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Identification, expression and functional characterisation of *CYP1A* in grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

In mammal, CYP1A has attracted special attention due to its important roles in the oxidative metabolism. In fish, the researches on CYP1A are more focus on its roles in pollution in water environments, but the immune function is unclear. In the study, *CiCYP1A* gene was cloned from grass carp (*Ctenopharyngodon idella*). Tissue distribution exhibited an overwhelmingly high basal expression levels in the liver. After GCRV infection, *CiCYP1A* showed a potent response, indicating *CiCYP1A* was involved in GCRV-induced immunity. Subcellular localisation showed CiCYP1A was distributed in the cytoplasm. Besides, dual-luciferase activity assays revealed CYP1A was relevant for *IFN-I* signaling pathway modulation, furthermore, overexpressed CYP1A potently suppressed the mRNA expression of *IRF3* and *IFN-I* but not *IRF7*. The results provide new sights into exploring immune function of *CiCYP1A* in teleosts.

1. Introduction

Grass carp (*Ctenopharyngodon idella*) is a native economical aquaculture species in China, accounting for approximately 10% of total aquaculture production in 2017 [1], which contributes a lot to meet the growing demand for aquatic animal protein. However, the frequent outbreak of grass carp hemorrhagic disease has brought huge losses to aquaculture industry [2]. Grass carp hemorrhagic disease was caused by grass carp reovirus (GCRV), the GCRV-infected fish would initiate intense immune responses, such as defense response, inflammatory response, and innate immune response to resist invaders [3–5], but GCRV can still escape the host's immune system by blunting the transcription of host interferon (IFN) [6]. Currently, although the pathogenesis of hemorrhagic disease has not been well revealed, the completion of draft genome of grass carp provides great hope for breeding new disease-resistant varieties, and also helps to explore the gene function of fish [7,8].

Cytochrome P450 enzymes (CYPs) are a group of heme-thiolate monooxygenases, which are involved in the oxidative metabolism of endogenous compounds and xenobiotics, thereby playing critical roles in cholesterol and hormone synthesis, the synthesis and degradation of biogenic amines, synthesis and metabolism of vitamin D_3, the hydroxylation of retinoic acid and presumably other morphogens, drug deactivation, and xenobiotics detoxification [9–14]. Mammalian CYP enzymes consist of 57 genes, which are classified into 18 families according to their cDNA and amino acid sequence identities [15–17]. Over the past decade, the CYP families have been relatively well studied as its function of biotransformation reactions with activation of prodrugs or degradation of exogenous substances in the liver. Notably, the researches on the CYPs have recently become the subject of particular scientific interest because CYPs are involved in various diseases including cancer and immune regulation [18–22].

Among the numerous of CYP family members, CYP1A has attracted special attention due to its important roles in metabolically activating many environmental procarcinogens in the polyaromatic hydrocarbons (PAHs), polyhalogenated aromatic hydrocarbons (PHAHs), aryl- and alkyl-amine, heterocyclic amine, and dioxins [9,23]. In view of this, CYP1A is also extensively used as a biomarker to assess contamination of the aquatic environment [24–27]. CYP1A is regulated by the aryl hydrocarbon receptor (AHR), which can interact with nuclear factor κB (NFκB), oestrogen receptor-α (ESRα) and retinoblastoma protein 1 (RB1), leading to the transcription of genes involved in growth, cell cycle, apoptosis, and immunity [22,28–30]. In fish, a large number of researches about CYP1A are focus on its roles in pollution in water

