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Cloning of six *serpin* genes and their responses to GCRV infection in grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

Grass carp, an economically important aquaculture fish, is very sensitive to Grass Carp Reovirus (GCRV). Haemorrhagic disease caused by GCRV infection can cause large-scale death of first-year grass carp, thereby severely restricting the intensive culture. *Serpins* (serine protease inhibitors) belong to the protease inhibitor gene family and are involved in numerous physiological and pathological processes, particularly coagulation and anticoagulation. Reports on grass carp *serpins* are scarce. Thus, we cloned six grass carp *serpin* genes (*serpinb1*, *serpinc1*, *serpind1*, *serpinf1*, *serpinf2b* and *serping1*) in this study. Molecular evolution showed that serpins between grass carp and zebrafish or carp are the closest relatives. SERPIN domains in these 6 *serpins* and reactive centre loop (RCL) along with their cleavage sites of 5 *serpins* (*serpinb1*, *serpinc1*, *serpind1*, *serpinf2b* and *serping1*) were predicted. Real-time quantitative PCR (RT-qPCR) showed that these *serpins* displayed tissue significance. Among them, *serpinc1*, *serpind1*, *serpinf2b* and *serping1* had the highest expression levels in the liver. After GCRV infection, RT-qPCR showed that the liver-enriched serpins were significantly changed. Key procoagulant factor genes (*kng-1*, *f2*, *f3a*, *f3b* and *f7*) and anticoagulant genes (*tpa*, *plg*, *thbd*, *proc* and *pros*) also showed significant changes on the mRNA level. Comprehensive comparative analysis showed that the up-regulated expression of key clotting factor genes was more prominent than that of main anti-coagulation factor genes. Thus, the function of coagulation may be more dominant in grass carp during the GCRV infection, which may cause overproduction of thrombi. The *serpins* were involved in GCRV infection and liver-enriched *serpins* participate in the interaction between coagulation and anticoagulation. This study provided new insights into further research on the biological functions of grass carp *serpins* and clarifying the molecular mechanism of GCRV affecting the homeostasis of grass carp blood environment.

1. Introduction

In normal living organisms, numerous proteases and their corresponding protease inhibitors restrict each other and are in a state of dynamic equilibrium [1]. In 1894, Fermi and Pernossi et al. discovered the first *serpin* (serine protease inhibitor) in human blood. Shultze et al. isolated this *serpin* gene and named it α1PI in 1995 [2–4]. Hunt and Dayhoff subsequently compared the sequence similarities between α1PI, ovalbumin and ATIII and finally demonstrated the existence of the *serpin* gene [5]. According to the similarity between different *serpin* genes, the *serpins* are divided into 16 clades [6], which constitute a large family of serine protease inhibitors. More than 3000 *serpin* genes have been identified in many species, including animals, plants, viruses, bacteria and archaea [6].

A typical serpin protein has a conserved SERPIN domain that contains three β-sheets (β-sheet A, β-sheet B and β-sheet C), 7-9 α-helices and a reactive centre loop (RCL) [7,8]. RCL serves as a functional region responsible for the interaction with the target enzyme, and its sequence determines serpin specificity [6]. In the proteolysis process, serpin proteins inhibit the proteolytic activity of serine/cysteine proteases through a “suicide” mechanism. The cleavage site of the RCL in the serpin protein is then recognised, bound and cleaved by the corresponding target protease [8]. The terminal amino acid of RCL is inserted into the β-sheet A, which is called a transition from a “stressed

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(S) to relaxed (R)," and leads to the formation of an irreversible covalent complex between the target enzyme and the serpins, resulting in their simultaneous inactivation; thus, their corresponding biological functions are realised [9,10]. Serpin proteins involve in multiple basic biology processes, including blood coagulation, complement activation, fibrinolysis, angiogenesis, inflammation and tumour suppression by inhibiting the activity of serine proteases (SPs) or cysteine proteases (CPs) [7].

The balancing process between blood coagulation and anticoagulation is the key to the normal body maintenance of blood flow and prevention of blood loss [11]. Coagulation and anticoagulant factors can restrain each other to ensure that the coagulation and anticoagulation process are in a dynamic equilibrium [11–13]. The normal blood clotting process, which eventually causes the change in the blood from a liquid to a colloidal state, involves the formation of prothrombin activators, thrombin and fibrin [12,14]. According to the types of different coagulation factors, the process of coagulation cascade is divided into the intrinsic pathway (also known as contact activation pathway) and the extrinsic pathway (also known as tissue factor pathway) [15]. The high molecular weight kininogen (HMWK), derived from Kininogen-1 (KNG-1, encoded by *kng-1*), f12 and prekallikrein, are key molecules that initiate the intrinsic pathway [16,17]. When f12 is activated to f12a, it further converts f11 to f11a. Then, f11a activates f9 to form f9a. The f9a forms a tenase complex with f8a, which further activates f10 to form f10a [18]. In the extrinsic pathway, the tissue factor (TF, also known as f3) forms a complex with f7a. This complex can activate f9 and f10; then, f9a further promotes f10 activation to form f10a [15,19]. Finally, f10a and its cofactor f5a from both endogenous and exogenous pathways form a prothrombinase, which activates prothrombin (f2) to thrombin (f2a) [18]. Thrombin transforms fibrinogen into fibrin, and the blood clot formed by fibrin polymerisation exerts coagulation function [18].

