Fish and Shellfish Immunology 94 (2019) 485–496

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Sequence and expression analysis of the cytoplasmic pattern recognition receptor *melanoma differentiation-associated gene 5* from the barbel chub *Squaliobarbus curriculus*

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ARTICLE INFO

Keywords:
MDA5
Squaliobarbus curriculus
Grass carp reovirus
Expression level
Overexpression

ABSTRACT

MDA5 is a cytoplasmic viral double-stranded RNA recognition receptor that plays a pivotal role in the aquatic animal innate immune system. To decipher the role of *MDA5* of *Squaliobarbus curriculus* (*ScMDA5*) in the immune response, full-length cDNA of *ScMDA5* was cloned using the RACE technology, mRNA and protein expression levels of *ScMDA5* signalling pathway members in response to stimulation were detected and effects of overexpression of *ScMDA5* on the immune response were investigated. *ScMDA5* comprises 3597 bp and is composed of an open reading frame (2958 nucleotides long) that translates into a putative peptide of 985 amino acid residues. ScMDA5 possesses two N-terminal caspase-recruiting domains, DEAD-like helicases superfamily, helicase superfamily C-terminal and RIG-I_C-RD domains, and differences in these domains among species were mainly observed with respect to their length and location. ScMDA5 was closely clustered with those of *Carassius auratus*, *Ctenopharyngodon idellus* and *Mylopharyngodon piceus*. *ScMDA5* transcripts were most abundant in the spleen and the lowest in the liver. Expression levels of *ScMDA5* in healthy tissues were significantly correlated with those of *ScIRF3*, *ScIRF7* and *ScIFN*. Besides, mRNA expression levels of *ScIRF3* were significantly correlated with those of *ScIRF7* (0.956, $P < 0.01$). Expression level changes, including downregulation, upregulation and initial upregulation followed by downregulation, were found in *ScMDA5* signalling pathway molecules in tissues after grass carp reovirus infection. Protein levels of ScMDA5 were the highest in the liver and the lowest in the spleen in detected healthy tissues. Overexpression of *ScMDA5* led to significantly enhanced *CiIRF7* and *CiMx* transcription in grass carp ovary cells ($P < 0.05$). The results of this study helped to clarify the role of *ScMDA5* in the immune reaction against grass carp reovirus and provided fundamental information for fish breeding to achieve strong resistance to infection.

1. Introduction

Various pathogens, such as bacteria and viruses, exist in the aquatic environment. The innate immune system of fish plays a significant role in defence against microorganism invasion by recognition of conserved pathogen-associated molecular patterns through pattern recognition receptors [1–3]. Cells possess defence mechanisms that allow them to rapidly detect and respond to viruses, which partly rely on cytoplasmic receptors that alert the cells to the presence of abnormal RNA molecules [4].Initial sensing of infection is mediated by pattern recognition receptors, such as Toll-like receptors, retinoic acid-inducible gene I-like receptors (RLRs), NOD-like receptors and c-type lectin receptors [5]. The DExD/H box RNA helicases RIG-I, MDA5 and LGP2 are RLR members located in the cytoplasm, and they are responsible for the recognition of viral RNAs in fish [6].

MDA5 (also termed as Ifih1 or Helicard) is a cytoplasmic viral double-stranded RNA (dsRNA) recognition receptor in the vertebrate innate immune system, which plays an essential role in antiviral immunity via recognition of viral RNA [7]. MDA5 detects long-duplex RNAs in the genome of dsRNA viruses or replication intermediates of positive-strand viruses, such as encephalomyocarditis and vesicular stomatitis viruses [8–10]. MDA5 possesses a caspase-recruiting domain (CARD) that is required for the initiation of immune response [11]. The signalling adaptor interferon-β promoter stimulator 1 (IPS-1) contains an N-terminal CARD-like structure that could mediate interaction with MDA5 [12]. MDA5 signalling induces the activation of IPS-1 and then

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https://doi.org/10.1016/j.fsi.2019.08.077
Received 16 June 2019; Received in revised form 25 August 2019; Accepted 30 August 2019
Available online 05 September 2019
1050-4648/

Table 1
Primers used for RACE PCR, qPCR and overexpression experiments.

Primer name	Primer sequence 5′-3′	Usage	Efficiency
3′-RACE CDS Primer A	AAGCAGTGGTATCAACGCAGAGTAC (T)30VN (N:A, C, G, or T; V:A, G, or C)	RACE	
5′-RACE CDS Primer A	(T) 25V N-3' (N: A, C, G, or T; V:A, G, or C)	RACE	
UPM Long Primer	CTAATACGACTCACTATAGGGCAAGCAGTG	RACE	
UPM Short Primer	CTAATACGACTCACTATAGGGC	RACE	
MDA5-F	GATTACGCCAAGCTTCCCCGGTTGTCCC ATCTGCTTCTTCCTG	RACE	
MDA5-R	GATTACGCCAAGCTTATGTTCGGCGCAA ACACGGACCTTCTGA	RACE	
MDA5 YF	AATCTACTGATAGCCACCACT	qPCR	109.7%
MDA5 YR	CTGAATCATGGCGACCTCA	qPCR	
IRF3 YF	TGTGGACACTGACGGACCCTTC	qPCR	93.1%
IRF3 YR	CGGCTGCTGATATGCTGGAGAA	qPCR	
IRF7 YF	CGCCTGTGTTCGTCACTCGT	qPCR	101.5%
IRF7 YR	GGTGGTTGGAAAGCGTATTGG	qPCR	
Mx YF	CGACCACAGAAGCATTGCAGA	qPCR	107.8%
Mx YR	CCCTTCAGTGCCTTTATCCACCA	qPCR	
IFN YF	AATGCTCTGCTTGCGAATG	qPCR	105.1%
IFN YR	CCTGGAAATGACACCTTGG	qPCR	
β-actin YF	GCTATGTGGCTCTTGACTTCG	qPCR	104.5%
β-actin YR	GGGCACCTGAACCTCTCATT	qPCR	
EF1α YF	CGCCATTGTTGAGATGATCCCT	qPCR	101.4%
EF1α YR	GACACCAACAGCAACGGTCT	qPCR	
ScMDA5-OF	aattctgcagtcgacggtaccATGAGCAGC GATCAGGACGT	overexpression	
ScMDA5-OR	cgtcatggtggcggcggatccATCACAGTC CATGTCTTCTTCTGAGT	overexpression	
CiMDA5-OF	aattctgcagtcgacggtaccATGAGTAGTGATCAGGACGCCG	overexpression	
CiMDA5-OR	cgtcatggtggcggcggatccCTCTGTTCTGTGAAATTCACAGTTGTC	overexpression	

the TBK1–interferon regulatory factor (IRF) 3/7–type I interferon (IFN-I) and NF-kB signal pathways. The activated transcription factors NF-κB, IRF-3 and IRF-7 translocate to the nucleus and participate in the induction of antiviral genes, including type I IFNs (IFN-α and IFN-β). Finally, transcription of IFNs leads to the production of a large number of interferon-stimulated genes, which possess diverse effector and regulatory functions [13]. Activated NF-kB is also transferred to the nucleus and induces the expression of cytokines involved in the inflammatory response [4,14,15].

To date, molecule identification and antiviral function studies of *MDA5* have been reported in many fish species. MDA5 from rainbow trout (*Oncorhynchus mykiss*) was constitutively produced in fibroblast and macrophage cell lines and upregulated by RNA virus infection. The overexpression of MDA5 has significantly upregulated the expression of Mx and enhanced protection against viral infection [16]. The full-length MDA5 gene in Japanese flounder (*Paralichthys olivaceus*) was cloned, and its transcript abundance was significantly increased in whole kidneys infected with viral haemorrhagic septicaemia virus (VHSV). MDA5-overexpressing Hirame natural embryo cells showed a lower cytopathic effect against VHSV, Hirame rhabdovirus and infectious pancreatic necrosis virus infection, and MDA5-overexpressing cells showed 24–75-fold lower virus titres than normal cells [17]. After grass carp reovirus (GCRV) stimulation, overexpression of *CiMDA5* significantly induced the expression of *CiIFN-1*, *CiIL-1β* and *CiMx1*; repressed GCRV replication and decreased viral titre in *Ctenopharyngodon idellus* kidney cells [11].

