid residues. The full-length 2907 bp cDNA of *CiPLG* contained an open reading frame of 2133 bp and encoded a putative polypeptide of 710 amino acid residues. *CiFVIII* was closely clustered with that of *Clupea harengus*. *CiPLG* was first clustered with those of *Cyprinus carpio* and *Danio rerio*. *CiFVIII* transcripts were most abundant in the liver and least in the skin. The highest expression level of *CiPLG* was observed in liver and the lowest in muscle. Expression levels of *CiFVIII* in gill, head kidney and spleen, and expression levels of *CiPLG* in gill, intestine and liver all reached the maximum at 72 h post GCRV infection. In spleen, expression levels of *CiFVIII* and *CiPLG* were significantly positively correlated. The activities of T-AOC, LSZ and IgM in R♂ were significantly higher than those in O♂. Likewise, T-AOC and LSZ activities in F1 were significantly higher than f1 individuals ($P < 0.01$). These results indicated that *CiFVIII* and *CiPLG* may play important roles in the immune response to GCRV infection. In addition, antioxidant ability and serum immune factor activity may confer a different viral resistance to *C. idella*.

1. Introduction

The grass carp (*Ctenopharyngodon idella*) is a fish species belonging to the subfamily Leuciscinae. Fish in this taxa represent the largest portion of global fish production over the past decade [1]. However, fish in this group are highly to die from infection by a double-stranded RNA grass carp reovirus (GCRV). As a pathogenic aquareovirus, GCRV infection leads to external and internal hemorrhage and caused tremendous loss of grass carp fingerlings during rearing. The hemorrhage of internal organs is the primary clinical sign of GCRV infection, including hemorrhage of the oral cavity, fins and gill covers, as well as internal hemorrhage of the musculature, liver, spleen and intestine [2].

Hemorrhage sites are open wounds that represent an ideal entry port for pathogens to invade the closed circulatory system. These sites bear a great risk of systemic infections. Therefore, remodeling of tissue integrity and repair at the wounded site is an important part of the innate immune system [3]. It is becoming increasingly clear that the mechanism of coagulation, which ensures fast and efficient wound sealing, and the innate immune system of subsequent wound healing have coevolved from a common ancestral during eukaryotic development [4]. Crosstalk between coagulation and the complement system has been shown to be a survival defense after tissue injury [5]. Soluble

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https://doi.org/10.1016/j.fsi.2018.12.024
Received 23 September 2018; Received in revised form 6 December 2018; Accepted 12 December 2018

Table 1
Primers used for cDNA cloning and quantitative real-time PCR (qPCR).

Name	Usage	Primer sequence (5′-3′)
SMARTII A Oligonucleotide	RACE	AAGCAGTGGTATCAACGCAGAGTACGCGGG
3′-RACE CDS Primer A	RACE	AAGCAGTGGTATCAACGCAGAGTAC(T) 30VN (N: A, C, G, or T; V:A, G, or C)
5′-RACE CDS Primer A	RACE	(T) 25V N-3' (N: A, C, G, or T; V: A, G, or C)
10 × UPM Long Primer	RACE	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
10 × UPM Short Primer	RACE	CTAATACGACTCACTATAGGGC
FVIII F	RACE	ACAGTCCTCAGTCCCACCAG
FVIII R	RACE	GGCTCCACTCCAAATCAAAG
PLG F	RACE	GTCCGTGAGAACGAGATGTG
PLG R	RACE	CGCATTGGTATGATGACTCC
FVIII YF	qPCR	CATTTCATGTCTGCATCGAGG
FVIII YR	qPCR	CAGATTTCTCCTGCAACACC
PLG YF	qPCR	AAGTCCCTGCCTGTCAATG
PLG YR	qPCR	GAACATCTGTGCTTTGGGTC
β-actinYF	qPCR	GGCTGTGCTGTCCCTGTATG
β-actinYR	qPCR	CTCTGGGCACCTGAACCTCT
18 s YF	qPCR	ATTTCCGACACGGAGAGG
18 s YR	qPCR	CATGGGTTTAGGATACGCTC

RACE: rapid-amplification of cDNA ends.

factors secreted by organs help the formation of a stable clot in order, to seal the wound and prevent the loss of body fluids. These are major functions of the coagulation system [3].

The process begins by the binding of blood clotting factors (such as *IXa*, *VIII* and *prothrombin*) to phosphatidylserine-exposing procoagulant activated platelets. Next, membrane-dependent enzyme complexes form which lead to blood coagulation [6]. *Coagulation factor VIII* (*FVIII*) is a large plasma glycoprotein that functions in the intrinsic pathway of blood coagulation. It has been demonstrated that the secretion of *FVIII* is regulated by resident proteins in endoplasmic reticulum, and its absence will lead to hemophilia A [7,8]. *FVIII* is also involved in the immune process of the complement system by participating in activation of complement component 3 [9]. The coagulation system also interacts with the fibrinolysis system to maintain normal blood circulation [10–12]. Fibrinolysis system functions in maintaining blood fluidity and vascular integrity, which includes the following factors: *plasminogen* (*PLG*), *fibrinogen*, *plasminogen activators*, *plasmin* and *plasmin inhibitors*. The activation of *PLG* is a central event in the fibrinolytic response [13,14].

As important immune-related genes, the structure and function of *FVIII* and *PLG* have been studied in depth in human, mouse and other mammals [15,16]. However, *FVIII* and *PLG* genes are not well known in fish, especially in grass carp that are susceptible to hemorrhage disease. In order to study these two genes and to clarify their immune function after GCRV infection, the full-length cDNAs of *FVIII* and *PLG* from grass carp (*C. idella*) were cloned, and their expression levels in different tissues after viral infection were investigated. Levels of immune factors in *C. idella* individuals that showed different GCRV resistance were also compared. The results provide a basis for understanding the immune response to hemorrhage, as well as disease-resistant in *C. idella.*

2. Materials and methods

2.1. Experimental fish

A total of 282 individuals of wild grass carp (*C. idella*, body length: 10∽15 cm) from Xiangjiang river were collected in July 2010 and injected with GCRV 918 (0.2 μL/g body weight, at 1.0×10^7 50% tissue culture infective dose (TCID50)/mL, kindly provided by professor Zeng Lingbing from the Yangtze River Fisheries Research Institute of Chinese Academy of Fishery Sciences). One hundred and seventy-seven individuals were died within two weeks of infection (mortality rate: 62.76%), and the remaining 105 surviving individuals continued to be cultured (Lishanba Fish Base, Zhuzhou, China) as parent fish. In May 2013, five sexually mature male fish from the surviving individuals (male parent with GCRV resistance, designated as R♂) were selected to mate with ordinary *C. idella* female fish (purchased from Wulong Fishery Base, Liuyang, China) to yield F1 progeny. Control hybrids consisted of mating ordinary sexually mature male fish (designated as: O♂) and female fish to obtain f1 progeny.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from the tissue sample (30 mg) of *C. idella* using TaKaRa MinBEST Universal RNA Extraction Kit, following the manufacturer's instructions. For quality control, the concentration of RNA was measured with a spectrophotometer (Eppendorf BioSpectrometer basic, Hamburg, Germany), and the integrity of RNA was analyzed by 1% agarose gel electrophoresis. RNAs with an $OD_{260/280}$ value ranged from 1.8 to 2.0 were used for cDNA synthesis. For full-length cDNA sequence cloning, the cDNA was synthesized using the SMARTer rapid amplification of cDNA ends (RACE) kit (Clontech, Mountain View, USA). For quantitative real-time PCR (qPCR) analysis, RNA (after treatment with DNase I) was used to synthesize cDNA using the ReverTra Ace-first-strand cDNA synthesis kit (Toyobo, Osaka, Japan), according to the manufacturer's instructions. .