Most of the coagulation factors involved in blood coagulation in the living body are serine proteases, such as f2a, f5a, f7a, f8a, f9a, f10a and f11a [20]. Serine proteases are also involved in the anticoagulant process, such as protein C (PROC, encoded by the *proc* gene) [21], plasmin [22], tissue plasminogen activator (tPA, encoded by the *tpa* gene) and the urokinase plasminogen activator (uPA, encoded by the *upa* gene) [23]. The complex formed by the combination of thrombin and the thrombomodulin (TM, encoded by the *thbd* gene) activated protein C. With the help of protein S (PROS, encoded by the *pros* gene) [21], the activated protein C (APC) inactivates f5a and f8a, thus exerting anticoagulation. During fibrinolysis, both tPA and uPA activate plasminogen (PLG, encoded by the *plg* gene) into plasmin, which degrades water-insoluble fibrin in the thrombus and dissolves the thrombus [23]. Serpin is one of the most important protease inhibitors in the body [24]. It is involved in the process of coagulation and anticoagulation. Studies have shown that in the presence of heparin, serpinc1 and serpind1, the most important factors regulating blood anticoagulation cascades, can rapidly inhibit thrombin. Of the two, serpinc1 is particularly important [9,10]. Serpinf2 (α2-antiplasmin) acts as an inhibitor of APC and can bind irreversibly with APC to exert its coagulation function [21]. Serpinf2 also acts as the most important inhibitor of plasmin and is involved in the inhibition of fibrinolysis [23]. Serping1 participates in blocking endogenous clotting pathways [25]. Therefore, there is a mutual restriction between serine protease and serpin in the mutual antagonism of coagulation and anticoagulation.

Grass carp is an important aquaculture species with huge economic value and widespread distribution. It is very sensitive to GCRV. Grass carp infected by GCRV can develop haemorrhagic disease and large-scale death of infected first-year grass carp take place, thereby severely restricting the intensive culture [26]. Previous studies in our laboratory suggested that during the process of GCRV infection, differentially expressed genes (including the *serpin* genes) in the four tissues (gill, liver, spleen and intestine) are mainly concentrated in the "complement and coagulation" cascade signalling pathway [27]. Abnormal blood clotting can cause pathological bleeding and thrombosis in organisms [12]. Thus, grass carp haemorrhagic disease caused by GCRV may be related to coagulation abnormalities.

Serpins are among the most widely studied protease inhibitors [28]. However, the serpins in grass carp are rarely studied. Only the biological function of serpinf1 in fatty inflammation has been studied in grass carp [29]. The cloning and expression analysis of these 6 *serpins* in grass carp facilitate the further research on the structure and related biological functions of the *serpin* gene family in grass carp and may provide new insights into the molecular mechanism of GCRV affecting the homeostasis of blood environment in grass carp.

2. Materials and methods

2.1. Experimental fish

Six-month-old grass carp (15 ± 3 cm, 40 ± 10 g) for gene cloning and GCRV infection experiments were provided by the Guanqiao Experimental Base of the Institute of Hydrobiology, Chinese Academy of Sciences. Prior to the grass carp GCRV infection experiment, the experimental fish were placed in a 500 L circulating water system at 28 °C with adequate dissolved oxygen, penicillin (100 U/ml) and streptomycin (100 μg/ml). Normal feeding was conducted for 1 week, and experimental fish without abnormal conditions were used for subsequent experiments. The absence of abnormalities on the one hand referred to no abnormalities in outward appearance, feeding and activity; on the other hand, we used the PCR method to detect the GCRV and found no GCRV infection.

2.2. Cloning of full-length cDNA from grass carp serpins

Respectively, the homologous *serpin* sequences of zebrafish (*Danio rerio*) from the NCBI database (*D. rerio serpinb1*, Accession no. NM_001002653; *D. rerio serpinc1*, Accession no. NM_182863; *D. rerio serpind1*, Accession no. NM_182880; *D. rerio serpinf2b*, Accession no NM_001080010; and *D. rerio serping1*, Accession no. NM_001123285) were downloaded. A comparison between these sequences and the grass carp genome database was performed to obtain matched sequences [30]. Furthermore, the CDS sequence of grass carp *sepinf1* was downloaded directly from NCBI. The flanking non-coding region sequences of all these grass carp *serpin* genes were obtained by the rapid amplification of cDNA ends (RACE) PCR according to the SMARTer® RACE 5′/3′ Kit instructions. The 5′ RACE and 3' RACE PCR products were recovered by gel cutting and purification and were ligated respectively into pMD18-T vector for sequencing and analysis. The entire *serpin* gene sequences were obtained by comparing PCR products with partial cDNA from genome database and discarding the obtained overlapping region sequence and vector sequence. All primers designed for gene cloning and sequence verification are listed in Supplemental Table 1.

2.3. Bioinformatics analysis of grass carp serpins

Based on DNAMAN analysis of full-length cDNA sequences and genomic sequences of *serpin*, the schematic diagrams of the genomic structure of grass carp *serpins* were drawn. Signal peptide prediction was performed using Signal 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The SMART analysis service (http://smart.embl-heidelberg.de/) was used to perform domain predictions. Tridimensional structure predictions of the grass carp serpin protein were performed using SWISS-MODEL (https://swissmodel.expasy.org/interactive/), and schematics were obtained using Swiss-PdbViewer 4.1

software. Using DNAMAN, the cleavage sites of the serpin proteins of grass carp were identified by comparing them with the cleavage sites of the RCLs of human homologous serpin proteins, which were attained from the literature search [9]. Based on the NCBI database (http://www.ncbi.nlm.nih.gov/sites/gquery), CDS region fragments of homologous *serpin* genes in zebrafish (*Danio rerio*), carp (*Cyprinus carpio*), African clawed frog (*Xenopus tropicalis*), chicken (*Gallus Galus*), mouse (*Rattus norvegicus*), cow (*Bos taurus*) and human (*Homo sapiens*) were attained. The accession numbers of the related sequences were located in Supplemental File S1. Multiple sequence alignment analysis of serpin protein sequences between multiple species was performed using the ClustalW 2.1 program (http://www.ebi.ac.uk/tools/clustalw2.1). The amino acid sequences of these species were first analysed using MEGA (7.0) and then the phylogenetic tree (Bootstrap = 1000) was constructed using the neighbour-joining (NJ) method [31].