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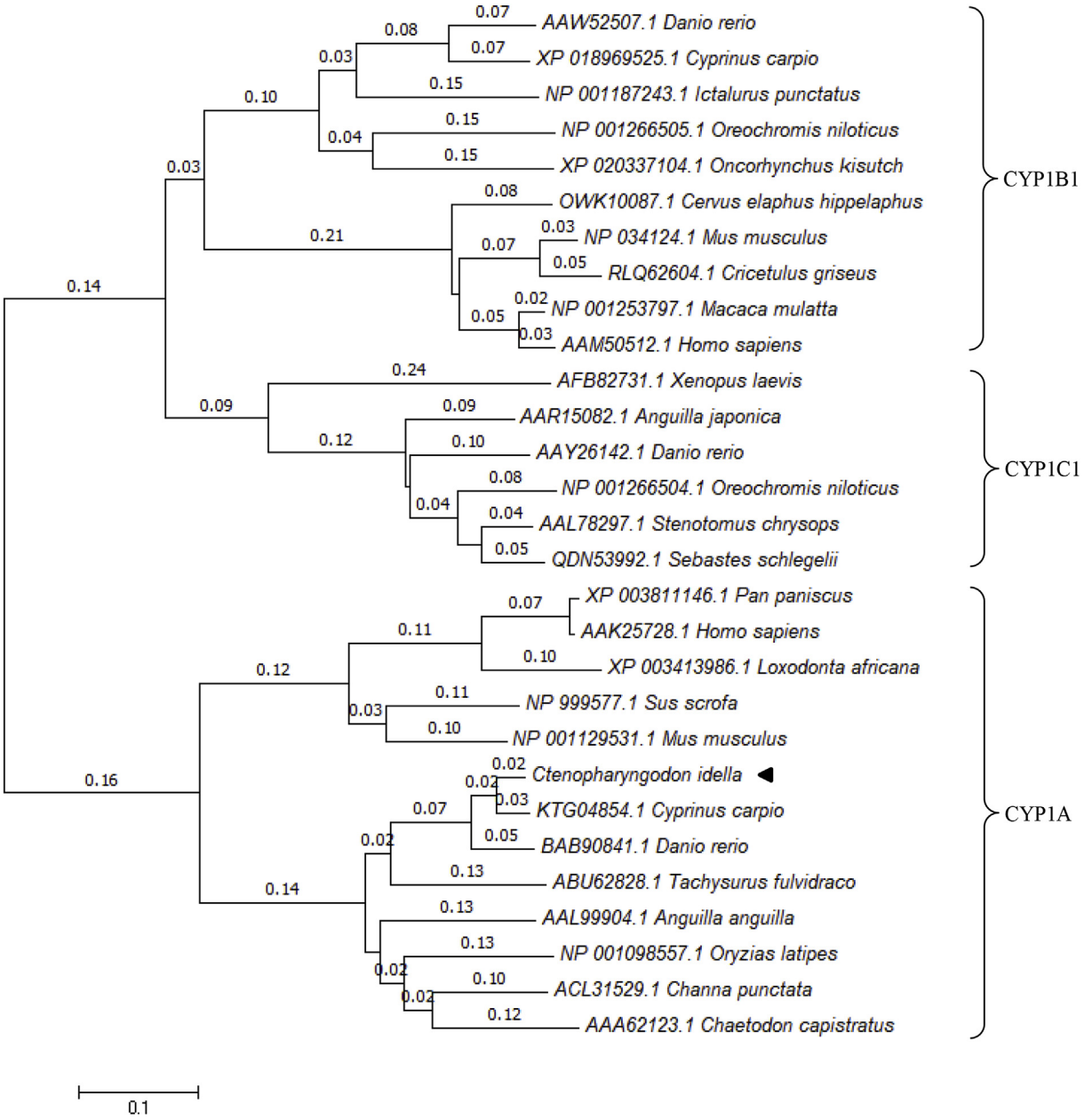

Fig. 1. Neighbour-joining phylogenetic tree analysis of CiCYP1A. The tree was constructed based on amino acid sequences of CiCYP1A and 28 other CYP1 family members using MEGA 7 software and the bootstrap values of the branches were obtained by testing the tree 1000 times.

environments [31–34]. However, little studies about CYP1A on immunity were reported. In this study, *CYP1A* was identified from *Ctenopharyngodon idella* (named as *CiCYP1A*) and functionally characterised. The expression profiles of *CiCYP1A* and subcellular localisation were analysed. Besides, the regulation of *Ci*CYP1A on *IFN-I* signaling pathway was explored. The results provided further insights into the gene function of *CiCYP1A* in teleost.

2. Materials and methods

2.1. Experimental fish and cells

Grass carp (weight, 40 ± 10 g; length, 15 ± 3 cm) were obtained from GuanQiao Experimental Station, Institute of Hydrobiology, Chinese Academic of Sciences, and acclimatized in aerated freshwater at 28 ± 1 °C for 1 week prior to use in experiments. The *Ctenopharyngodon idella* kidney (CIK) and ovary (CO) cells (China Center for Type Culture Collection, China) used in the study were maintained in low glucose Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with 10% fetal bovine serum at 28 °C in a humidified atmosphere with 5% CO_2 and 1% (v/v) penicillin-streptomycin. The 293 T cells (a kind gift from Professor Wei Hu, Institute of Hydrobiology, Chinese Academy of Sciences) were maintained at 37 °C and the other condition was the same as above.