2.3. Full-length cDNA cloning of CiFVIII and CiPLG

Gene specific primers, FVIII F, FVIII R, PLG F and PLG R, for *CiFVIII* and *CiPLG* cDNA cloning were designed by Oligo7 software based on the unigene sequences of corresponding genes from the spleen transcriptome data of *C. idella*. The above synthesized cDNA was used as the template for the RACE PCR. The total volume of the PCR reaction mixture was 50 μL, including 1 μL cDNA, 25 μL 2 × EasyTaq PCR SuperMix, 1 μL of each gene specific primer FVIII F or FVIII R (PLG F or PLG R), 1 μL Universal Primer A Mix and 22 μL ddH_2O. The PCR programs were run as follows: 5 cycles at 94 °C for 30 s, 72 °C for 3 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; 25 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. The PCR products were purified, ligated with pUCm-T vector (Kanglang, Shanghai, China), transformed into *Escherichia coli DH5α* cell and sent for sequencing (Sangon Biotech Company, Shanghai, China). Primers for RACE PCR are listed in Table 1.

2.4. Bioinformatics analysis

The open reading frames and deduced amino acid sequences of

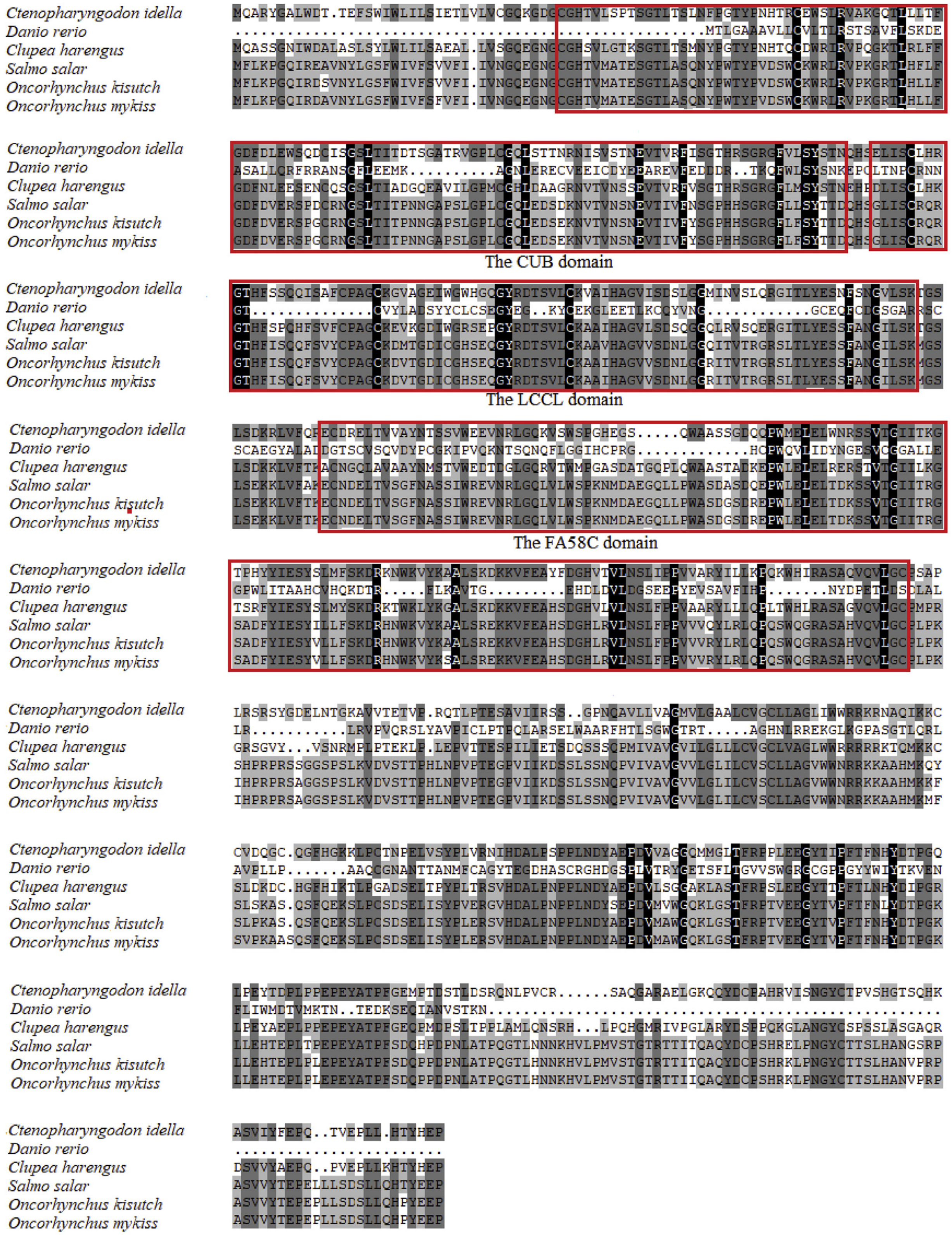

Fig. 1. Multiple sequence alignment of *CiFVIII* with other homologues. The amino acid residues shaded in black represent conserved sites, and functional domains are indicated in the red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CiFVIII and *CiPLG* were analyzed with ExPASy-Translate tool (http://web.expasy.org/translate/). Molecular weights and theoretical isoelectric points were calculated by ExPASy-Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The protein domains were analyzed by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) and Pfam database (http://pfam.sanger.ac.uk/search). Multiple sequence alignment was performed with DNAman 7 software and neighbor-joining phylogenetic trees for *CiFVIII* and *CiPLG* were constructed using MEGA 6.06 software, and a bootstrap value of 1000 was set to assess the repeatability of the results. *FVIIIs* from *C.*