2.4. GCRV infection and sampling

The GCRV-GD108 virus solution used in this experiment was prepared by our laboratory [32]. Before the GCRV infection experiment was conducted, three fish were randomly selected as a control group. Sampling on a total of 11 tissues including gill, liver, spleen, intestine, kidney, head kidney, muscle, skin, blood, brain and heart were performed. At the same time, the experimental fish were intraperitoneally injected at a dose of 2% (vol/g) virus fluid (1.20×10^3 copy/μl) to fish quality. Subsequently, the liver tissues were sampled. First, the infected group after the challenge was sampled for six consecutive days and then continued to be sampled on the 8th, 11th and 14th days. The fish (n = 3) were randomly selected in the incubation period (8 days before), and live grass carp (n = 3) with obvious symptoms were collected from the onset (8 days later). All samples were triturated with TRIzol reagent (Invitrogen, USA) and stored at −80 °C for cryopreservation.

Prior to sampling, eugenol was used to anesthetise the experimental fish, and utmost care was done to minimise injuries to them. The feeding and sampling of all experimental fish mentioned above were carried out in accordance with the relevant policies and regulations of the Guidelines for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006), and the experimental procedures were recognised by the Academic committee of the Institute of Hydrobiology, Chinese Academy of Sciences (CAS).

2.5. Tissue distribution of grass carp serpins

In order to understand the tissue distribution of *serpins* in healthy grass carp, the mRNA expression levels of *serpins* were measured in 11 tissues of control grass carp group mentioned in 2.4 using RT-qPCR. The expression level of each gene in the muscle tissue (0-day) was set as the baseline (1.0-fold). RT-qPCR was performed by the CFX96™ Real-Time PCR Detection System (Bio-Rad, USA) using AceQ qPCR SYBR® Green Master Mix (Vazyme Biotech Co., Ltd). Taking *β-actin* as an internal control, the RT-qPCR data were analysed using the $2^{-\triangle\triangle Ct}$ method [33,34]. The primers are listed in Supplemental Table 2.

2.6. Expression of serpins, coagulation and anticoagulation factor genes in the liver of grass carp after GCRV-GD108 infection

In order to further investigate the expression level of grass carp *serpins* before and after GCRV infection, RT-qPCR was also used to detect the transcript expression patterns of liver-enriched *serpins* at different time points before and after GCRV infection (0-day, 1-day, 2-day, 3-day, 4-day, 5-day, 6-day, 8-day, 11-day and 14-day). To discuss the constraining relationship between coagulation and anticoagulation in the blood system, we detected key coagulation factors (*kng-1*, *f2*, *f3a*, *f3b*, *f7*, *f9* and *f10*) and anticoagulants in the liver (*tpa*, *plg*, *thbd*, *proc* and *pros*). During data processing, *β-actin* was used as an internal reference gene for expression analysis [33], and the expression level (1.0-fold) of each gene of control group (0-day) in the liver tissues was set as a benchmark for comparative expression analysis. The analysis of relative gene expression levels was performed using the $2^{-\Delta\Delta Ct}$ method [34]. The primers are listed in Supplemental Table 2 and Supplemental Table 3.

2.7. Data analysis

In the present study, the data generated by RT-qPCR were calculated by GraphPad Prism 5.0 and were represented as mean ± standard deviation of three independent experiments. Significant differences in data were assessed by the two-tailed *t*-test using GraphPad instat 3.0. $P < 0.05$ was considered to be statistically significant difference.

3. Results

3.1. Cloning and characterisation of serpins from grass carp

Six full-length cDNA sequences (*serpinb1*, *serpinc1*, *serpind1*, *serpinf1*, *serpinf2b* and *serping1*) of the *serpin* gene family were cloned in grass carp (*Ctenopharyngodon idella*). The cDNA lengths and sequences of the six grass carp *serpins* genes are shown in Supplementary Table S4 and Fig. S1. The open reading frames (ORFs) ranged from 1143 bp to 1806 bp. The genomic structures of six *serpins* were analysed to obtain the exons and introns of each cDNA sequence and the number and length of related sequences (Fig. S2). The linking region between the exons and introns satisfied the "GT-AG" rule for eukaryotic interrupted gene.

Signal peptide predictions showed that serpinc1 (aa 1–20), serpind1 (aa 1–19), serpinf1 (amino acids (aa) 1–19), serpinf2b (aa 1–19) and serping1 (aa 1–17) have signal peptides at the N-terminus (Fig. S3). This signal peptide was not predicted in serpinb1. The SERPIN domains was identified in all six serpin proteins, namely, serpinb1 (aa 13–379), serpinc1 (aa 76–446), serpind1 (aa 146–507), serpinf1 (aa 53–403), serpinf2b (aa 114–453) and serping1 (aa 250–600) (Fig. S3). The serping1 protein was found to contain one Ig domain (aa 16–95) and one IgC2 domain (aa 117–203). The SWISS-MODEL was used to predict the tertiary structure of grass carp six serpin proteins, and the SPDBV software was used to display the predicted tertiary structure diagram (Fig. S4). All six serpin proteins were conserved, and the SERPIN domain contained typical 7-9 α-helices and three β-sheets (β-sheet A, β-sheet B and β-sheet C). Combined with literature search [9], the cleavage sites of five serpin proteins (serpinb1, serpinc1, serpind1, serpinf2b and serping1) in grass carp were identified (Supplementary Table S5).