2.2. Cloning and identification of CiCYP1A

The total RNA was extracted from the liver of healthy grass carp and then reverse-transcribed to obtain cDNA as described previously [35]. Partial fragments of *CiCYP1A* were obtained from grass carp whole-genome sequence database [7]. 5′ and 3′ untranslated regions (UTRs) of the *CiCYP1A* gene were obtained by the rapid amplification of cDNA ends (RACE) PCR according to the instructions of 5′ and 3′ Full RACE Kit (TaKaRa, Japan). The coding sequences (CDS) were amplified using

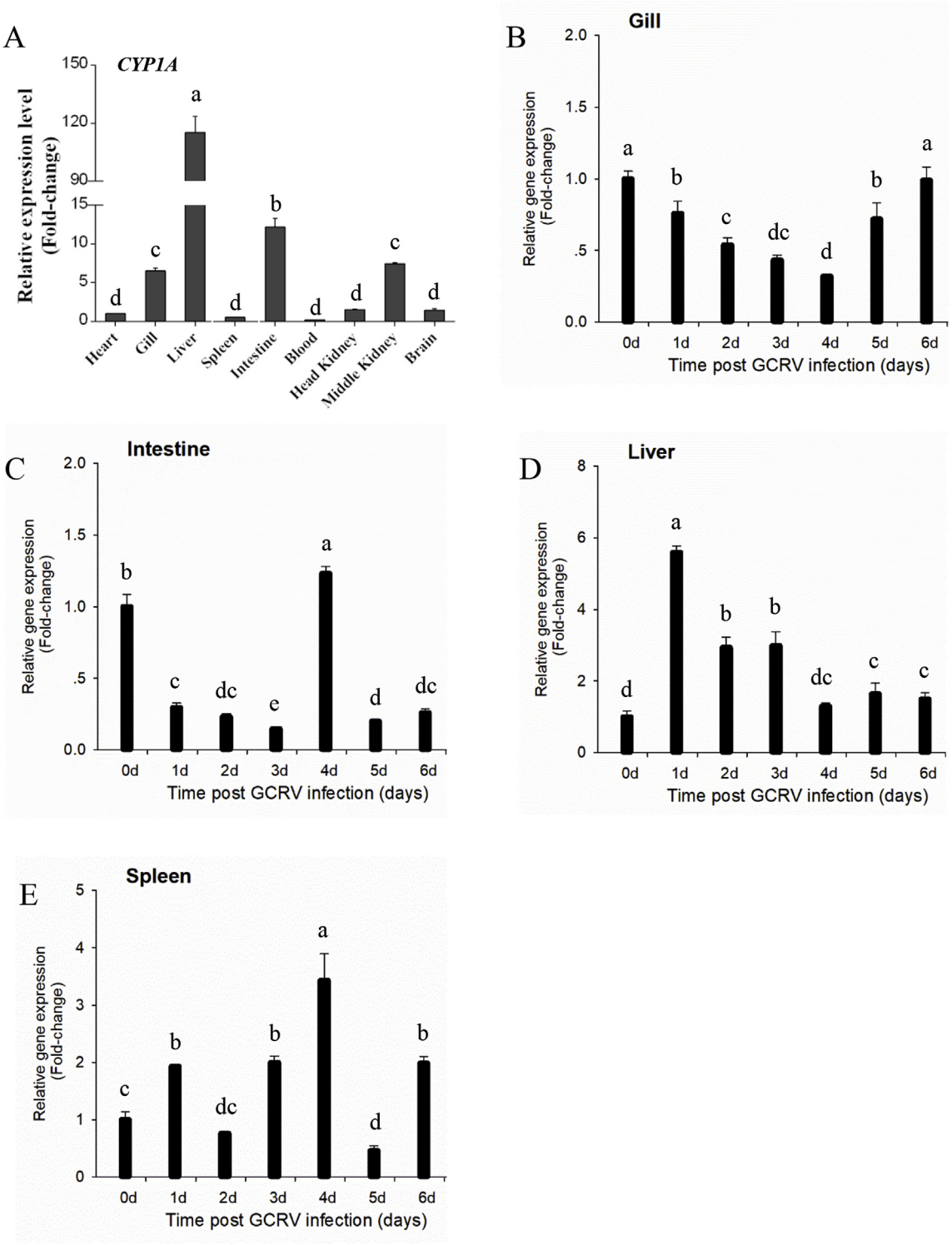

Fig. 2. Tissue distribution of *CiCYP1A* and the expression pattern in response to GCRV infection. RNA extraction from healthy grass carp tissues (heart, gill, liver, spleen, intestine, blood, head kidney, middle kidney, and brain) was subjected to qRT-PCR analysis. A. Relative mRNA expression levels of *CiCYP1A* were calculated on the basis of the ratio of gene expression in the different tissues relative to that of the heart. The expression levels of *β-actin* were used as an internal control. B-E. The mRNA expression levels of *CiCYP1A* on day 0 were set to 1 and *β-actin* was used as an internal control to normalize *CiCYP1A* mRNA expression from the gill (B), intestine (C), liver (D), spleen (E) after GCRV infection. Error bars indicate standard deviation. The data (expressed as mean ± standard deviation) were analysed by one-way analysis of variance, followed by Dunnett's test for multiple comparisons using SPSS Statistics 19 software. Different hours labelled with different letters indicate statistically significant differences in mRNA levels ($p < 0.05$).