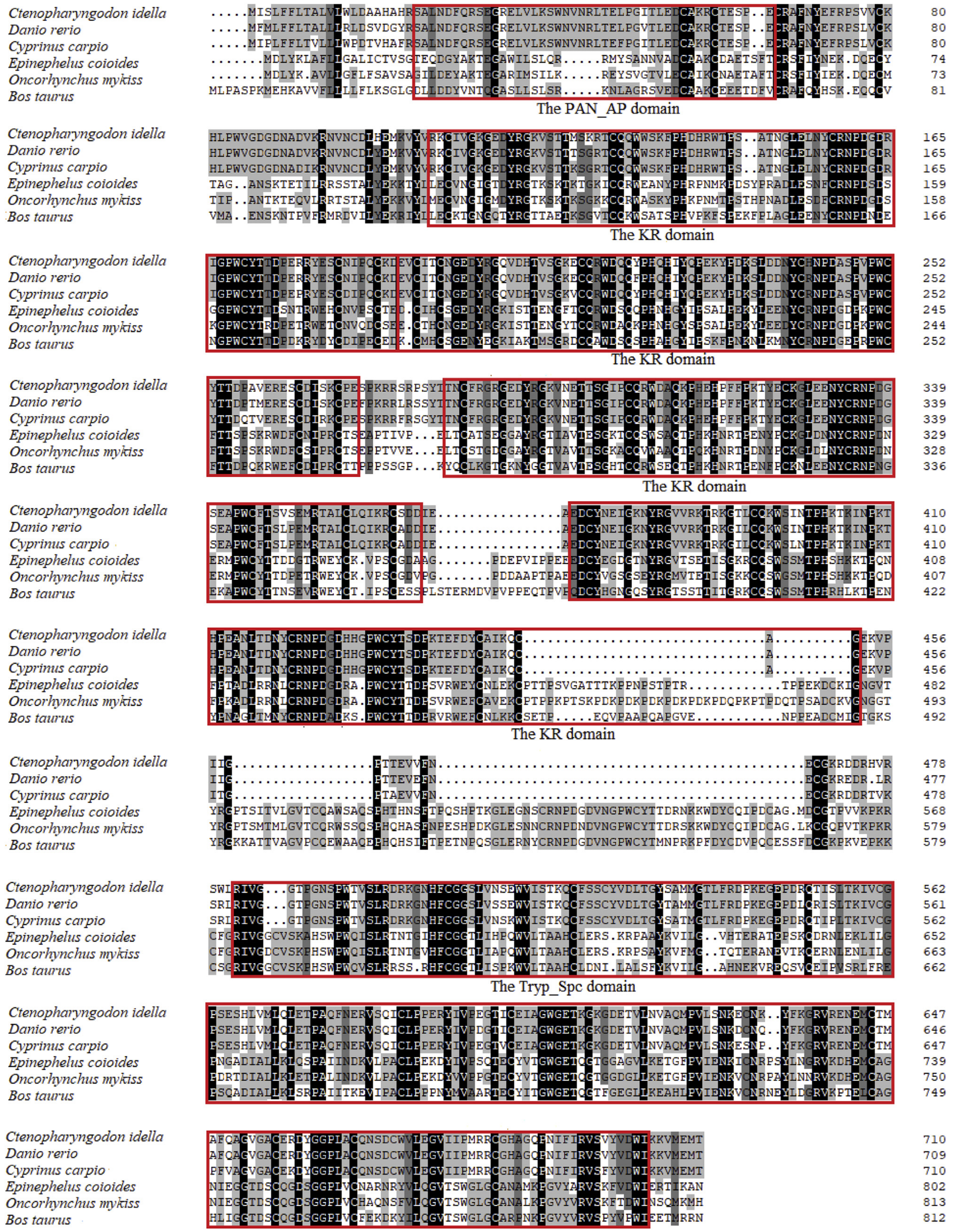

Fig. 2. Multiple sequence alignment of *CiPLG* with other homologues. The amino acid residues shaded in black are conserved sites, and functional domains are indicated in the red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

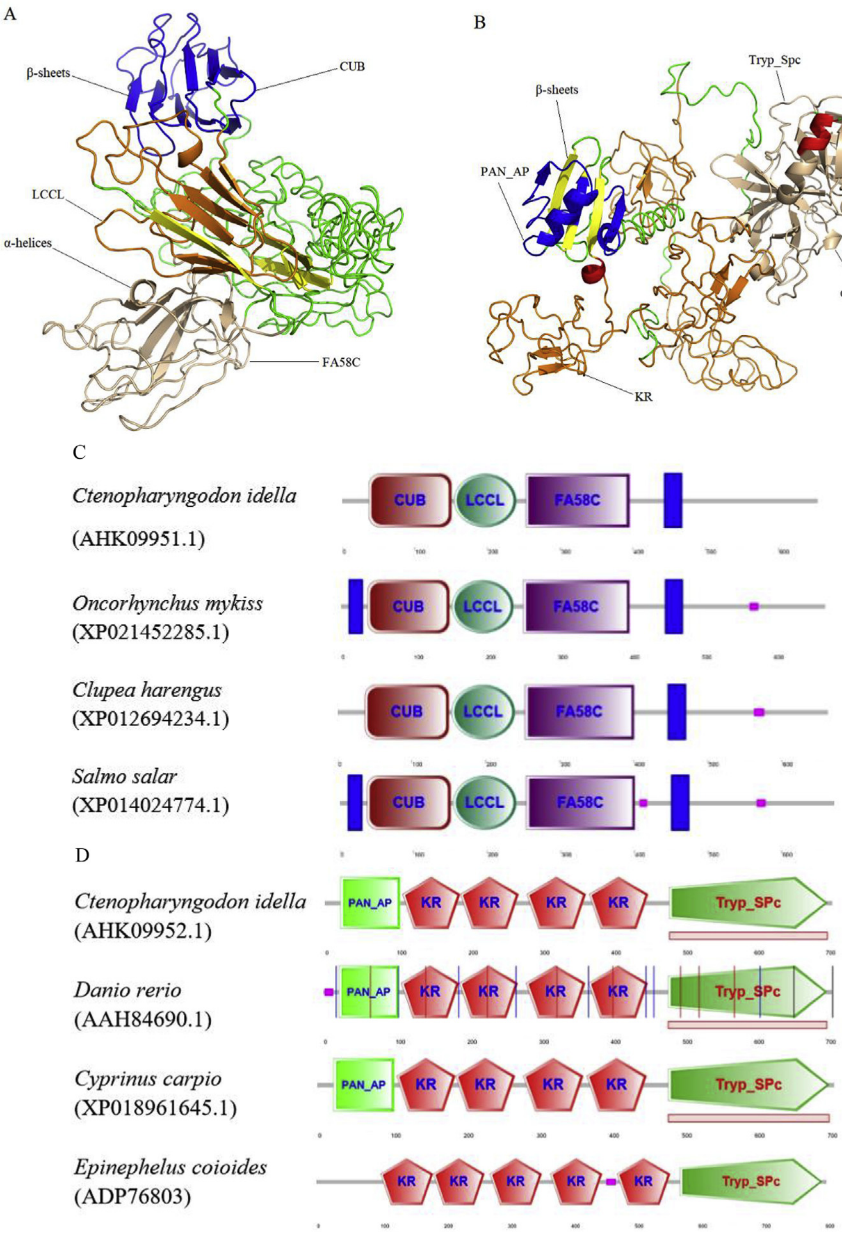

Fig. 3. Three-dimensional structure of *CiFVIII* (A) and *CiPLG* (B), and protein domains of FVIIIs (C) and PLGs (D) in species of fish.