The comparison of amino acid sequences of homologous serpin proteins in grass carp (*C. idella*), zebrafish (*D. rerio*), carp (*Cyprinus carpio*), African clawed frog (*Xenopus tropicalis*), chicken (*Gallus Galus*), mouse (*Rattus norvegicus*), cow (*Bos taurus*), and human (*Homo sapiens*) shows that the serpin sequences of grass carp are closest to that of the zebrafish or carp. Overall, the similarity between the amino acid sequences of the six *serpin* genes in grass carp and zebrafish or carp was more than 82% (Table 1).

3.2. Phylogenetic analysis of serpin proteins of grass carp

A phylogenetic tree was constructed between the amino acid sequences of serpins through the NJ method at a bootstrap value of 1000 in multiple species, namely zebrafish (*D. rerio*), carp (*C. carpio*), African clawed frog (*X. tropicalis*), chicken (*G. gallus*), mouse (*R. norvegicus*), cow (*B. taurus*), and human (*H. sapiens*). This evolutionary tree (1000 bootstraps) was used to represent the evolutionary history of the

Table 1
Similarity comparison of serpin protein sequences between grass carp and other species.

protein	*Dre*	*Ccr*	*Bta*	*Gga*	*Rno*	*Xtr*	*Hsa*
serpinb1	90.79		53.46	55.82	54.50/52.25	53.46	56.08
serpinc1	88.64	86.44	57.3	61.07	58.65	56.82	57.66
serpind1	90.53	89.02	50.1	56.26	53.44	55.49	51.81
serpinf1	90.15	92.65	40.3	38.61	39.26	38.77	38.61
serpinf2b	86.01		37.9	38.01	36.16	38.09	37.07
serping1	67.95		30.8	29.11	31.03		30.8

Notes: *Dre*: *Danio rerio*, zebrafish; *Ccr*: *Cyprinus carpio*, carp; *Bta*: *Bos taurus*, cow; *Gga*: *Gallus gallus*, chicken; *Rno*: *Rattus norvegicus*, rat; *Xtr*: *Xenopus tropicalis*, African clawed frog; *Hsa*: *Homo sapiens*, human. *Serpinb1* has two members in *Rattus norvegicus*, namely *serpinb1a* and *serpinb1b*. In the *Rno* column, the values on the left are comparisons of the *Cidserpinb1* with the *Rnoserpinb1a*, and the values on the right are comparisons of the *Cidserpinb1* with the *Rnoserpinb1b*. Only *serpinf2* was found in humans. There were no *serpinf2a* and *serpinf2b*. Therefore, grass carp *serpinf2b* was compared with human *serpinf2*. The blanks indicated that the related sequences of the species have not been submitted to the NCBI, and they have not been compared.

analysed taxa. According to the constructed phylogenetic tree, the serpin proteins of these species can be divided into three major branches. The one labelled purple includes serpinb1 and serpinc1 members, and the one labelled green contains serpind1 members (Fig. S5). A branch of red is composed of serpinf1, serpinf2 (including serpinf2b) and serping1. Among them, the members of serpinf2 (including serpinf2b) and serping1 were first clustered into a small branch and then merged into a large branch with serpinf1. Among the species studied, the serpins of grass carp, zebrafish, and carp have the closest genetic relationship. Each of their corresponding serpin members first clustered into one small branch and then merged with the corresponding serpin members of other species. The evolutionary information of serpins suggests a high reliability of the serpin sequences of grass carp.

3.3. Distribution of normal tissue of serpins at the mRNA level of grass carp

Using RT-qPCR, muscle tissues were used as the reference tissues to analyze the relative expression levels of the six *serpins* in grass carp among 11 different tissues (skin, blood, brain, kidney, head kidney, spleen, intestine, heart, muscle, gill and liver). As shown in Fig. 1, *serpinb1* was mainly enriched in the muscle tissue and had low expression levels in the blood, brain, kidney, head kidney and spleen. *Serpinf1* was highly expressed in the skin but lowly expressed in the blood, head kidney, intestine and heart. *Serpinc1*, *serpind1*, *serpinf2b* and *serping1* exhibited the highest mRNA expression levels in the liver. Low levels of *serpinc1*, *serpind1*, *serpinf2b* and *serping1* were observed in the skin, blood, kidney, intestine, muscle and gill. Furthermore, *serpinc1* displayed low levels in the brain; *serpinf2b* displayed low levels in the head kidney and heart; and *serping1* showed low levels in the brain and heart. Overall, these serpins had different expression patterns in different tissues, whereas some *serpins* clearly had a tissue-significant expression pattern.

3.4. Changes of serpins, coagulation and anticoagulant factor genes in the liver before and after GCRV infection

After GCRV infection, the expression of liver-enriched *serpins* was firstly detected by RT-qPCR (Fig. 2). The *serpinc1* was higher than the original expression level both in latency and early onset and reached the highest level on the eighth day of the disease (1.86-fold, $p < 0.05$). However, *serpinc1* was down-regulated at the late stage (Fig. 2A). The *serpind1* reached the highest expression level (2.70-fold, $p < 0.05$) on the 2nd day after challenge, after which it went through a process of down-regulation and reached the lowest expression level on day 5 (Fig. 2B). The *serpinf2b* was up-regulated on the first and second days after the challenge and showed low-level expression in all other detected time points ($p < 0.05$). It reached the lowest expression level ($p < 0.05$) on the 8th day (Fig. 2C). Afterward, the changes of *serpinf2b* in a slightly up-regulated expression are presented (Fig. 2C). *Serping1* showed a significantly high level of expression in the latent stages of GCRV infection and reached the highest expression level (1.83-fold, $p < 0.05$) on the first day after challenge. Then, it was gradually down-regulated and reached the lowest expression level on day 5 (Fig. 2D).