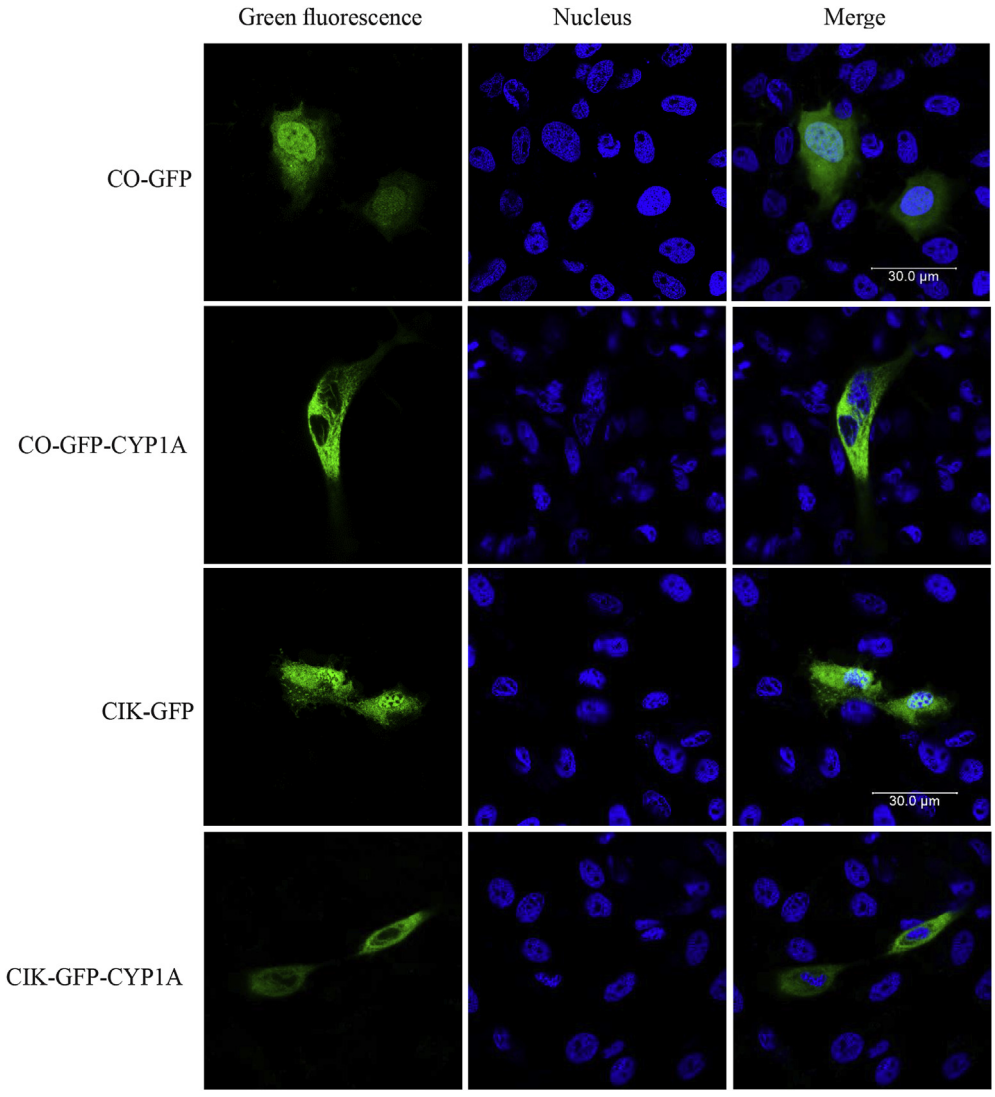

Fig. 3. Subcellular localisation of CiCYP1A in CO and CIK cells. Cells were transfected with CYP1A-GFP plasmids, and fluorescence was observed at 24 h after transfection. Green fluorescence showed the distribution of CYP1A and blue fluorescence showed the nucleus stained with Hoechst 33342 under a 63 × oil immersion objective lens (scale bar, 30 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

PCR with primers within the 5′- and 3′-UTRs. Primers for gene cloning were listed in Table S1.