Both barbel chub (*Squaliobarbus curriculus*) and grass carp (*C. idellus*) are economically important freshwater fish species belonging to the subfamily Leuciscinae [18]. The double-stranded RNA virus GCRV causes severe haemorrhagic disease that results in the high mortality of *C. idellus*. *S. curriculus* shows strong resistance to GCRV and can mate with *C. idellus* to produce progeny with conferred resistance to GCRV [19,20]. To obtain molecular information of *ScMDA5* from GCRV-resistant fish and decipher its response to GCRV invasion and immune function, the full-length cDNA of *ScMDA5* was cloned, mRNA and protein expression levels in the tissue in response to GCRV infection were investigated and effects of heterologous expression of *ScMDA5* on immune response in grass carp ovary (GCO) cells were also analysed. The results of this study will help clarify the molecular characterisation and role of *ScMDA5* in the immune reaction against GCRV and provide fundamental information for fish anti-disease breeding.

2. Materials and methods

2.1. Experimental fish and sample collection

S. curriculus were purchased from the fishery base of Wulong, Liuyang, China. All fish were maintained in tank for 1 month at constant temperature of 28 °C and fed twice daily at 2% of their body weight. The fish were euthanised by an overdose of tricaine methanesulfonate (200 mg/L) before sampling. To detect tissue expression levels of *ScMDA5*, the liver, skin, spleen, gill, kidney and head kidney were collected from three healthy *S. curriculus* individuals. An experimental group and a control group were set up for the GCRV infection experiment. Individuals in the experimental group (24 individuals) were intraperitoneally injected with 200 μL of GCRV. Those in the control group (24 individuals) were intraperitoneally injected with 200 μL of PBS. In each group, samples (liver, skin, spleen, gill and kidney) from three individuals were separately taken at 12, 48 and 96 h after treatment. All samples were stored in liquid nitrogen for further experiments. GCRV106 with 1.78×10^7 50% tissue culture infective dose ($TCID_{50}$)/mL was obtained from the Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science (Wuhan, China).

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from *S. curriculus* tissues using the TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Japan), according to the manufacturer's instructions. The concentration of RNA was measured using a spectrophotometer (Eppendorf BioSpectrometer Basic, Germany), and RNA integrity was analysed by 1.3% agarose gel

electrophoresis. RNA with an $OD_{260/280}$ value ranging from 1.8 to 2.0 was used for cDNA synthesis. RNA from the spleen was used for preparing the full-length cDNA template and was synthesised using the SMARTer RACE cDNA amplification kit (Clontech, USA). For quantitative real-time PCR (qPCR), tissue RNA was treated with DNase I, and cDNA was synthesised using the ReverTra Ace-first-strand cDNA synthesis kit (Toyobo, Japan), as per the manufacturer's instructions.

2.3. Full-length cDNA cloning of ScMDA5

Based on the highly conserved sequences of *MDA5s* from other fish species, primers MDA5-F and MDA5-R were designed using Oligo 7.0 software for full-length cDNA cloning (Table 1). Total volume of the RACE PCR system was 50 μL, including 21 μL Premix Ex Taq (TaKaRa, Japan), 2 μL 10 pmol/μL primer MDA5-F or MDA5-R, 2 μL Universal Primer A Mix, 2 μL cDNA template and 23 μL ddH_2O. The PCR programme was as follows: five cycles at 94 °C for 30 s and 72 °C for 3 min; five cycles at 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min; five cycles at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min and 25 cycles at 94 °C for 30 s, 66 °C for 30 s and 72 °C for 3 min. The PCR products were purified, ligated into the pMD19-T vector, transformed into *Escherichia coli DH5α* cells and sent to the Sangon Biotech Company (Shanghai, China) for sequencing.

2.4. Bioinformatics analysis

The nucleotide sequence homology analysis of *ScMDA5* was performed via the BLAST program (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequence of *ScMDA5* was analysed using the Expert Protein Analysis System (http://www.expasy.org/). The isoelectric point and molecular weight of deduced amino acid sequences were predicted using the ExPAsy site (http://web.expasy.org/compute_pi/), and protein domains were analysed by the Simple Modular Architecture Research Tool (http://smart.emblheidelberg.de/). Multiple sequence alignment was performed using the DNAMAN 7.0 software. A phylogenetic tree was constructed using the MEGA 6.0 software, and 1000 bootstraps were set to assess the repeatability of the results. MDA5s from *S. curriculus* (APB09201.1), *C. idellus* (ACT68336.2), *Carassius auratus* (AEN04473.1), *M. piceus* (ARO77472.1), *Danio rerio* (NP_001295492.1), *Cyprinus carpio* (AIX47136.1), *Epinephelus coioides* (AEX01716.1), *P. olivaceus* (ADU87114.1), *Oreochromis niloticus* (AUN88445.1), *Kryptolebias marmoratus* (XP_017279885.1), *Larimichthys crocea* (ANQ31758.1), *Lates calcarifer* (AOV82292.1), *Oplegnathus fasciatus* (AHX37214.1), *Siniperca chuatsi* (ATQ36109.1), *Monopterus albus* (XP_020450399.1), *Anser cygnoides* (AGH30275.1), *Cairina moschata* (AHN92039.1), *Nipponia nippon* (XP_009460278.1), *Pterocles gutturalis* (KFV08725.1), *Gallus gallus* (AEM00325.1), *Sylvilagus bachmani* (AHB62713.1), *Lepus granatensis* (AHB62714.1), *Sus scrofa* (AWH63112.1), *Sousa chinensis* (TEA34307.1), *Physeter catodon* (XP_007105350.1), *Delphinapterus leucas* (XP_022425599.1), *Equus caballus* (XP_001494380.1), *Pan paniscus* (XP_003820983.1), *Macaca mulatta* (NP_001040588.1) and *Homo sapiens* (AAG34368.1) were used for phylogenetic tree construction.

2.5. mRNA expression levels of ScMDA5 signalling pathway members

To detect transcription levels of *ScMDA5* and of the members in its signalling pathway (i.e. *ScIRF3*, *ScIRF7*, *ScIFN* and *ScMx*) in healthy and PBS- or GCRV-treated individuals, qPCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Amplifications were performed in triplicate in a total volume of 10 μL containing 1 μL of diluted cDNA template (1:5), 5 μL of SYBR Premix Ex Taq II, 0.4 μL primer YF, 0.4 μL Primer YR and 3.2 μL ddH_2O. The PCR programme was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 40 s. *β-actin* and *EF1α* were used as reference genes, and the relative expression levels of the target genes were calculated by the $2^{-\triangle\triangle Ct}$ method. For qPCR experiments, the primers used were MDA5 YF and MDA5 YR for *ScMDA5*. Primers for the reference gene *β-actin* were β-actin YF and β-actin YR and for *EF1α* were EF1α YF and EF1α YR. The primers for *ScIRF3*, *ScIRF7*, *ScIFN* and *ScMx* are listed in Table 1.