The coagulation factor gene *kng-1*, reached the highest expression level on the first day after the challenge (4.50-fold, $p < 0.05$). Then, it underwent a gradual down-regulation and was adjusted to the initial level (Day 0) (Fig. 3A). The key activation factors (*f3a* and *f3b*) of exogenous coagulation pathway were highly expressed on the whole after the challenge. They were up-regulated on the first day after the infection (7.05-fold, 6.67-fold; $p < 0.05$) and reached the highest expression levels of 17.71-fold and 9.16-fold ($p < 0.05$) on days 3 and 2, respectively. Subsequently, *f3a* and *f3b* showed fluctuating down-regulation expression with a second peak of expression on day 5 (13.08-fold, 6.53-fold; $p < 0.05$). In addition, other coagulation factors (*f2*, *f7*, *f9* and *f10*) were basically up-regulated in fluctuating expression, with the peaks of expression appearing successively on Days 1, 5 and 8 (Fig. 3A).

The expression profiles of *tpa* and *plg* were similar in the anticoagulant factor genes. *Plg* and *tpa* reached the highest expression levels on the first day after the challenge (3.21-fold, 2.41-fold, $p < 0.05$), and gradually adjusted from the 6th day to the same level as the initial 0th day (Fig. 3B). The mRNA expression profiles of *thbd*, *proc*, and *pros* were also similar (Fig. 3B). *Proc* and *thbd* reached the highest expression levels on day 1 after the challenge (4.60-fold, 2.04-fold; $p < 0.05$), whereas *pros* reached the highest level on day 4 (2.37-fold, $p < 0.05$). From the 8th day, the mRNA expression levels of *thbd*, *proc* and *pros* displayed down-regulation. Fig. 3C showed a comprehensive analysis of expression profile changes in the clotting factor genes (red marker) and anticoagulant factor genes (black marker) during the entire GCRV infection. At the early stage of GCRV infection, the up-regulation levels of the key activation factor genes (*f3a* and *f3b*) in the coagulation pathway were significantly higher than those of clotting factor genes. On the 8th day after obvious infection symptoms of grass carp infected with GCRV, the expression of anticoagulant genes (*tpa*, *plg*, *thbd*, *proc* and *pros*) was down-regulated in the liver, and the *f3b*, one of the initial activation factor genes of the coagulation pathway and another important coagulation factor *f7* that formed a complex with it were up-regulated from the 8th day. The *f3a*, the other initiation factor gene of the coagulation pathway, showed a tendency of up-regulated expression from the 11th day. Overall, the up-regulated expression of coagulation factors was high during GCRV infection.

4. Discussion

In normal living organisms, coagulation and anticoagulation are two vital processes, which depends on the restriction between numerous proteases and their corresponding protease inhibitors [1,10]. Serpin and coagulation factors as serine proteases are mainly derived from the liver [19,35,36]. Here, we cloned the full-length cDNA sequence of six members of the *serpin* gene family of grass carp. The distribution of mRNA expression levels showed that four *serpins* (*serpinc1*, *serpind1*, *serpinf2b* and *serping1*) were mainly expressed in the liver. Information on the tissue expression patterns of these *serpins*, coagulation, and anticoagulation factor genes may be useful for in-depth researches on the function of each member in the blood circulation system in grass carp.

In the coagulation process, the acute phase protein Kininogen-1 (encoded by *kng-1*), an activation factor of the endogenous coagulation pathway, is a precursor protein of the high-molecular-weight kininogen (HMWK) [15]. Fig. 3A showed that the expression of *kng-1* was up-regulated on the first day after infection (4.50-fold, $p < 0.05$), which