2.3. Sequence analysis

Primers were designed by primer premier 5 and nucleotide sequences were assembled by DNAMAN 7. Amino acid sequences were predicted using open reading frame (ORF) Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) and protein domain features were predicted by SMART (http://smart.embl-heidelberg.de/). Neighbour-joining (NJ) phylogenetic trees were constructed on the basis of the amino acid sequences by using MEGA7 software (http://www.megasoftware.net/index.html) and the bootstrap values of the branches were obtained by testing the tree 1000 times.

2.4. Tissue distribution of CiCYP1A in grass carp

Nine tissues (heart, gill, liver, spleen, intestine, blood, head kidney, middle kidney, and brain) were sampled from six healthy grass carp. Total RNA was extracted from the tissues and then reverse-transcribed to obtain cDNA as described previously [35]. In order to explore the tissue distribution of *CiCYP1A* in grass carp, qRT-PCR and the CFX96™ real-time PCR detection system (Bio-Rad, USA) were used to measure the mRNA expression levels of *CiCYP1A*. The housekeeping gene *β-actin* was used as a reference gene. Relative expression levels were calculated as the ratio of gene expression in each tissue relative to that of the heart. The specific primers for qRT-PCR were listed in Table S1. Relative expression levels of *CiCYP1A* were calculated using the $2^{-\triangle\triangle Ct}$ method [36].

2.5. GCRV challenge

The GCRV preparation and GCRV (2.97 × 103 copy/μL) challenge experiment were carried out as described previously [35]. Briefly, healthy grass carp were intraperitoneally injected with 200 μL PBS as control group, the other group were injected with an equal volume of GCRV. Tissues (gill, intestine, liver, and spleen) were also randomly sampled from six fish for consecutive days after intraperitoneal injection (D0-D6). All samples were homogenized in TRIzol reagent (Invitrogen, USA) and reverse-transcribed into cDNA for expression pattern analysis of *CiCYP1A* in respond to GCRV infection.