idella (AHK09951.1), *Danio rerio* (XP009305924.1), *Homo sapiens* (AAA52420.1), *Oryctolagus cuniculus* (ACA42556.1), *Anas platyrhynchos* (XP012952193.1), *Mus musculus* (EDL29229.1), *Piliocolobus tephrosceles* (XP023049378.1), *Canis lupus familiaris* (NP001003212.1), *Xenopus tropicalis* (NP001072171.1), *Chlamydotis macqueenii* (KFP36707.1), *Corvus brachyrhynchos* (KFO61062.1), *Podiceps cristatus* (KFZ48540.1), *Opisthocomus hoazin* (KFR16165.1), *Phaethon lepturus* (KFQ72067.1), *Sus scrofa* (NP999332.2), *Bos taurus* (NP001138980.1), *Clupea harengus* (XP012694234.1), *Oncorhynchus kisutch* (XP020308701.1), *Oncorhynchus mykiss* (XP021452285.1) and *Salmo salar* (XP014024774.1), and *PLGs* from *C. idella* (AHK09952.1), *Epinephelus coioides* (ADP76803), *Oncorhynchus mykiss* (NP001117863), *Bos taurus* (NP776376), *Rattus norvegicus* (NP445943), *Macaca mulatta* (NP001036540.1), *Homo sapiens* (AAA36451.1), *Anas platyrhynchos* (EOB03995.1), *Sus scrofa* (NP001038055.1), *Mus musculus* (NP032903), *Pongo abelii* (NP001126035.1), *Branchiostoma belcheri tsingtauense* (ACE86411.1), *Salvelinus alpinus* (XP023830244.1), *Myotis lucifugus* (XP006106065.1), *Danio rerio* (AAH84690.1), *Cyprinus carpio* (XP018961645.1), *Pygocentrus nattereri* (XP017563904.1), *Acanthochromis polyacanthus* (XP022063671.1), *Ictalurus punctatus* (XP017335876.1), *Amphiprion ocellaris* (XP023143192.1) and *Larimichthys crocea* (XP019118483.1) were used to construct phylogenetic trees, respectively.

2.5. Tissue expression level changes in CiFVIII and CiPLG after GCRV infection

The gill, head kidney, liver, spleen, kidney, intestine, muscle and skin from five *C. idella* individuals were collected for tissue gene expression level analysis. A total of 200 individuals were intraperitoneally injected with 200 μL of GCRV918, and set as the experimental group. The gill, head kidney, spleen, muscle, intestine and liver tissues of five individuals in the experimental group at time points of 12, 24, 48, 72, 96, 120, 144 and 168 h post infection were sampled, separately. Tissues from five healthy individuals of *C. idella* were also sampled and set as the control group. qPCR was performed on a CFX96 Touch Real-Time

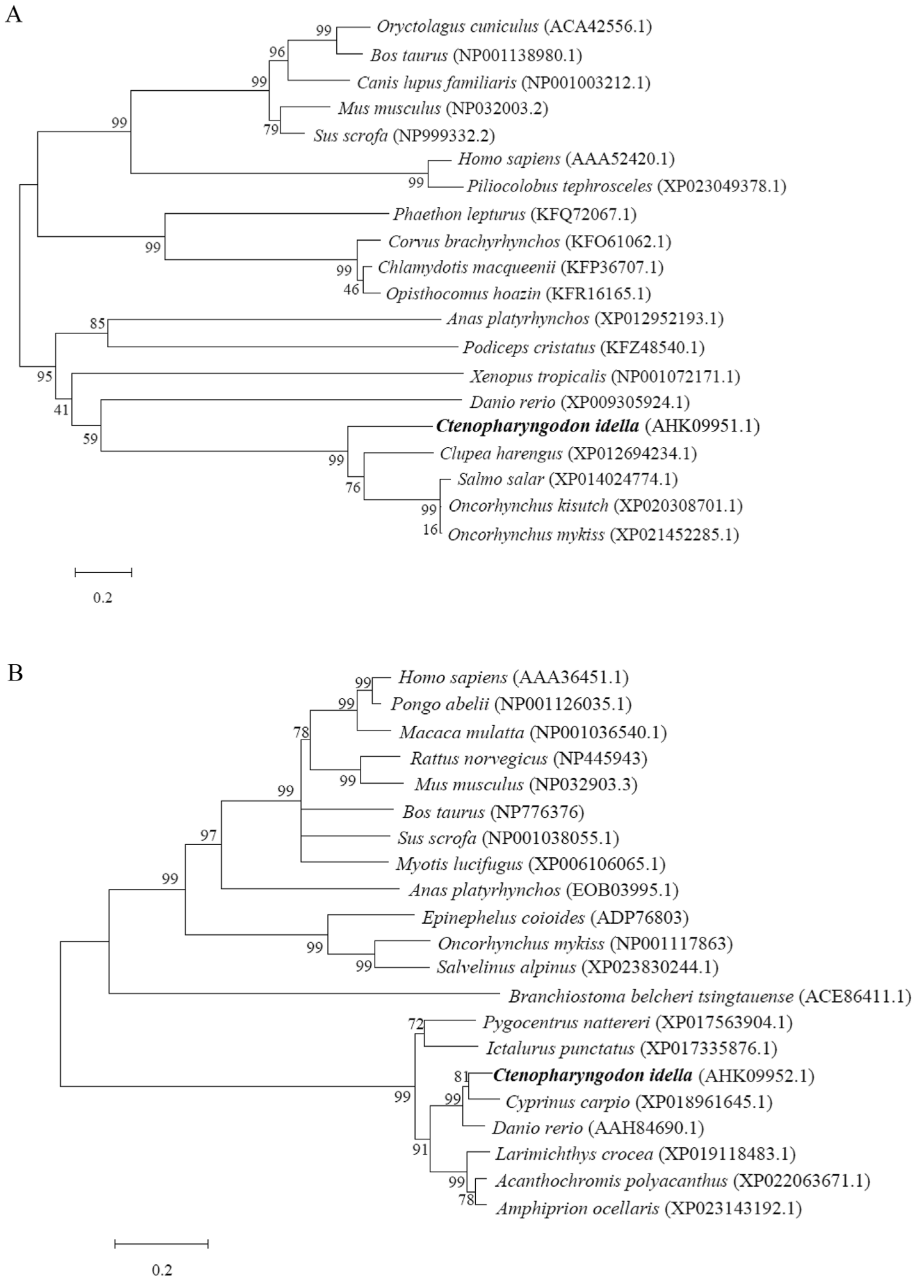

Fig. 4. Phylogenetic relationships of FVIII (A) and PLG (B) proteins.