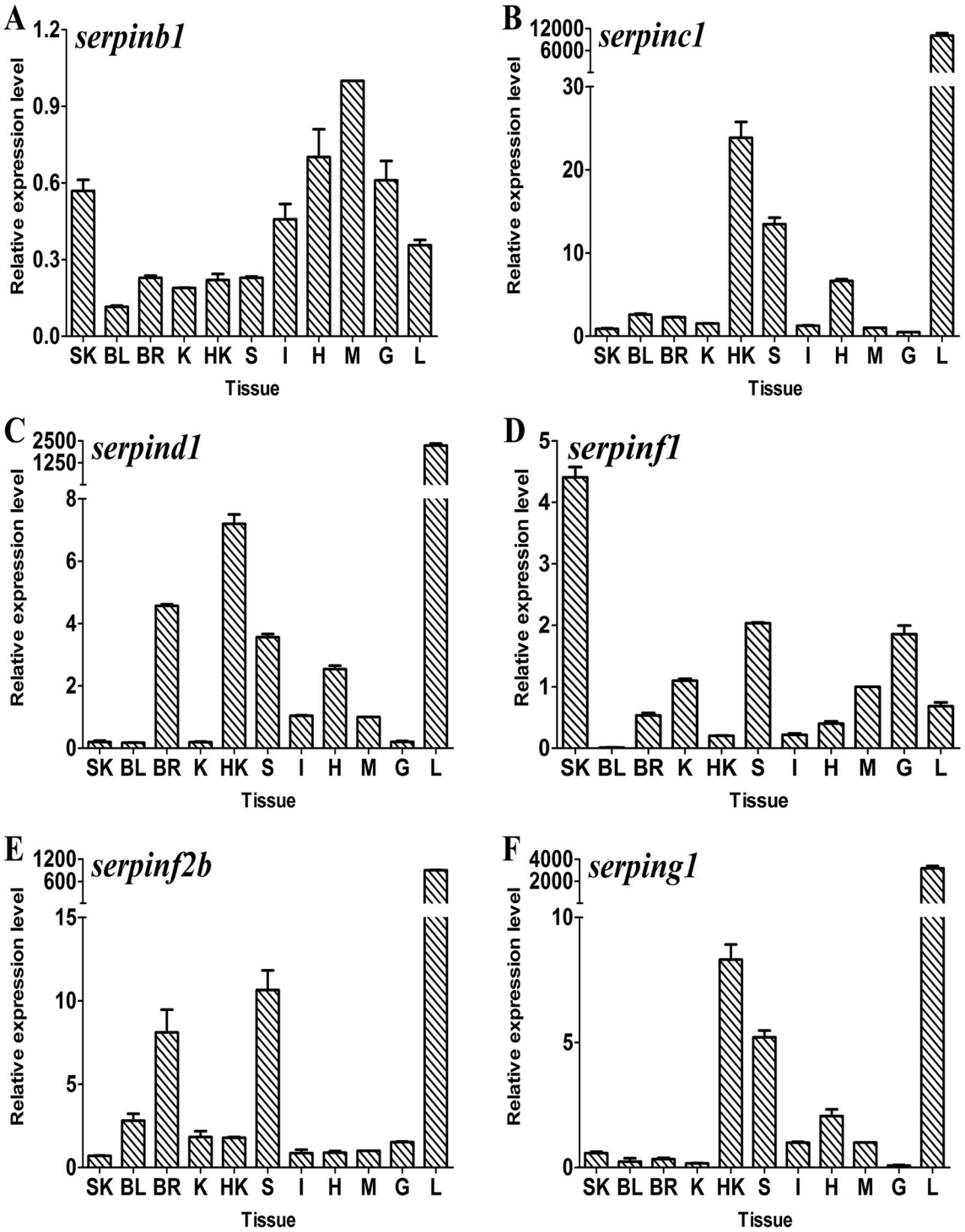


Fig. 1. Tissue distribution of *serpin* mRNA expression in healthy grass carp. RNA was isolated from 11 different tissues, namely, skin (SK), blood (BL), brain (BR), kidney (K), head kidney (HK), spleen (S), intestine (I), heart (H), muscle (M), gill (G) and liver (L). The expression level of *β-actin* was used as an internal control for the RT-qPCR. The relative expression was calculated based on the ratio of gene expression in the different tissues relative to that in the muscles. (A) *serpinb1*, (B) *serpinc1*, (C) *serpind1*, (D) *serpinf1*, (E) *serpinf2b* and (F) *serping1*. The results were based on three independent experiments and were expressed as mean ± standard deviation. Error bars indicate standard deviation (n = 3).

suggested that the endogenous coagulation pathway may be activated in grass carp after GCRV infection. F3, the initial activation factor of the exogenous coagulation pathway, is a receptor for f7 on the cell surface and is a cofactor for f7a [37]. Activated f3 and activated f7 may form complexes to activate the up-regulated f9 and f10, which initiated exogenous coagulation [15,19]. The up-regulated expressions of *f3 genes* (*f3a* and *f3b*) and *f7* (7.05-fold, 6.67-fold and 3.23-fold; $p < 0.05$) after GCRV infection suggested that GCRV infection may also activate the exogenous coagulation pathway of grass carp (Fig. 3A).