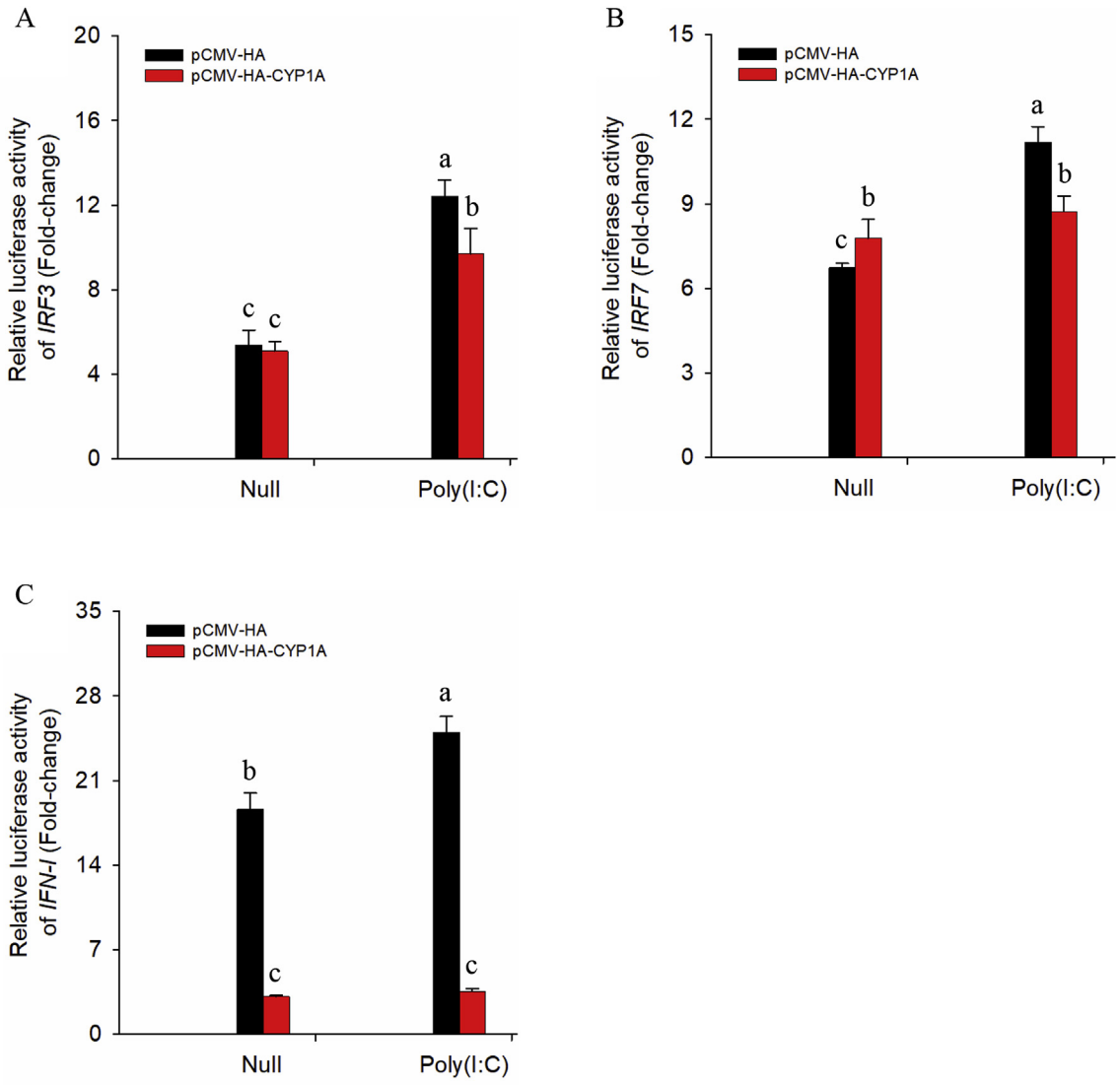

Fig. 4. CiCYP1A suppressed the promoter activity of *IFN-I* signaling pathway after poly(I:C) infection. 293 T cells were seeded in 24-well plate for 16–18 h. Subsequently, the cells were transfected with 300 ng of pIRF3pro-Luc, or pIRF7pro-Luc, or pIFN-Ipro-Luc, 300 ng of pCMV-HA-CYP1A or pCMV-HA, and 10 ng of pRL-TK Renilla plasmid (Promega, USA) for 6 h. Then the medium was renewed by DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and stimulated with poly(I:C) or PBS for 24 h. Renilla luciferase activity was examined as the internal control and relative luciferase activity levels were expressed as fold increase of luciferase activity. Error bars indicate standard deviation. The data (expressed as mean ± standard deviation) were analysed by one-way analysis of variance, followed by Dunnett's test for multiple comparisons using SPSS Statistics 19 software. Different hours labelled with different letters indicate statistically significant differences in mRNA levels ($p < 0.05$).

2.6. Construction of plasmid vectors

To analyse the roles of CiCYP1A in *IFN-I* signaling pathway, plasmid pCMV-HA-CYP1A and pIFN-Ipro-Luc were constructed. The restriction sites *Eco*RI and *Kpn*I (NEB, USA) were introduced to construct pCMV-HA-CYP1A as described previously [37]. Briefly, plasmid pCMV-HA (Clontech, USA) was linearized with *Eco*RI and *Kpn*I, then linearized pCMV-HA together with CiCYP1A ORF which amplified by primers designed by CE Design (http://www.vazyme.com) was incubated at 37 °C for 30 min and then incubated on the ice straightway according to the manufacturer's instructions of ClonExpress kit (Vazyme, USA). Plasmid pIFN-Ipro-Luc were constructed as following method. The promoter region of *IFN-I* were cloned, digested with *Kpn*I and *Xho*I and then subcloned into the same restriction sites of pGL3-Basic luciferase reporter vector (Promega, USA). Plasmids pIRF3pro-Luc and pIRF7pro-Luc were constructed and kept in our lab [37] (primers were listed in Table S1).

In order to analyse the subcellular localisation of CiCYP1A, CYP1A-GFP vector was constructed as the same method described above. The restriction sites *Eco*RI and *Kpn*I (NEB, USA) were introduced and subcloned into the same digested restriction sites of pEGFP-N3 vector (Clontech, USA). All the sequences of the resulting plasmids were verified by DNA sequencing (Qing Ke, China).

2.7. Subcellular localisation of CiCYP1A

To explore the subcellular localisation of the CiCYP1A, CO and CIK cells were plated evenly in six-well plates with glass bottom for 24 h to 70–80% confluence. Then, 2500 ng of CYP1A-GFP vectors were transiently transfected into the cells. After 24 h transfection, all cells were fixed with 4% paraformaldehyde, and stained with Hoechst 33342 (Beyotime, China). The cells were observed with confocal system (SP8, Leica, Germany) and a 63 × oil immersion objective lens.