2.6. Overexpression of ScMDA5 in grass carp ovary (GCO) cells

For the overexpression experiment, the open reading frames (ORFs) of *ScMDA5* and *CiMDA5* were amplified using primer pairs ScMDA5-OF and ScMDA5-OR, and primers CiMDA5-OF and CiMDA5-OR, respectively (Table 1). The total volume of PCR reactions was 20 μL, including 10 μL 2× Taq Master Mix (Vazyme, China), 1 μL primer MDA5-OF, 1 μL primer MDA5-OR, 1 μL cDNA template (spleen-originated template for qPCR) and 7 μL ddH_2O. The PCR programme for ORF amplification was as follows: 1 cycle at 94 °C for 5 min and 30 cycles at 94 °C for 30 s, 68 °C for 1 min and 72 °C for 5 min and 1 cycle at 72 °C for 7 min. The pEGFP-N1-Flag vector (Clontech, Palo Alto, CA, USA) and ORF PCR product were digested with *Bam*HI and *Kpn*I, respectively. Recombinant vectors, the pEGFP-N1-Flag-ScMDA5 and the pEGFP-N1-Flag-CiMDA5, were constructed by cloning the purified *MDA5* ORF products into the pEGFP-N1-Flag vector, and the recombinant plasmids were confirmed by sequencing analysis. pEGFP-N1-Flag-ScMDA5 was introduced into GCO cells. The experiment procedures were as follows: GCO cells were seeded into 5-cm^2 cell culture flasks for 24 h (at a density of 2×10^6 cell/mL) with minimum essential medium containing 10% FBS. The cells of each flask were transfected with a mixture containing 8 μg plasmid and 20 μL lipofectamine 2000 (Invitrogen, USA) in 500 μL minimum essential medium. Then, the transfection reagent was replaced with fresh minimum essential medium at 6 h after transfection. The cells were observed under fluorescence microscopy (fluorescence microscopy Olympus IX53, Japan) and collected at 24 h after transfection. Total mRNA and protein levels of MDA5 and mRNA levels of *C. idellus IRF3* (*CiIRF3*), *CiIRF7*, *CiIFN* and *CiMx* in pEGFP-N1-Flag-ScMDA5-overexpressed GCO cells (the GCO cells, the pEGFP-N1-Flag-overexpressed GCO cells and the pEGFP-N1-Flag-CiMDA5-overexpressed cells were set as controls) were detected using qPCR and western blots.

2.7. ScMDA5 antibody preparation and western blot detection

The recombinant plasmid (pEGFP-N1-Flag-ScMDA5) was transformed into *E. coli* BL21 (DE3) cells (Novagen, Germany). Positive clones were confirmed by sequencing and cultured in LB medium with 50 μg/mL of kanamycin. After incubation at 37 °C to the mid-log phase of growth, the expression of pEGFP-N1-Flag-ScMDA5 was induced by adding 0.5 mM isopropyl-β-D-1-thiogalactosidase (IPTG) to the cells and incubation for 6 h at 37 °C. DE3 cells containing pEGFP-N1-ScMDA5 without IPTG induction were set as negative controls. The recombinant protein was purified by HisTrap HP Ni-Agarose (GE healthcare, China), according to the manufacturer's instructions. The induced bacterial lysate and purified recombinant protein were analysed by SDS-PAGE and Coomassie Brilliant Blue R-250 staining (Beyotime, China). The concentration of the recombinant protein was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific, USA). For the production of rabbit anti-ScMDA5 polyclonal antibody, the purified recombinant protein was used to immunise New Zealand white rabbits. The titre of the polyclonal antibody against ScMDA5 was determined by indirect enzyme-linked immunosorbent assay, and the non-immune rabbit serum was used as a negative control.

For western blotting experiments, tissues of the liver, spleen, kidney, head kidney, gill and skin were separately sampled from three healthy *S. curriculus* individuals. Tissues from three individuals were collected at 12 h after GCRV infection for protein level detection. Proteins were extracted from the tissues and their concentrations were

measured using the BCA protein assay kit (Thermo Fisher Scientific, USA). The extracted proteins (20 μg) were subjected to 12% SDS-PAGE and then transferred onto a PVDF membrane (Biosharp, China). The membrane was washed three times with 1× Tris-buffered saline containing 0.05% Tween 20 (TBST). The membrane was incubated with ScMDA5 polyclonal antibody (diluted 1:1000 in PBS) for 2 h at room temperature. The membrane was again washed three times with the TBST buffer and then incubated with the secondary antibody HRP-conjugated goat anti-rabbit IgG (Abclonal, China, diluted 1:2000 in PBS) for 1 h at room temperature. After washing thrice with the TBST buffer, the membrane was dyed with BeyoECL Moon (Beyotime, China) and scanned using the Genesys Imaging System (Alcatel, France). Band intensity from the western blot was calculated using Image J.

2.8. Statistical analysis

The differences in mRNA and protein expression levels between tissues and cells were compared by one-way analysis of variance followed by Duncan's multiple range tests, using SPSS Statistics 22.0 software. The SPSS independent sample *t*-test was applied to compare the difference in protein levels of ScMDA5 between tissues of healthy and GCRV-infected *S. curriculus* individuals. Correlations between mRNA expression levels of genes were analysed by applying Pearson correlation analysis. A p-value ≤ 0.05 was considered to be statistically significant and lower or equal to 0.01 was considered extremely statistically significant.

3. Results

3.1. ScMDA5 sequence information

The cloned full-length cDNA sequence of *ScMDA5* comprises 3597 bp (GenBank accession number: KU955846.1). This transcript comprises an ORF that is 2958 nucleotide long and translates into a putative peptide of 985 amino acid residues, with a 107-bp 5′-terminal untranslated region (UTR) and a 532-bp 3′-UTR. The predicted molecular weight of the *ScMDA5* ORF is 111.68 kDa, with a theoretical isoelectric point of 5.56 (Fig. 1).

3.2. Sequence alignment, functional domain and phylogenetic analysis

BLAST analysis results showed that *ScMDA5* closely matched with homologues from the Cyprinidae fish. *ScMDA5* shared the highest identity (96.82%) with that of *Carassius auratus* (JF970226.1), followed by homologues from *C. idellus* (96.72%, FJ542045.2) and *Mylopharyngodon piceus* (96.27%, KX871189.1). Multiple amino acid sequence alignments showed that *MDA5s* from Cyprinids were highly conserved. MDA5s from fish and mammalian species possessed two N-terminal CARD, a DEAD-like helicases superfamily domain (DEXDc), a helicase superfamily C-terminal domain (HELICc) and a RIG-I_C-RD domain in the C-terminus. There were 43, 113, 57 and 27 identical amino acid residues among all selected species in CARD, DEXDc, HELICc and RIG-I_C-RD domain, respectively (Fig. 2). Comparison analysis also showed that differences between species were mainly concentrated on the length and location of the functional domains. To determine the phylogenetic relationship of ScMDA5 with those of other species, a phylogenetic tree was constructed. ScMDA5 closely clustered with those of *Carassius auratus* (AEN04473.1), *M. piceus* (ARO77472.1) and *C. idellus* (ACT68336.2) (Fig. 3).