When the body produces a coagulation reaction, it simultaneously

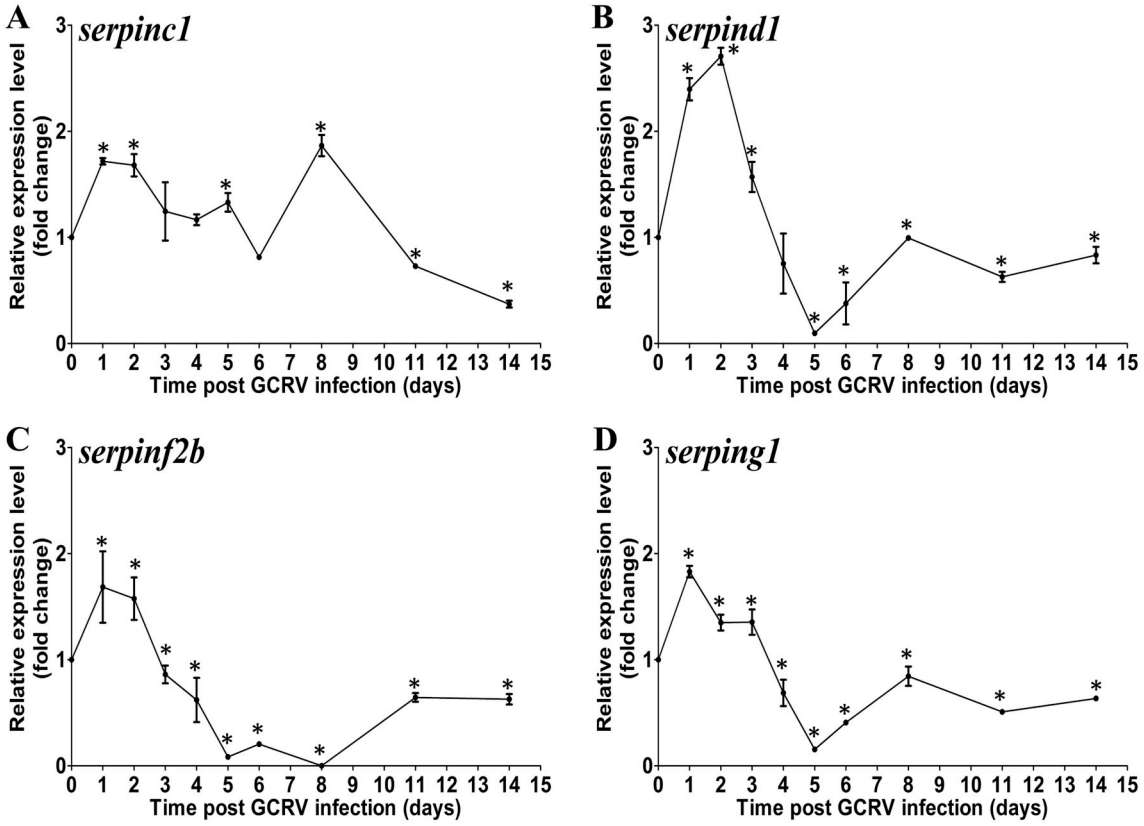


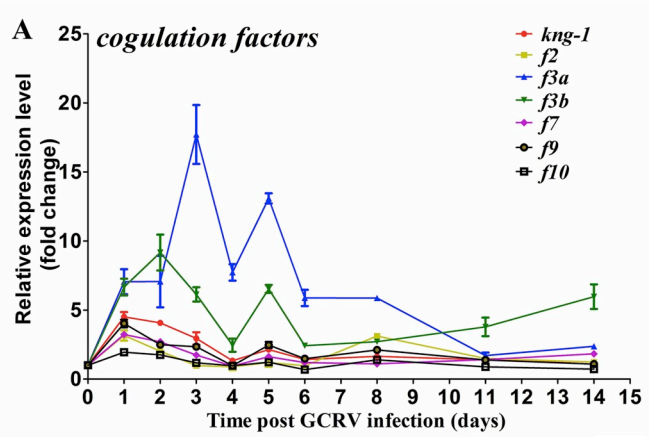
Fig. 2. Expression patterns of the liver-enriched *serpins* in the liver of grass carp following a GCRV infection. (A) *serpinc1*, (B) *serpind1*, (C) *serpinf2b* and (D) *serping1*. The expression of these liver-enriched *serpins* in the untreated groups (day 0) was set at 1.0. *β-actin* and was used as the internal control to normalise the relative expression level of the target gene. The results were based on three independent experiments and were expressed as mean values ± standard deviation. Asterisks (*) indicate significant differences between experimental and control groups ($p < 0.05$). Error bars indicate standard deviation (n = 3).

activates the fibrinolytic system with anticoagulant function and degrades the extra thrombus, thereby avoiding excessive aggregation of fibrin [8,10,11]. The degradation of fibrin is accomplished by plasmin, which is derived from plasminogen (PLG, encoded by the *plg* gene) activated by tissue plasminogen activator (tPA, encoded by the *tpa* gene) and the urokinase plasminogen activator (uPA, encoded by the *upa* gene) [23]. The up-regulated expressions of *plg* and *tpa* suggested that the plasmin of the fibrinolytic system may also be activated along with the coagulation system and produce anticoagulant activity post GCRV infection (Fig. 3A and C). For protein C (PROC, encoded by the *proc* gene), the combination of PROC with thrombomodulin (TM, encoded by the *thbd* gene) further promotes its activation, thereby exerting better anticoagulant function with the help of protein S (PROS, encoded by the *pros* gene) [20]. On the first day after GCRV challenge, *proc*, *thbd* and *pros* were up-regulated (4.60-fold, 2.04-fold and 2.00-fold; $p < 0.05$) at the same time (Fig. 3C), thereby suggesting that PROC, TM and PROS were activated and showed anticoagulant activity. However, the low level expression of *proc*, *thbd* and *pros* in the late period (after 6 days) may reduce the inactivation of thrombin.

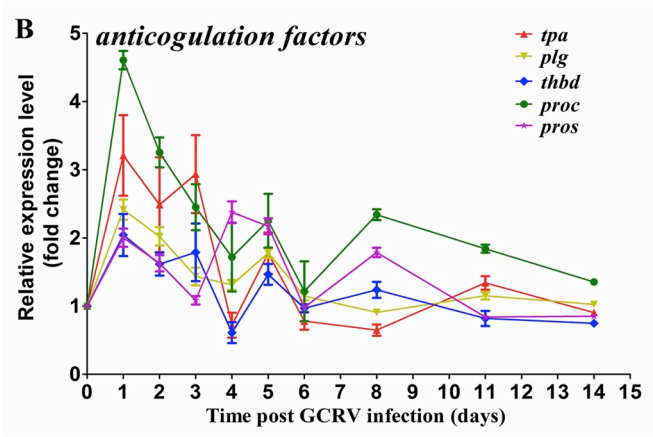
Studies have shown that serpinc1 and serpind1 are the most important inhibitors of f2 [8,9]. The serping1 can prevent endogenous coagulation pathways [24]. Anticoagulation factor serpinf2b, as inhibitors of plasmin and PROC [20,23], has an important coagulation function. The *serpinc1* and *serpind1* were up-regulated early in the infection (1.86-fold on Day 8 of *serpinc1*, 2.70-fold on Day 2 of *serpind1*) (Fig. 2), but this did not affect the peak expression of *f2* (3.12-fold) on Day 8 (Fig. 3). The anticoagulant effects exerted by grass carp serpinc1 and serpind1 were more obvious in the early stage of GCRV infection, but their inhibitory effect was not obvious at the later stage. Although the target enzyme of grass carp serping1 was not known, its early up-regulated expression also did not inhibit the up-regulated expression of f3b and f7 in the late phase of infection (Fig. 3). The *serpinf2b* produced up-regulated expression (1.68-fold) on the first day after GCRV infection (Fig. 2C), but did not exceed the up-regulation level of *plg* and *proc* (2.41-fold and 4.60-fold) (Fig. 3B). Subsequently, *serpinf2b* began to decrease in level, but was also lower than that of *plg* and *proc*. Grass carp serpinc1, serpind1 and serping1, which may have anticoagulant function, and grass carp serpinf2b, which may have coagulation function, possibly do not exert sufficient anticoagulant or coagulation functions during GCRV infection.