PCR Detection System (Bio-Rad, Hercules, USA). Amplifications were performed in triplicate in a total volume of 10 μL, containing 5 μL of 2 × SYBR Premix Ex Taq (Toyobo, Osaka, Japan), 1 μL of diluted cDNA, 0.4 μL of each primers and 3.2 μL ddH$_2$O. The cycle conditions were as follows: 1 cycle of 95 °C for 3 min, 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. The specificity of amplification products was verified by melting curve analysis. The relative expression levels of genes were analyzed using the Ct method ($2^{-\triangle\triangle}$Ct method). The primers were FVIII YF and FVIII YR for qPCR of *CiFVIII*, and PLG YF and PLG YR for *CiPLG*, and β-actinYF and β-actinYR for the reference gene of *β-actin* and 18 s YF and 18 s YR for the reference gene of *18 s*. The primers for qPCR are listed in Table 1.

2.6. Comparison of serum immune factor levels

The tail vein blood from five individuals of each group (R♂, O♂, F1 and f1) was collected, centrifuged at 3000 rpm for 10 min, and stored at −80 °C. Antioxidant ability and serum immune factor level analysis was performed in R♂, O♂, F1 and f1 individuals of *C. idella*. The total antioxidant capacity (T-AOC), total superoxide disumutase (T-SOD), catalase (CAT) and lysozyme (LSZ) were measured by commercial kits (Jiancheng bioengineering institute, Nanjing, China), according to the manufacturer's instructions. The complement 3 detection kit (ERKN company, Wenzhou, China), and serum SOD, immunoglobulin M (IgM), interferon α (IFN-α) and interleukin 1 beta (IL-1β) detection kits (Bomei Biotechnology, Hefei, China) were used for level analysis of corresponding factors, following the manufacturer's instructions.

2.7. Data analysis

In order to compare control vs the experimental group, differences in the relative expression levels of *CiFVIII* and *CiPLG* among tissues and over different time points after GCRV infection, the one-way ANOVA and Duncan's Multiple Range tests were performed using SPSS Version

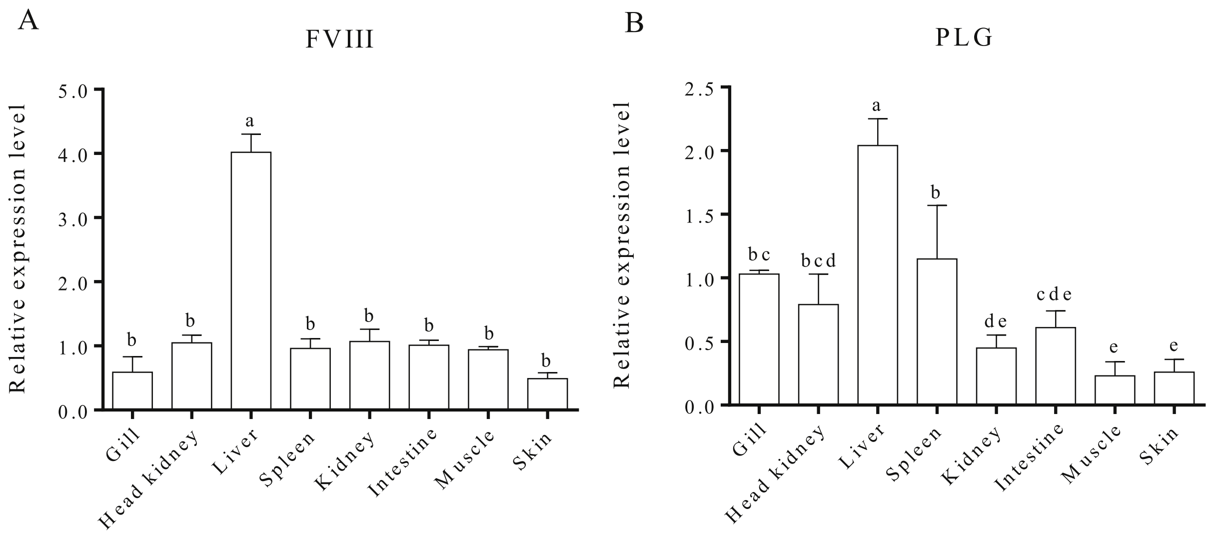

Fig. 5. Tissue expression levels of *CiFVIII* (A) and *CiPLG* (B) of *C. idella*. The letters a, b, c, d and e indicate the significant difference ($P < 0.05$) of gene expression levels between tissues.

25.0 (SPSS Inc., Chicago, USA) with a significance level of $P < 0.05$. Correlation between expression levels of *CiFVIII* and *CiPLG* was analyzed by applying Pearson's correlation analysis and a significance level of $P < 0.05$ was adopted.

3. Results

3.1. Full-length cDNA sequences and protein domains of CiPLG and CiFVIII

Both 5′-RACE and 3′ RACE PCR were performed to obtain the full-length cDNA sequences of *PLG* and *FVIII*, respectively. The full-length cDNA sequence of *CiFVIII* (GenBank accession number: KF937389) is 2478 base pair (bp), which consisted of a 118 bp 5′-terminal untranslated region (UTR), an open reading frame (ORF) of 1965 bp, and a 395 bp 3′-UTR (Fig. S1). The ORF encoded a polypeptide of 654 amino acids with a predicted molecular weight of 71.99 kDa, and a theoretical isoelectric point of 7.53. A 2907 bp nucleotide sequence representing the full-length cDNA of *CiPLG* was obtained (GenBank accession number: KF937390). The cDNA sequence of *CiPLG* consisted of a 40 bp 5′-terminal UTR, an ORF of 2133 bp, and a 289 bp 3′-UTR. The full-length cDNA sequence and the deduced amino acid sequence of *CiPLG* are shown in Fig. S2. The ORF encoded a 710 amino acid polypeptide with a predicted molecular weight of 81 kDa and a theoretical isoelectric point of 6.70. For protein domain prediction, the CiFVIII contained conserved domains of a CUB (interval: 37–150 aa), a LCCL (interval: 154–239 aa), and a FA58C domain (interval: 253–395 aa) (Figs. 1 and 3). The CiPLG contained the following conserved domains: a PAN-AP (interval: 22–65 aa), four KR (109–189, 190–271, 283–365 and 370–452 aa) and a Tryp_SPc (interval: 482–703 aa) (Figs. 2 and 3).