3.3. mRNA levels of ScMDA5 signalling pathway members

qPCR was performed to investigate mRNA levels of *ScMDA5*, *ScIRF3*, *ScIRF7*, *ScIFN* and *ScMx* in *S. curriculus* tissues. The relative expression levels of *ScMDA5* in the liver, skin, spleen, gill, kidney and head kidney were 0.240 ± 0.101, 0.410 ± 0.290, 0.827 ± 0.120, 0.370 ± 0.111, 0.363 ± 0.176 and 0.520 ± 0.178, respectively. Among these tissues, *ScMDA5* transcripts were mostly abundant in the spleen followed by the head kidney and skin, with the lowest levels in the liver (Fig. 4). The relative expression levels of *ScIRF3* and *ScIRF7* were the highest in the spleen (significantly higher than those in all other tissues, $P < 0.05$). The relative expression levels of *ScIFN* and Mx were the highest in the spleen and in the gill, respectively. Pearson correlation analysis showed that mRNA levels of *ScMDA5* in healthy tissues were significantly correlated with those of *ScIRF3* (0.928, $P < 0.01$), *ScIRF7* (0.913, $P < 0.05$) and *ScIFN* (0.842, $P < 0.05$). mRNA expression levels of *ScIRF3* were significantly correlated with those of *ScIRF7* (0.956, $P < 0.01$), and transcription levels of *ScIRF7* were significantly correlated with those of *ScIFN* (0.945, $P < 0.01$). qPCR was also performed to investigate transcription levels of *ScMDA5*, *ScIRF3*, *ScIRF7*, *ScIFN* and *ScMx* in the liver, spleen, kidney, gill and skin of *S. curriculus* at different time points after GCRV infection or PBS treatment. In the liver, mRNA expression levels of *ScMDA5*, *ScIRF3* and *ScMx* showed an overall trend of downregulation from 12 to 96 h after infection. In the spleen, expression levels of *ScIRF7* exhibited an overall trend of initial upregulation and then downregulation, whereas expression levels of *ScMx* were significantly downregulated from 12 to 96 h after infection ($P < 0.05$). In the gill, expression levels of *ScMDA5*, *ScIRF7*, *ScIFN* and *ScMx* were all significantly upregulated after GCRV infection. In the skin, an overall trend of initial upregulation and then downregulation were observed for expression levels of *ScIRF7* and *ScIFN*, whereas those of *ScIRF3* were significantly downregulated from 12 to 48 h after GCRV infection (Fig. 4).

3.4. Effects of overexpression of ScMDA5 on the expression of ScIRF3, ScIRF7, ScIFN and ScMx

To test the effects of heterologous expression of *ScMDA5* on transcription levels of *CiIRF3*, *CiIRF7*, *CiIFN* and *CiMx*, overexpression experiments of *ScMDA5* in GCO cells were conducted. mRNA expression levels of MDA5 (total levels of *ScMDA5* and *CiMDA5*), *CiIRF7* and *CiMx* were significantly higher in pEGFP-N1-Flag-ScMDA5-overexpressed GCO cells than in GCO cells and pEGFP-N1-Flag-overexpressed GCO cells ($P < 0.05$). In addition, the MDA5 expression levels in pEGFP-N1-Flag-CiMDA5-overexpressed GCO cells were significantly upregulated, and the expression levels of *CiIRF7* and *CiMx* were significantly higher in pEGFP-N1-Flag-ScMDA5-overexpressed cells than those in pEGFP-N1-Flag-CiMDA5 cells. For the expression levels of *CiIFN*, overexpression of pEGFP-N1-Flag, pEGFP-N1-Flag-CiMDA5 and pEGFP-N1-Flag-ScMDA5 resulted in significantly lower mRNA levels than that in GCO cells (Fig. 5).

3.5. Western blot analysis of protein levels of ScMDA5

Western blot analysis showed that protein levels of ScMDA5 in the liver, spleen, kidney, head kidney, gill and skin were 2.254 ± 0.193, 0.518 ± 0.137, 2.080 ± 0.234, 0.848 ± 0.087, 1.083 ± 0.136 and 1.940 ± 0.347, respectively. Protein expression levels of ScMDA5 were the highest in the liver tissue followed by those in the kidney and skin and the lowest levels were identified in the spleen. Protein levels of ScMDA5 in GCRV-infected liver, spleen, kidney, head kidney, gill and skin (12 h after infection) were 1.555 ± 0.210, 0.297 ± 0.130, 0.435 ± 0.145, 0.362 ± 0.097, 0.432 ± 0.150 and 0.738 ± 0.147, respectively. At 12 h after GCRV infection, the protein level of ScMDA5 was the highest in the liver and the lowest in the spleen (Fig. 6). Compared with protein levels of ScMDA5 in healthy tissues, those of ScMDA5 in tissues infected with GCRV were downregulated, although no significant difference was found.

4. Discussion

Early host response to pathogens is mediated by several distinct

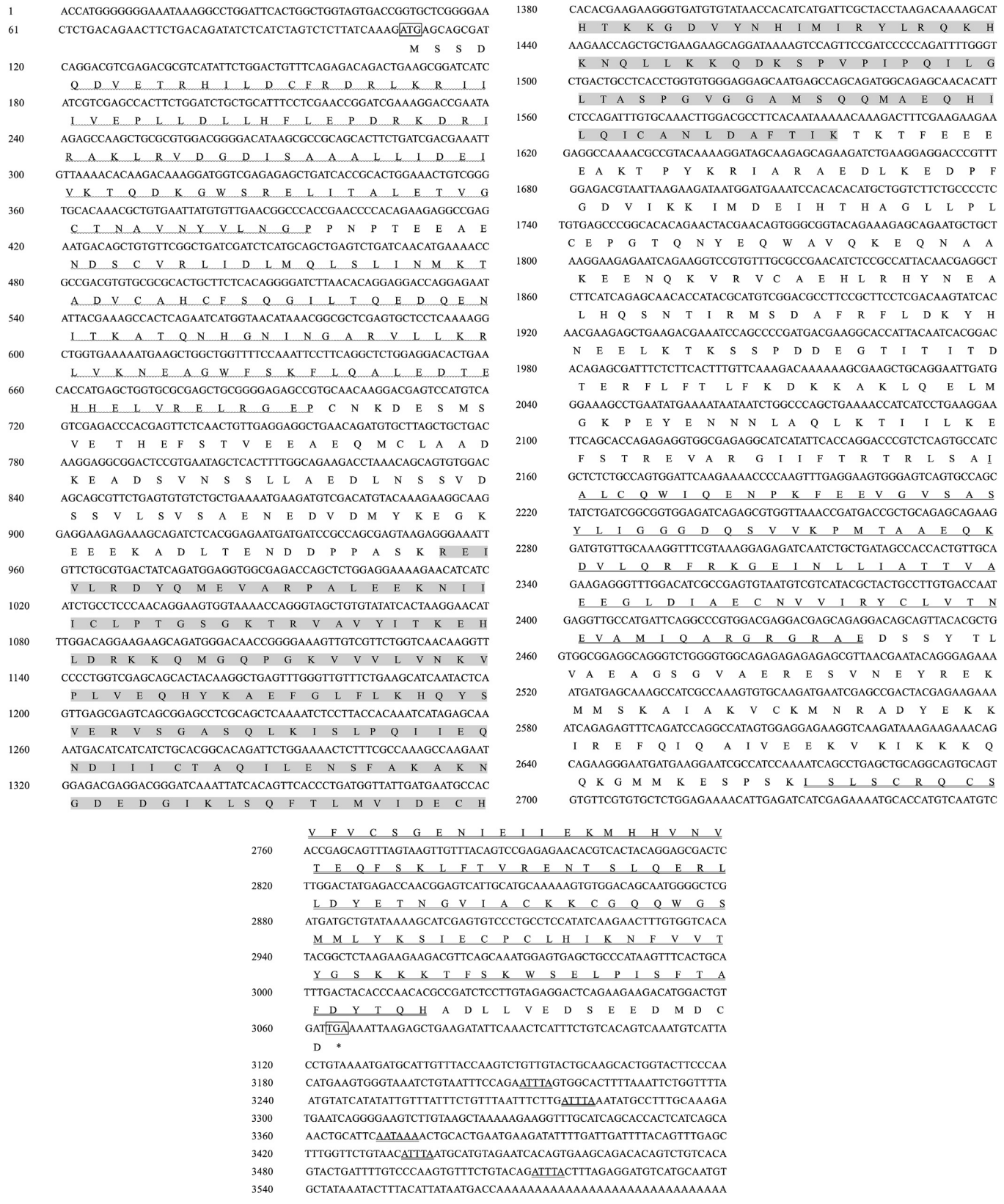

Fig. 1. ScMDA5 cDNA and amino acid (aa) sequences.
The start codon (ATG) and stop codon (TGA) are indicated in boxes. The CARD aa sequence is underlined (wavy), DEXDc is highlighted in grey, HELICc is underlined (single) and the RIG-I_C-RD. domain (856–970 aa) is underlined (double). The mRNA instability motif (attta) and the polyadenylation signal motif (aataaa) are underlined (double).