For the overall expression level of coagulation and anticoagulants (Fig. 3C), the coagulation system always dominated, although the two opposite systems of coagulation and anticoagulation had an antagonistic effect in the early stages of GCRV infection. Presumably, a possibility of excessive thrombosis is present during GCRV infection. Ong et al. found that the overproduction of thrombi may form disseminated intravascular coagulation in the blood circulation, and consequent vascular rupture may cause systemic hemorrhage [38], which may explain the reason why grass carp shows bleeding throughout the whole-body post GCRV infection.

In summary, this study preliminarily described the expression patterns of several members of the *serpin* gene family in GCRV-infected grass carp. With the interaction of serine protease and serine protease inhibitor as the entry point, the relationship between coagulation and anticoagulant system in the liver tissue of grass carp infected with GCRV was discussed. The *serpins* were speculated to play a very important role in the blood system of grass carp. In response to GCRV infection, different serpin members may have different division of labor



gene \ day	0	1	2	3	4	5	6	8	11	14
kng-1	1.00	4.51*	4.07*	2.95*	1.33*	2.14*	1.42*	1.65*	1.39*	1.24*
f2	1.00	3.16*	2.02*	0.97	0.88	1.2	0.97	3.12*	1.5*	1.23*
f3a	1.00	7.05*	7.07*	17.72*	7.73*	13.09*	5.88*	5.88*	1.71*	2.38*
f3b	1.00	6.67*	9.16*	6.13*	2.45*	6.53*	2.42*	2.71*	3.78	5.97*
f7	1.00	3.23*	2.7*	1.74*	0.94	1.62*	1.2	1.11	1.41*	1.83*
f9	1.00	4.02*	2.49*	2.35*	0.96	2.47*	1.48*	2.11*	1.38*	1.09
f10	1.00	1.95*	1.75*	1.18	0.97	1.22*	0.68*	1.39*	0.89*	0.73*



gene \ day	0	1	2	3	4	5	6	8	11	14
tpa	1.00	3.21*	2.49*	2.94*	0.72*	1.78*	0.78*	0.65*	1.34*	0.91
plg	1.00	2.41*	2.02*	1.43*	1.31*	1.78*	1.15	0.91	1.15	1.03
thbd	1.00	2.04*	1.62*	1.79*	0.61*	1.47*	0.97	1.24*	0.82*	0.75*
proc	1.00	4.61*	3.25*	2.45*	1.72*	2.25*	1.22*	2.34*	1.84*	1.35*
pros	1.00	2.00*	1.63*	1.09	2.38	2.17*	0.98	1.79*	0.84*	0.85*

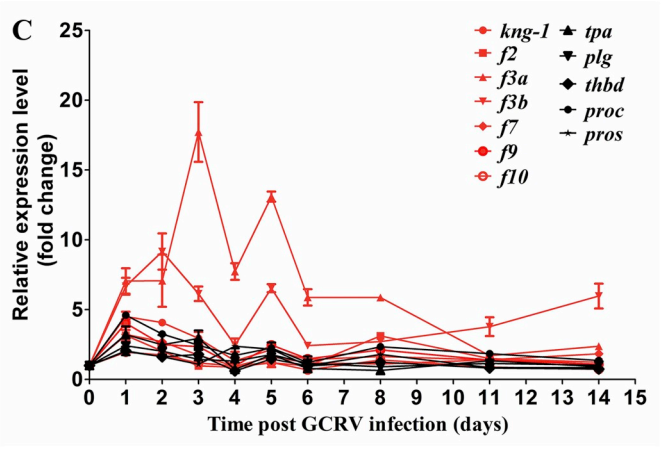


Fig. 3. Expression patterns of key coagulation factor and anticoagulant genes of liver form grass carp following GCRV infection. (A) coagulation factor genes, (B) anticoagulant factor genes and (C) Comprehensive expression analysis of coagulation (red marker) and anticoagulant factor (black marker) genes. The expression of these genes in the untreated groups (day 0) was set at 1.0. *β-actin* was used as the internal control to normalise the relative expression level of the genes. The results were based on three independent experiments and were expressed as mean values ± standard deviation. Asterisks (*) indicate significant differences between experimental and control groups ($p < 0.05$). Error bars indicate standard deviation (n = 3). Detailed values are listed as tables at the bottom of the separated figures. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and cooperate to regulate the homeostasis of the blood microenvironment. This study provided new insights to further clarify the molecular mechanism of GCRV affecting the homeostasis of grass carp blood environment.

Competing financial interest statement

The authors declare that there are no competing financial interests.

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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.2018.11.008.

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