3.2. Phylogenetic trees of FVIIIs and PLGs

To determine the phylogenetic relationship of *FVIIIs* and *PLGs*, phylogenetic trees for the two genes were constructed. For the *FVIIIs*, *CiFVIII* was the most closely clustered with that of *Clupea harengus* (XP012694234.1), and then clustered with salmonoid fishes of *Salmo salar* (XP014024774.1), *Oncorhynchus kisutch* (XP020308701.1) and *Oncorhynchus mykiss* (XP021452285.1). For the *PLGs*, *CiPLG* was firstly clustered with that of *Cyprinus carpio* (XP018961645.1) and *Danio rerio* (AAH84690.1) followed by those of *Larimichthys crocea* (XP019118483.1), *Acanthochromis polyacanthus* (XP022063671.1) and *Amphiprion ocellaris* (XP023143192.1) (Fig. 4).

3.3. Tissue expression profile of CiFVIII and CiPLG

qPCR was carried out to investigate mRNA expression levels of *CiFVIII* and *CiPLG* in different tissues of *C. idella*. The relative expression levels of *CiFVIII* in gill, head kidney, liver, spleen, kidney, intestine, muscle and skin were 0.651 ± 0.270, 1.048 ± 0.238, 4.015 ± 0.550, 0.960 ± 0.296, 1.074 ± 0.329, 1.008 ± 0.110, 0.944 ± 0.079 and 0.493 ± 0.155, respectively. Among these tissues, *CiFVIII* transcripts were most abundant in the liver (significantly higher than all of other tissues, $P < 0.05$) followed by the kidney and head kidney, and the lowest in the skin (Fig. 5A). The relative expression levels of *CiPLG* in gill, head kidney, liver, spleen, kidney, intestine, muscle and skin were 1.034 ± 0.058, 0.794 ± 0.421, 2.039 ± 0.359, 1.151 ± 0.415, 0.448 ± 0.179, 0.613 ± 0.224, 0.230 ± 0.085 and 0.259 ± 0.017, separately. The highest expression level of *CiPLG* was observed in liver (significantly higher than all of other tissues, $P < 0.05$) and the lowest expression level was detected in muscle (Fig. 5B).

3.4. Expression level change of CiFVIII and CiPLG after GCRV challenge

qPCR was performed to detect mRNA expression level changes of *CiFVIII* and *CiPLG* in different tissues of *C. idella* at different time points after viral infection. After GCRV challenge, the relative expression levels of both *CiFVIII* and *CiPLG* in all detected tissues exhibited an overall trend of first up-regulation and then down-regulation (Figs. 6 and 7). The *CiFVIII*, expression levels in gill, head kidney and spleen all reached the maximum level at 72 h post infection, and were significantly higher than those of all other time points (except in the spleen). In liver, the expression level of *CiFVIII* reached the maximum at 48 h post-challenge, and was significantly higher than those of all other time points (except 72 h) post-infection ($P < 0.05$) (Fig. 6). The *CiPLG*, expression levels in gill, intestine and liver all reached the peak at 72 h after challenge. In liver, the transcription levels of *CiPLG* at the time points of 72 and 96 h post-infection were significantly higher than those of all other time points ($P < 0.05$, Fig. 7). Correlations between expression levels of *CiFVIII* and *CiPLG* in tissues of *C. idella* after GCRV infection were investigated. The results showed that expression levels of *CiFVIII* and *CiPLG* were significantly positively correlated in spleen (Pearson correlation coefficient: 0.729, *P*: 0.026). The expression levels of the two genes were also positively correlated in muscle, gill, head kidney, liver and intestine, while no significant correlations were observed.

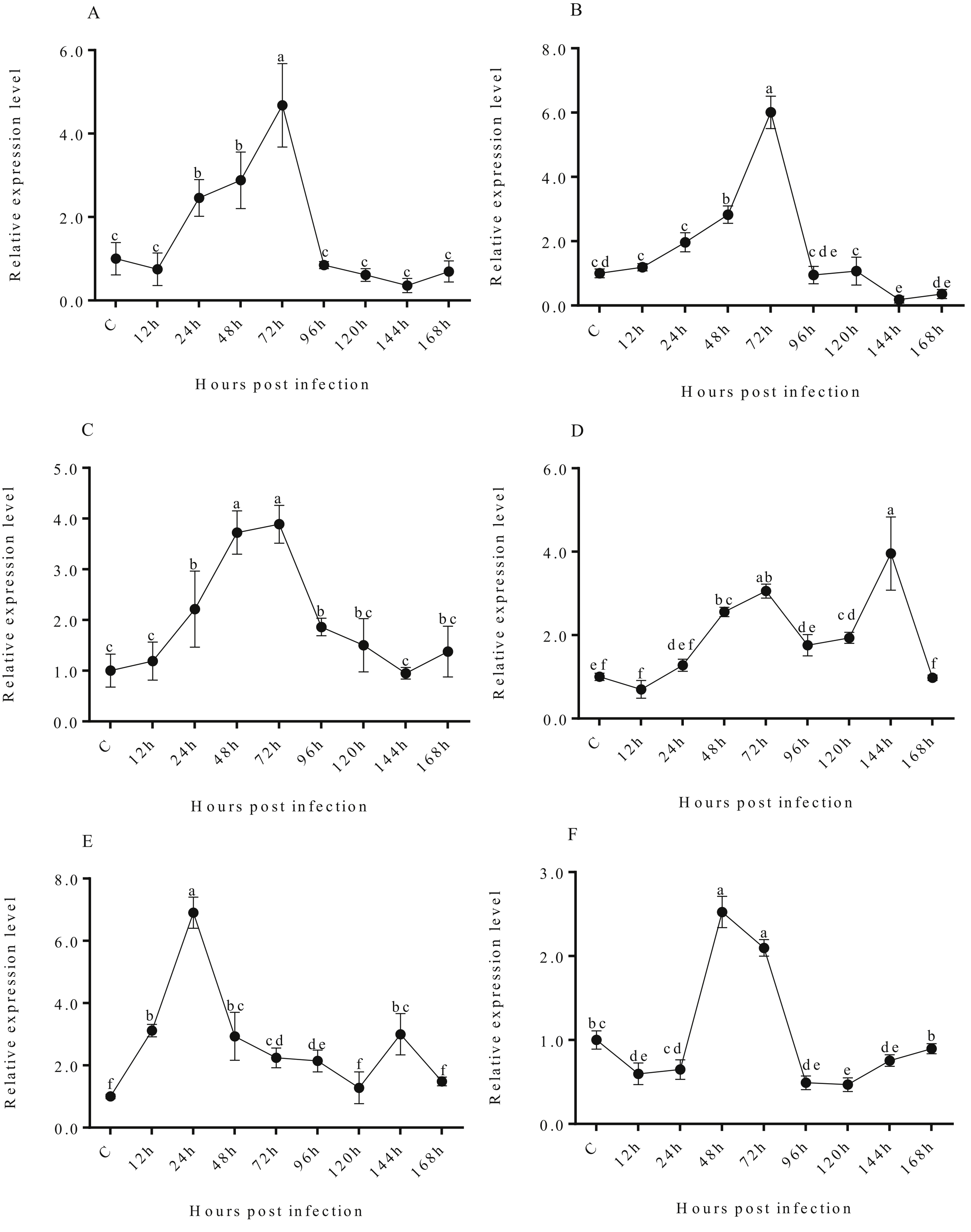

Fig. 6. Expression levels of *CiFVIII* in tissues after GCRV infection. A: expression level analysis of *CiFVIII* in gill; B: expression level analysis of *CiFVIII* in head kidney; C: expression level analysis of *CiFVIII* in spleen; D: expression level analysis of *CiFVIII* in muscle; E: expression level analysis of *CiFVIII* in intestine; F: expression level analysis of *CiFVIII* in liver. Different letters a, b, c, d, e, and f indicate the significant difference of gene expression levels between different time points ($P < 0.05$).