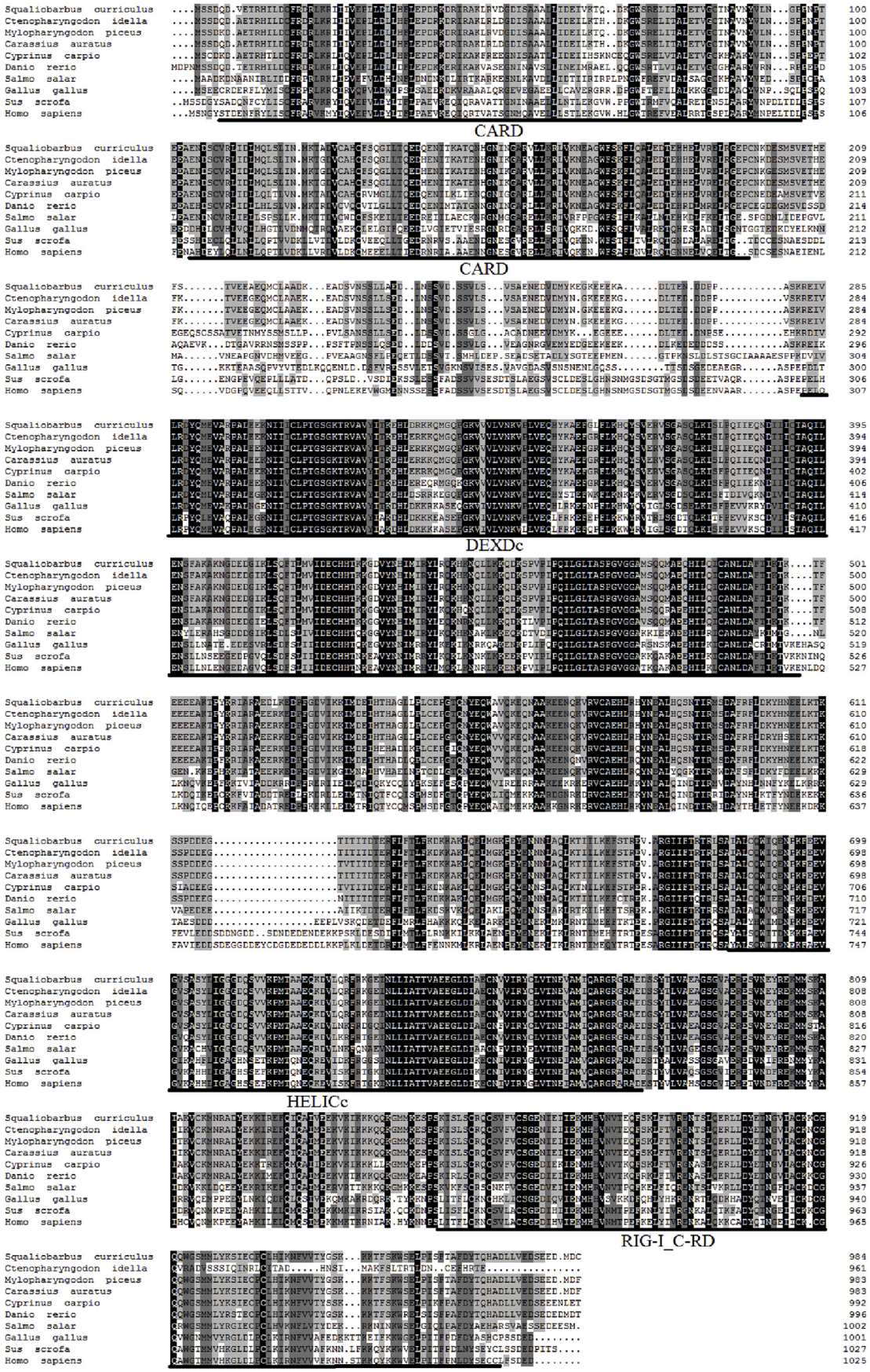

Fig. 2. Alignment of the ScMDA5 domains with those of other fish and mammalian species.

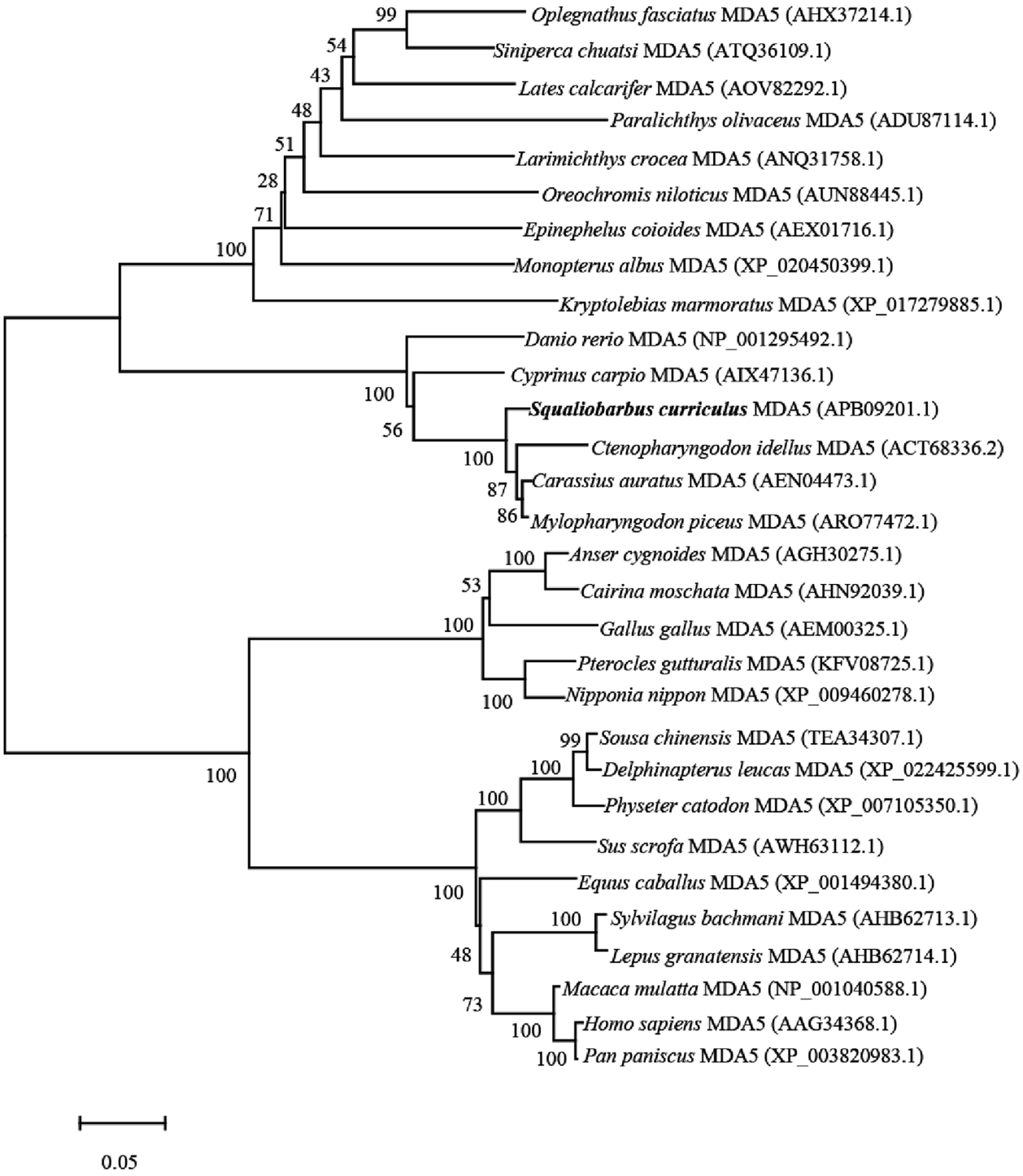

Fig. 3. Phylogenetic relationship of MDA5 proteins.