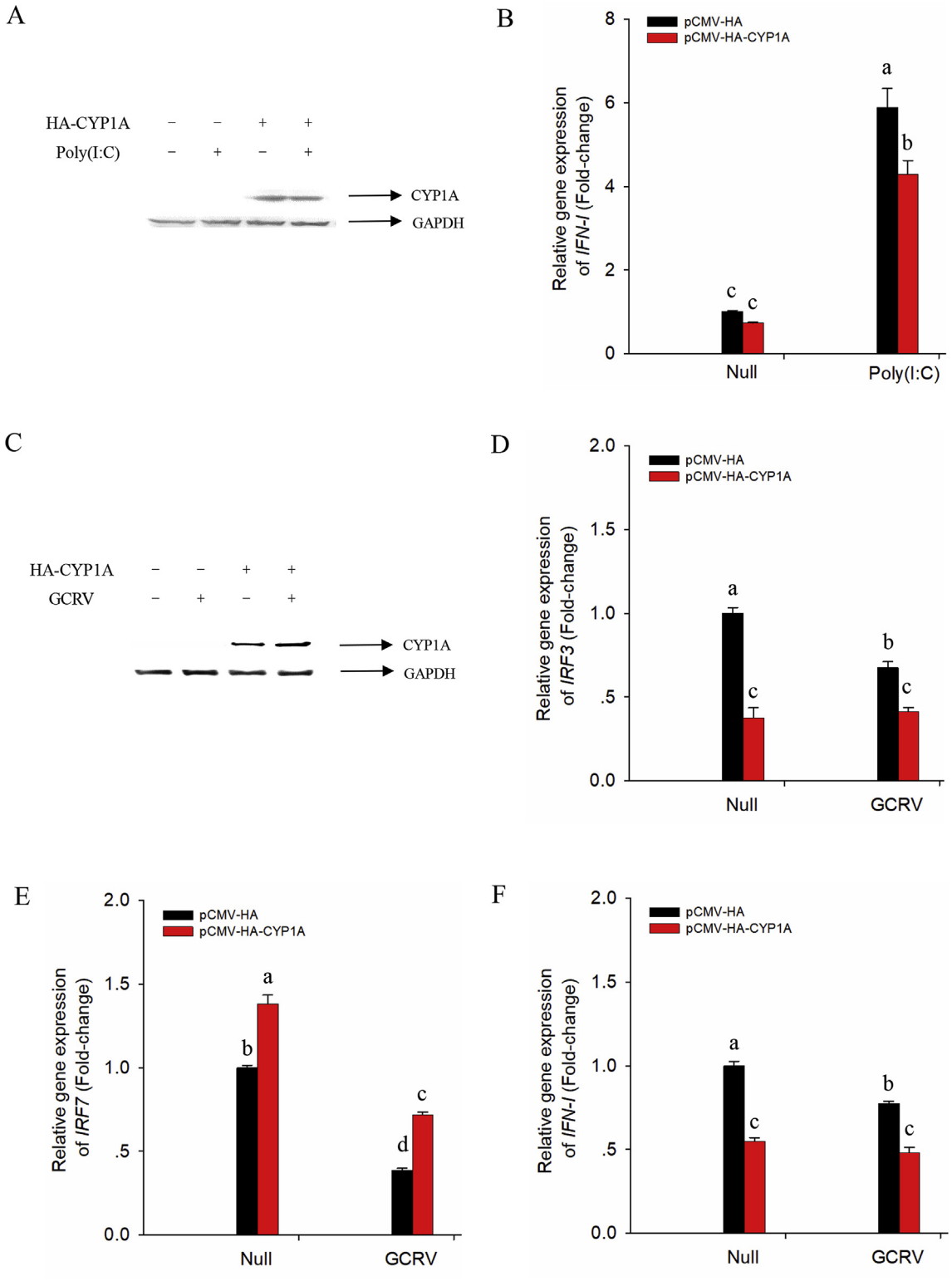

Fig. 5. CiCYP1A suppressed the expression levels of *IFN-I*. CO cells seeded in 6-well plate were transfected with pCMV-HA-CYP1A or empty vector, at 24 h post-transfection, cells were infected with poly(I:C) or GCRV or PBS. At 24 h post-infection, total RNA were extracted to examine the mRNA expression levels of *IFN-I* signaling pathway genes (B, D, E, and F). The expression of CiCYP1A was confirmed by Western blot (A and C). Error bars indicate standard deviation. The data (expressed as mean ± standard deviation) were analysed by one-way analysis of variance, followed by Dunnett's test for multiple comparisons using SPSS Statistics 19 software. Different hours labelled with different letters indicate statistically significant differences in mRNA levels ($p < 0.05$).