3.5. Comparison of serum-immune-factor level

The antioxidant ability and serum immune factors in R♂, O♂, F1 and f1 individuals were detected. For antioxidant ability comparison, the activity of T-AOC in R♂ (male parent with GCRV resistance) was significantly higher than those in **O**♂ (ordinary male parent fish) ($P < 0.01$). The T-AOC activities in F1 progeny was also significantly higher than those in f1 individuals ($P < 0.01$) (Table 2). For serum immune factor analysis, the levels of LSZ and IgM in R♂ fishes were significantly higher than those in ordinary individuals (**O**♂) ($P < 0.01$). The activity of LSZ in F1 individuals was significantly higher than that in f1 individuals ($P < 0.01$), and also higher than that

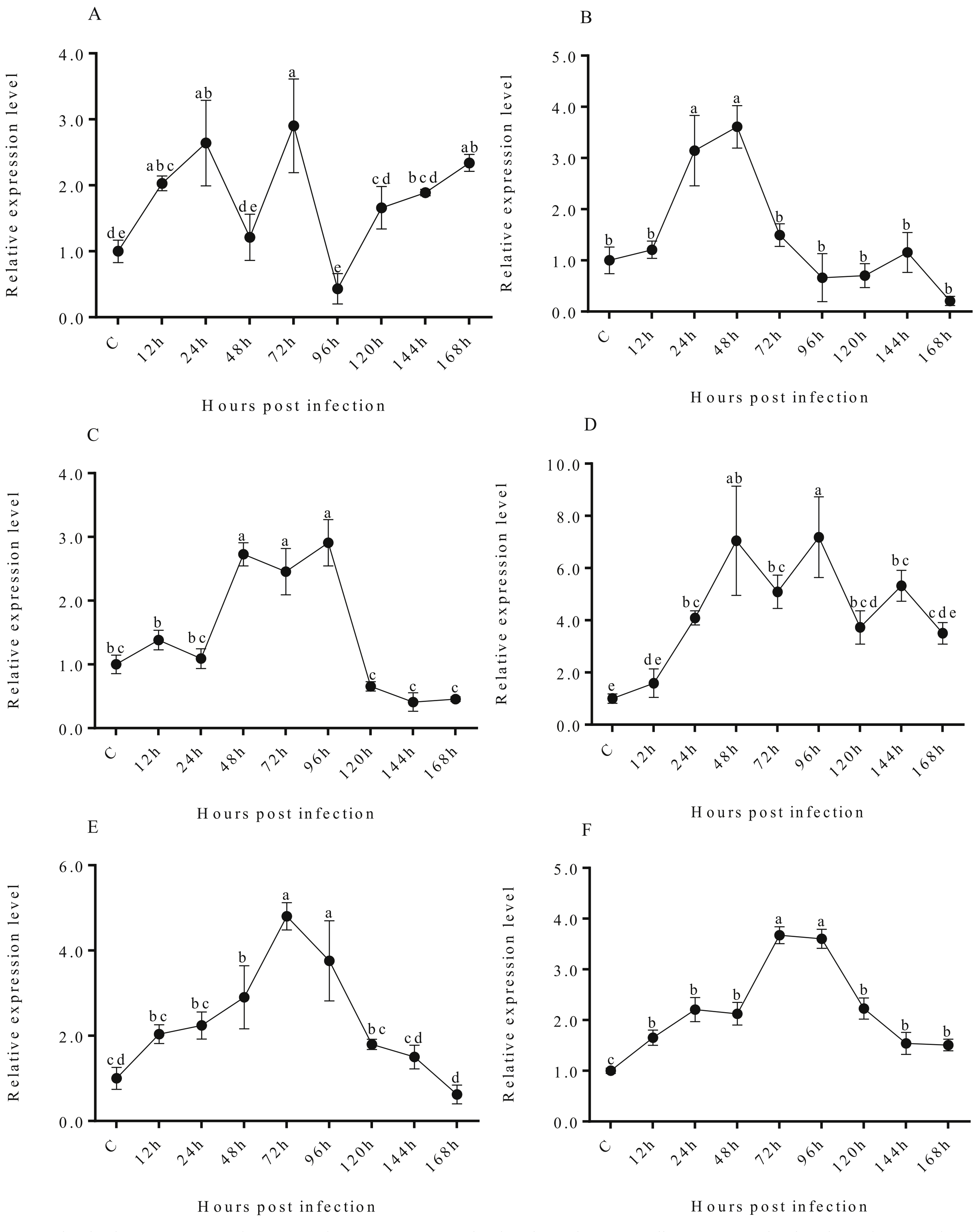

Fig. 7. Expression levels of *CiPLG* in tissues after GCRV infection. A: expression level analysis of *CiPLG* in gill; B: expression level analysis of *CiPLG* in head kidney; C: expression level analysis of *CiPLG* in spleen; D: expression level analysis of *CiPLG* in muscle; E: expression level analysis of *CiPLG* in intestine; F: expression level analysis of *CiPLG* in liver. The letters.
a, b, c, d, e, and f indicate the significant difference of gene expression levels between different time points ($P < 0.05$).

in **O**♂. The level of IgM in F1 individuals was significantly higher than those in **O**♂ and f1 individuals ($P < 0.01$). On the contrary, the activities of C3 and IL-1β in R♂ were significantly lower than those in O♂, F1 and f1 individuals ($P < 0.01$) (Table 2).

4. Discussion

Grass carp (*C. idella*) is one of the most important freshwater cultured fish species in China. However, the cultivation industry of this species has been greatly hampered by a severe hemorrhagic disease caused by GCRV [17,18]. After GCRV infection, oxidative stress is

Table 2

Serum immune factor level detection.