pattern recognition receptors. Cytoplasmic RNA helicases, including RIG-I and MDA5, have shown to respond to viral RNA by inducing interferon production [21]. The deduced amino acid sequence of ScMDA5 contained functional domains, including CARD, DEXDc and RIG-I_C-RD, and these domains shared many identical amino acid residues with those from other fish species. In the cytoplasm, intracellular antiviral responses were initiated by the recognition of dsRNA generated by the replication of infected viruses. As for MDA5 from other fish, ScMDA5 may also sense intracellular viral infection and trigger innate antiviral responses through its DEXDc and transmit a signal downstream of CARD [22,23]. The RIG-I_C-RD domain is responsible for the binding of viral RNA, and differences in this domain are the basis of distinct dsRNA recognition and binding ability [24,25]. MDA5 CTD (with a dsRNA binding site) binds to the dsRNA stem and then forms an open ring structure around the dsRNA stem in the cytoplasm [24,26]. In the present study, domain differences (focussed on sequence length and location) were found between ScMDA5 and those from other fish species, indicating that viral recognition and binding ability of MDA5s may be different.

Upon activation, MDA5 interacts with downstream adaptor IPS-1 and initiates signalling to IRF3, IRF7 and NF-κB transcription factors, resulting in the induction of antiviral response by IFN production [27].

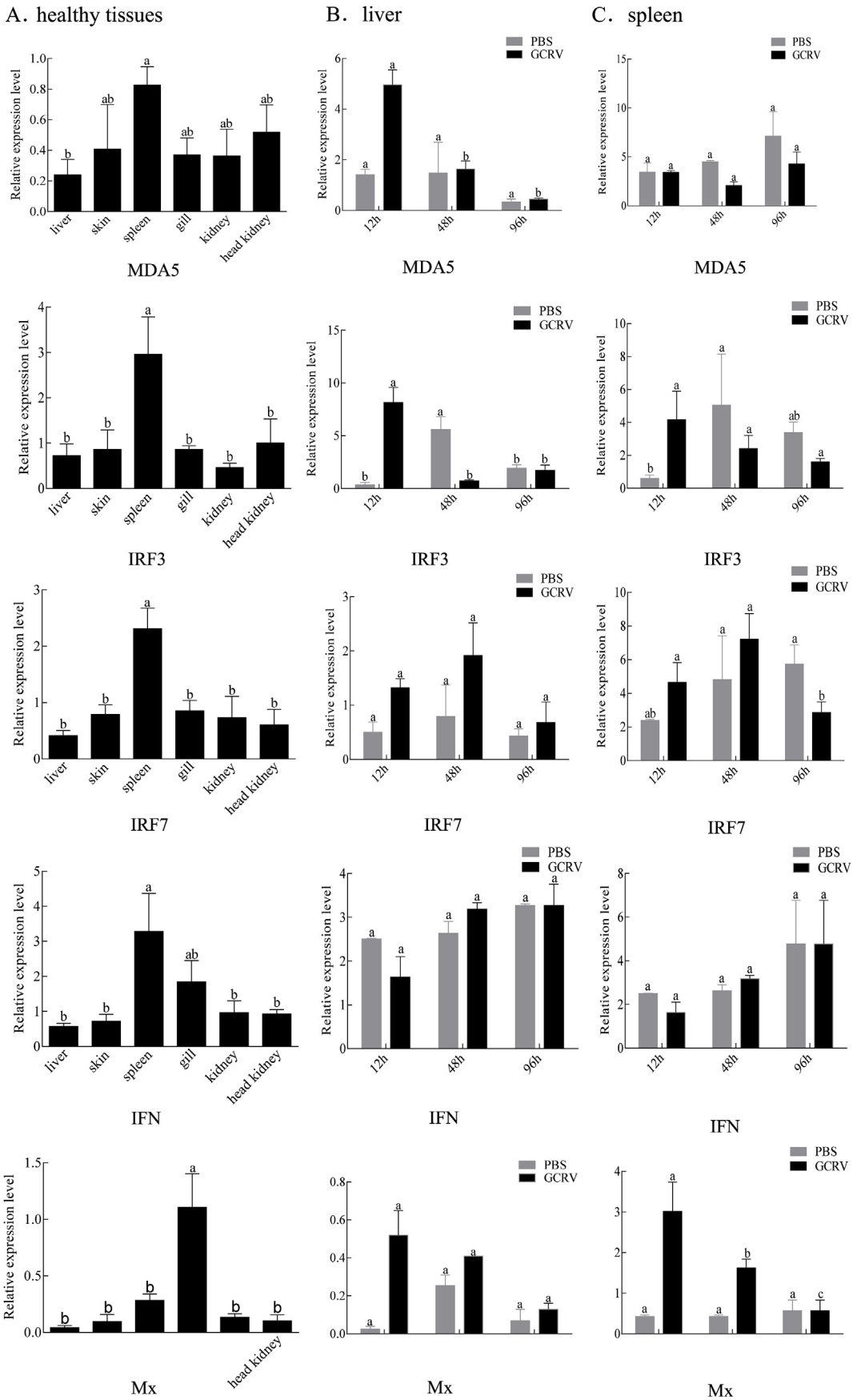

Fig. 4. Expression levels of *ScMDA5*, *ScIRF3*, *ScIRF7*, *ScIFN* and *ScMx* in tissues after GCRV infection or PBS treatment. The letters a, b and c indicate the significant difference in gene expression levels between different time points for either treatment ($P < 0.05$).

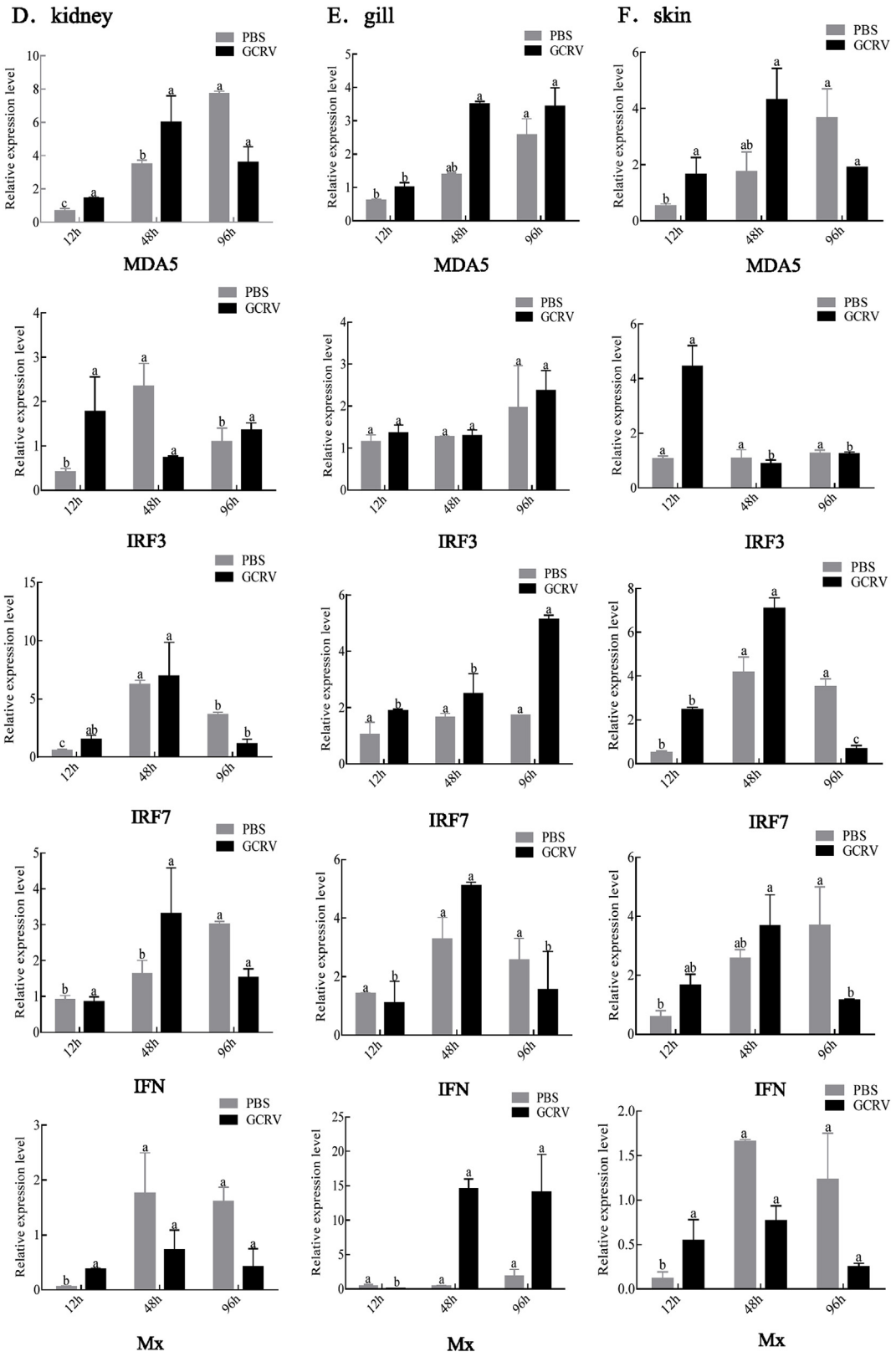

Fig. 4. (*continued*)

IFN signalling elicits the activation of a group of interferon-stimulated genes, including Mx, ISG56 and Trim39, that are responsible for limiting viral infection and replication within a cell [28]. In the present study, expression levels of *ScMDA5* in healthy tissues were significantly correlated with those of downstream signalling pathway members, such as *ScIRF3*, *ScIRF7* and *ScIFN*, indicating that they are in a same signal pathway and work together to deal with viral invasion. Among the detected molecules in the ScMDA5 signalling pathway, expression levels of *ScIRF3* and *ScIRF7* showed an extremely significant positive relationship. *IRF3* and *IRF7* are molecules with high structural

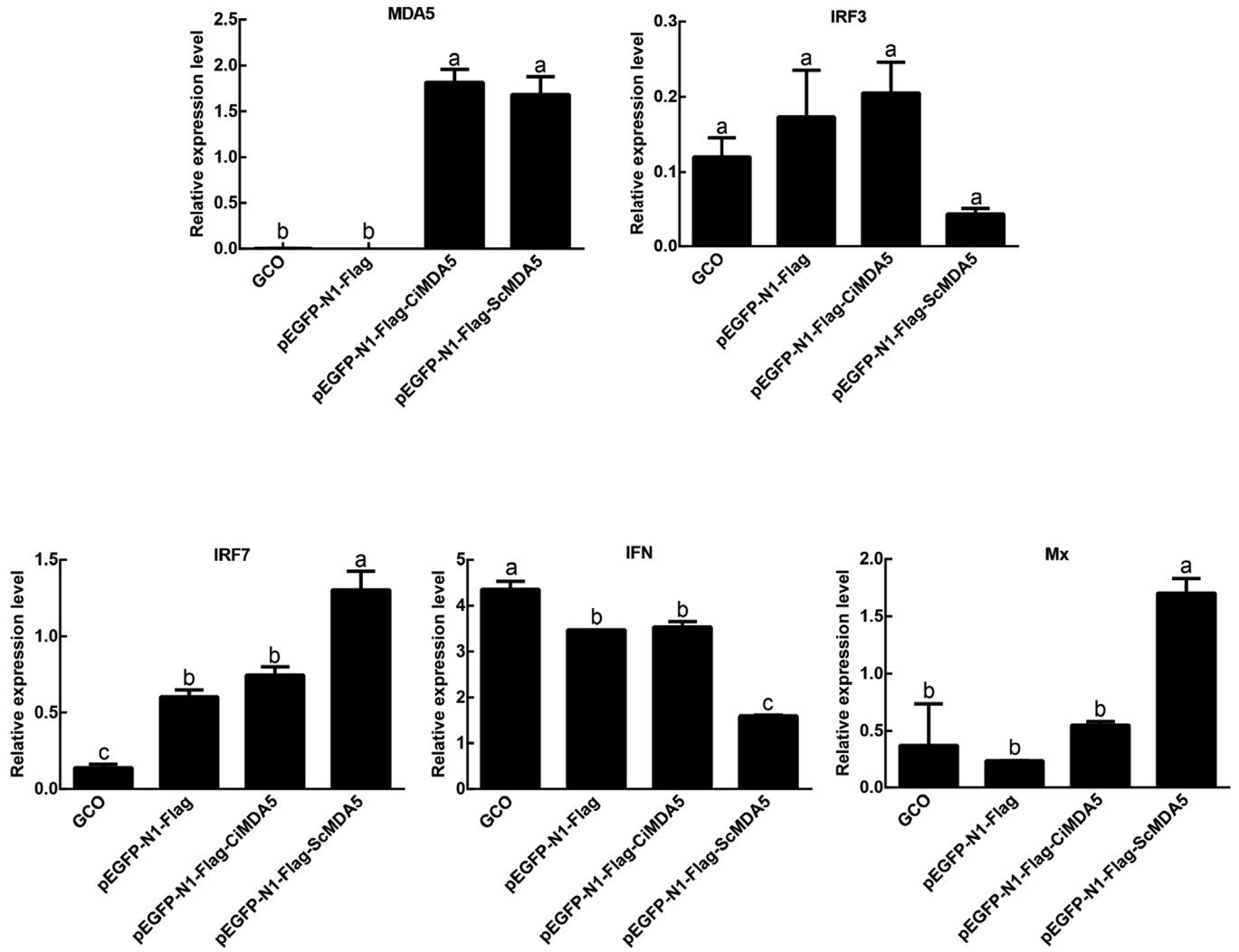

Fig. 5. Effect of overexpression of ScMDA5 on MDA5, CiIRF3, CiIRF7, CiIFN and CiMx in GCO cells at 24 h post infection. The letters a, b and c indicate the significant difference ($P < 0.05$) in mRNA expression levels between different treatments (GCO cells, GCO cells with pEGFP-N1-Flag, GCO cells with pEGFP-N1-Flag-CiMDA5 and GCO cells with pEGFP-N1-Flag-ScMDA5).

homology; expression levels of both were upregulated following nervous necrosis virus infection in the Asian seabass *L. calcarifer* [29]. *IRF3* and *IRF7* cooperatively act in myeloid dendritic cells downstream of mitochondrial antiviral protein signalling to regulate activation of the IFN gene [30,31]. The relationships between the expression levels of MDA5 signalling members were also found in *ScMDA5* overexpression experiments, and the upregulation of the expression of *ScMDA5* led to significantly enhanced *CiIRF7* and *CiMx* transcription in pEGFP-N1-Flag-ScMDA5-overexpressed GCO cells. The transcriptional factor *IRF-7* is essential for the induction of IFN-α/β genes after virus infection, and it is the master regulator of IFN-1-dependent immune responses [32,33]. Overexpression of *ScMDA5* induced the activation of the IFN-I regulator *IRF7* and the interferon-stimulated gene *Mx* in GCO cells, indicating that *ScMDA5* enhances the immune response in *C. idellus* cells.