Index	R ♂	O ♂	F1	f1
T-AOC (U/mL)	8.17 ± 0.16^{A}	5.71 ± 0.58^{B}	5.48 ± 0.61^{B}	4.14 ± 0.19^{C}
CAT (U/mL)	5.83 ± 0.10^{C}	5.18 ± 0.19^{D}	7.01 ± 0.21^{A}	6.49 ± 0.19^{B}
T-SOD (U/mL)	113.21 ± 8.84^{A}	105.43 ± 0.75^{A}	107.27 ± 3.16^{A}	96.21 ± 0.65^{B}
LSZ (U/mL)	292.01 ± 15.68^{A}	249.73 ± 9.34^{B}	258.14 ± 11.49^{B}	204.08 ± 14.68^{C}
C3 (mg/L)	274.17 ± 6.71^{C}	299.73 ± 4.39^{B}	300.31 ± 2.65^{B}	314.62 ± 4.51^{A}
IFN-α (ng/L)	22.06 ± 2.05^{B}	8.76 ± 1.06^{C}	33.34 ± 7.22^{A}	19.75 ± 4.31^{B}
IL-1β (ng/L)	17.21 ± 1.77^{C}	55.23 ± 3.22^{B}	53.31 ± 3.01^{B}	103.03 ± 3.23^{A}
IgM (ng/L)	17.44 ± 1.92^{A}	10.53 ± 0.69^{B}	16.36 ± 1.52^{A}	9.63 ± 0.50^{B}

R♂: male parent with GCRV resistance; O♂: ordinary male parent fish; F1: progeny of R♂ and ordinary female fish; f1: progeny of O♂ and ordinary female parent fish. Different superscript capital letters (A, B and C) indicate a significant difference of $P < 0.01$ between mean values in the same row.

induced by the process of viral replication, which causes extensive loss of antioxidants and formation of lipid peroxidation. In these fish, the immune factors *IL-1β* and *TNF-α* are also synthesized and released [19,20]. Two apoptotic pathways, the intrinsic pathway and the extrinsic pathway, were found to be triggered by different stimuli [21]. As one of the stimulus signals, oxidative stress triggered the intrinsic pathway and resulted in degradation of cellular substrates [22]. In the present study, we found that *IL-1β* levels in R♂ individuals was extremely lower than those in O♂ individuals, indicating that a more severe oxidative stress may exist in ordinary *C. idella* individuals than GCRV-resistance individuals. The protective effect against oxidative damage could be reflected by antioxidant enzymes, such as T-AOC [23]. Antioxidant enzymes and genes are the principal cellular protective components against oxidative stress in fish [24]. In the present study, antioxidant abilities in R♂, O♂, F1 and f1 individuals were compared. The results indicated that the activity of T-AOC in the male parent with GCRV resistance (R♂) was significantly higher than those in the ordinary male parent fish (**O**♂). The T-AOC activity in F1 individuals (progeny of R♂) was also significantly higher than those in f1 individuals (progeny of **O**♂). This indicated that *C. idella* individuals of GCRV-resistance may have stronger antioxidant ability than ordinary fish.

As one of the most effective systems to defend against pathogens, teleost fish are able to produce IgM class antibody by their B-cells. After activation by pathogens or antigens, the B-cells proliferate and differentiate into long lasting memory cells and plasma cells, which secrete specific antibodies [25–27]. As natural antibodies, IgM can present in relatively high concentration in fish serum, and its neutralization reaction may help to control the later stage of viral infections [28,29]. Lysozyme activity is one of the most represented biomarkers used for immune status evaluation of many fish species, where increase in activity boosts immunity strength of the fish [30,31]. In the present study, we found that the levels of IgM and LSZ in R♂ fish were significantly higher than those in ordinary individuals (**O**♂), and their levels in progeny of R♂ fish (F1) were also higher than those both in **O**♂ and its progeny (f1). The above results indicated that the antibody response ability induced by IgM and the LSZ levels may be the cause of viral resistance difference of *C. idella* individuals, and the T-AOC, IgM and LSZ levels may be able used as criteria for GCRV resistance grass carp breeding.

After an injury caused by infection, coagulation factors are essential for the formation of blood clots, which functions to protect the body by sealing off damaged blood vessels and preventing the loss of blood. The *FVIII* is a serum protein in the coagulation cascade that nucleates the assembly of a membrane-bound protease complex on the surface of activated platelets at the site of a vascular injury [32]. In response to injury, *FVIII* is activated from an inactive form and separates from von Willebrand factor (VWF) then interacts with coagulation factor IX to set off a chain of chemical reactions that form a blood clot [33]. *FVIII* and *PLG*, as two important members of the coagulation and fibrinolysis systems, are tightly connected with the innate immune system in response to pathogens. Clotting can be resolved by fibrinolysis, which triggers *PLG* to induce a distinct enzymatic cascade that leads to the removal of fibrin deposits [34–36].

In the present study, the full-length cDNAs of *CiFVIII* and *CiPLG* were obtained. *FVIII* is produced chiefly by liver sinusoidal cells and endothelial cells outside the liver throughout the body [33], which is in accordance with the highest expression of *CiFVIII* occurring in the liver tissue of *C. idella*. Conserved domains including CUB, LCCL and FA58C were identified in the CiFVIII. The CUB domain takes part in prevention of the elongation of ultra-large VWF strings on activated endothelial cells and inhibits platelet adhesion/aggregation on the collagen surface [37]. The LCCL domain has been found in various metazoan proteins in association with complement B-type domains, C-type lectin domains, von Willebrand type A domains and CUB domains [38–40]. Proteins that contain an LCCL domain may serve to protect the organism from pathogen infection by signaling to innate immune cells in the periphery, thus amplifying the cytokine response [41]. The FA58C domain present in blood coagulation factors and thought to be involved in cell adhesion [42]. For *CiPLG*, conserved domains of PAN-AP, KR and Try-SPC were also observed. Highly conservative domains and closely clustered relationships of *CiFVIII* and *CiPLG* with corresponding proteins in other fish species indicated that the main functions of the two genes may be similar to those of other fish. The functional study of domains identified in *CiFVIII* and *CiPLG* will be performed in future studies. Transcriptional levels of the *CiFVIII* and *CiPLG* were investigated after GCRV infection. The results showed that the relative expression levels of both *CiFVIII* and *CiPLG* in all detected tissues exhibited an overall trend of initial up-regulation with positive correlations between expression levels of *FVIII* and *PLG*. This indicated that both *CiFVIII* and *CiPLG* may be activated by GCRV infection, and a series of processes involved in coagulation, fibrinolysis and innate immune systems may be initiated.

In conclusion, we cloned and conducted molecular and expression analysis of *CiFVIII* and *CiPLG*. The antioxidant ability and serum immune factor levels were also compared between *C. idella* individuals of different GCRV resistance. It was found that *C. idella* individuals with GCRV-resistance may have less oxidative stress while stronger antioxidant ability than ordinary fish. Expression levels of *CiFVIII* and *CiPLG* can be activated by GCRV stimulation, and a combined process involved in coagulation, fibrinolysis and innate immune reactions may function simultaneously in viral defense.

Acknowledgments

The Project was funded by the National Natural Science Foundation of China (31802288, 31572615) and the China Postdoctoral Science Foundation (2017M612560, 2018T110833).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

doi.org/10.1016/j.fsi.2018.12.024.

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