The responses of MDA5-mediated signal pathway members to GCRV were different among the different tissues of *S. curriculus*. For example, mRNA levels of *ScMDA5* and *ScMx* were downregulated in the liver, whereas the two molecules were significantly upregulated in the gill. At least three expression level change modes, including downregulation, upregulation and initial upregulation followed by downregulation, were found for MDA5 signalling pathway molecules in *S. curriculus* tissues after GCRV invasion. Diverse immune response modes and discrepancy in expression levels of MDA5-mediated signalling pathway members indicated that there are obvious functional differentiations among *S. curriculus* immune tissues/organs regarding response to virus invasion [34,35]. Simultaneously, data of the current study showed that *ScMDA5*, *ScIRF3*, *ScIRF7* and *ScIFN* transcripts were most abundant in the spleen, suggesting that the spleen is a potential target tissue for immune functional studies and selective breeding of aquatic animals with increased disease resistance [36]. From our investigations into protein levels, we observed that in healthy or GCRV-infected tissues, protein levels of ScMDA5 were the highest in the liver and the lowest in spleen, which is nearly opposite to corresponding mRNA expression levels of *ScMDA5*. The reason for this discrepancy in the mRNA and protein levels of ScMDA5 may be the modifications or degradation of mRNA during the translation process, which ultimately affects the production of the ScMDA5 protein [37]. Based on the results of the present study, protein levels of ScMDA5 were more reduced in GCRV-infected tissues than in healthy tissues (although no significant change was observed in protein levels). Our previous study has shown that *S. curriculus* was resistant to GCRV, suggesting that a relative higher basal protein level of ScMDA5 can lead the fish to respond quickly and strongly enough to prevent viral replication [18]. The ScMDA5 protein may rapidly degrade to protect the fish from excessive immunity after the successful induction of type I IFNs and the inhibition of viral replication [38].

Taken together, the full-length cDNA of *ScMDA5* was obtained,

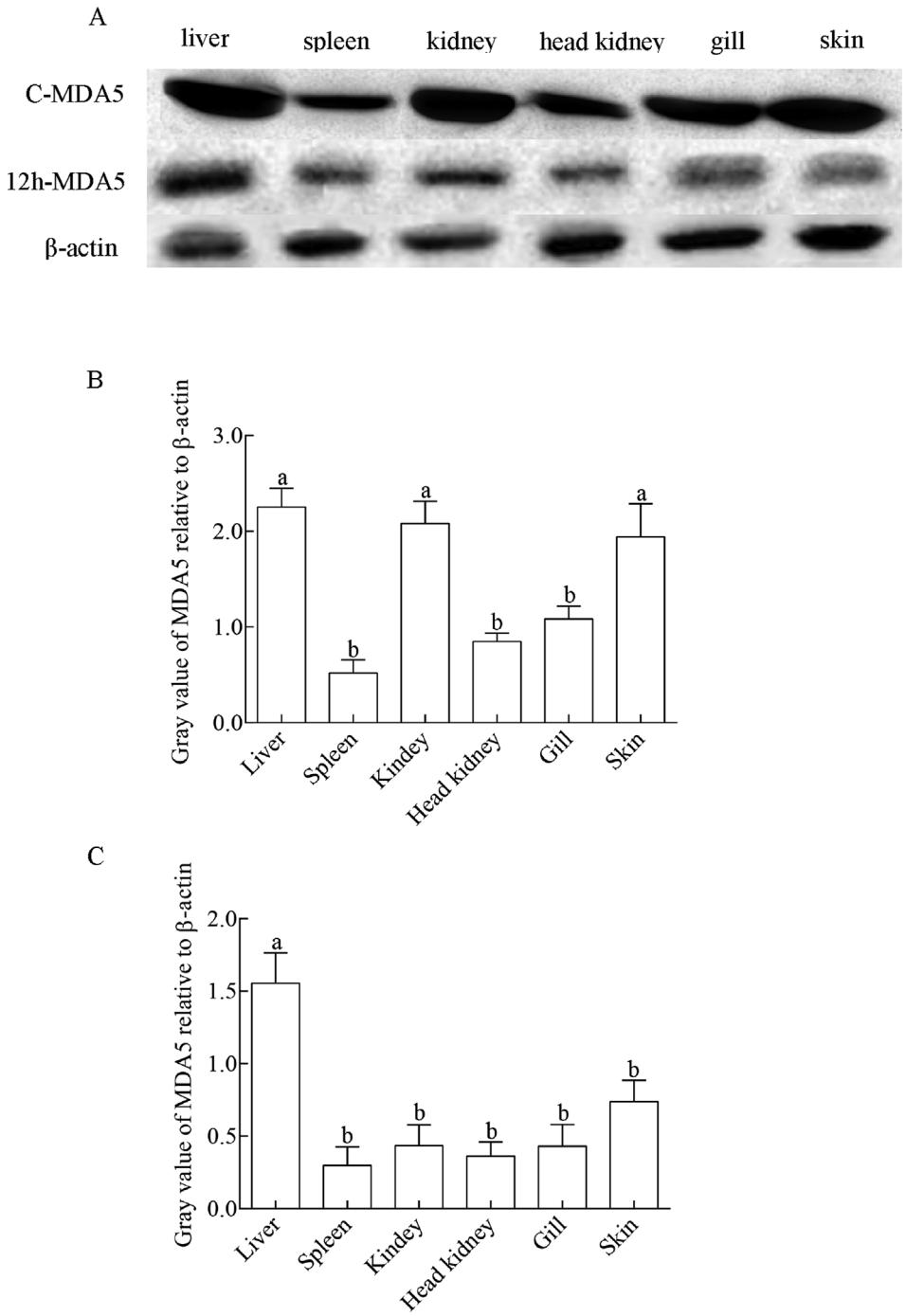

Fig. 6. Western blot analysis of ScMDA5 protein abundance in different tissues. A: C-MDA5, ScMDA5 protein detection in the control group; 12 h-MDA5, ScMDA5 protein detection in tissues 12 h after GCRV infection. B: tissue protein levels of ScMDA5 in the control group; C: tissue protein levels of ScMDA5 12 h after GCRV infection. The letters a, b and c indicate significant differences ($P < 0.05$) in protein levels between different tissues.

which comprises 3597 bp that encoded a putative peptide of 985 amino acid residues. CARD, DEXDc and RIG-I_C-RD were domains of ScMDA5 and may function in the recognition and binding of viral dsRNAs. The expression of *ScMDA5* in healthy tissues was significantly correlated with that of *ScIRF3*, *ScIRF7* and *ScIFN*, indicating that they are in the same signal pathway and work together to deal with viral invasion. Upregulation of the expression level of *ScMDA5* led to significant enhancement of the transcription of *CiIRF7* and *CiMx* in pEGFP-N1-Flag-ScMDA5-overexpressed GCO cells. The protein level of ScMDA5 was the highest in the liver and the lowest in the spleen. After GCRV infection, the protein level of ScMDA5 was downregulated, and this may be because it is rapidly degraded to protect fish from excessive immune responses. The results from this study provide a fundamental for fish anti-disease breeding regimens.

Acknowledgements

The Project was funded by the National Natural Science Foundation of China (31802288, 31572615), China Postdoctoral Science Foundation (2017M612560, 2018T110833), Hunan Provincial Natural Science Foundation of China (2019JJ50231) and Double first-class construction project of Hunan Agricultural University (SYL201802020).

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