2.8. Dual-luciferase activity assays

In order to explore the regulatory effect of *CiCYP1A* on the promoter activity of *IFN*-related genes, 293 T cells were seeded in 24-well plate and incubated at 37 °C overnight. Subsequently, the cells were transfected with 300 ng of pIRF3pro-Luc, or pIRF7pro-Luc, or pIFN-Ipro-Luc, 300 ng of pCMV-HA-CYP1A or pCMV-HA, and 10 ng of pRL-TK Renilla plasmid (Promega, USA) for 6 h. Then the medium was renewed by DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and stimulated with poly(I:C) or PBS for 24 h. Cells were lysed by 200 ml of 1 × Passive Lysis Buffer (Promega, USA) and luciferase activities were measured by using Dual-Luciferase Reporter Assay System (YEASEN, China).

2.9. Modulation of IFN-I related genes expression by CiCYP1A

In order to further elucidate the roles of *CiCYP1A* in the *IFN-I* signaling pathway, 2500 ng of pCMV-HA-CYP1A or empty vector were transfected into CO cells seeded in 6-well plate (the transfection efficiency in CO cells is much higher than that of CIK cells). At 24 h post-transfection, cells were infected with poly(I:C) or GCRV or PBS. At 20 h post-infection, total RNA were extracted to examine the mRNA expression levels of *IRF3*, *IRF7*, and *IFN-I*. Another group were washed twice with PBS and incubated with cells lysate (Beyotime, China) for Western blot to detect the over-expression levels of CiCYP1A protein.

2.10. Statistical analysis

The statistical results (expressed as mean ± standard deviation) were analysed by one-way analysis of variance, followed by Dunnett's test for multiple comparisons using SPSS Statistics 19 software. $P < 0.05$ was considered to be statistically significant. All experiments were repeated at least three times.

3. Results

3.1. Molecular characterisation of CiCYP1A

The *CYP1A* in grass carp (named *CiCYP1A* and Genbank accession number: MK852703) encoded a predicted polypeptide of 524 amino acids (Fig. S1) and protein domain features analysis revealed that CiCYP1A consisted a transmembrane region (TM, aa 15–27) in N-terminal and followed by a conserved low complexity region (aa 44–55). Evolutionary relationship analysis based on the full-length amino acid sequences of CiCYP1A and 28 other CYP1 family members revealed that CiCYP1A were closely related to that of *Cyprinus carpio* and *Danio rerio* (Fig. 1), which indicated CiCYP1A is an ortholog of CYP1A in mammals.

3.2. Tissue distribution of CiCYP1A in healthy grass carp

Nine tissue samples (heart, gill, liver, spleen, intestine, blood, head kidney, middle kidney and brain) were isolated from healthy grass carp for qRT-PCR to analyse the tissue distribution of *CiCYP1A* gene in grass carp. As shown in Fig. 2A, *CiCYP1A* was overwhelmingly enriched in the liver, which was almost ten times as that of the intestine, the second highest tissue, and expressed the lowest in the blood.

3.3. Analysis of CiCYP1A expression following GCRV infection

The relative mRNA expression levels of *CiCYP1A* on different days after GCRV infection were examined by RT-qPCR. As shown in Fig. 2, following GCRV stimulation, *CiCYP1A* mRNA expression levels were altered in all the detected tissues. In the gill and intestine, *CiCYP1A* were almost downregulated in all detected days (except day 4 in the intestine). In the liver, *CiCYP1A* expression levels were sharply up-regulated to the peak on day 1, subsequently, down-regulated in next three days, afterward increased at the late stage. In the spleen, the expression levels of *CiCYP1A* is particularly unstable. On day 1, it was significantly up-regulated and then immediately decreased to the former levels on day 2, subsequently, sharply up-regulated again and peaked on day 4, afterward dramatically decreased to the lowest levels on day 5, then up-regulated on day 6.

3.4. Subcellular localisation of CiCYP1A

The subcellular localisation of the CiCYP1A was investigated in the study. CO cells were transfected with CYP1A-GFP plasmids, and then fluorescence was observed at 24 h after transfection. As shown in Fig. 3, CiCYP1A was distributed in the cytoplasm, while the control group were evenly distributed in the cytoplasm and nucleus. In order to further confirm the results, CIK cells were also used to perform the experiment, and the results were consistent with the former.

3.5. CiCYP1A suppressed the promoter activity of IFN-I signaling pathway after Poly(I:C) infection

293 T cells were used to assess the effect of CiCYP1A on the transcriptional regulation of the promoter activity of *IRF3*, *IRF7*, and *IFN-I*. The plasmids pCMV-HA-CYP1A or pCMV-HA were co-transfected into 293 T cells with pIRF3pro-Luc, or pIRF7pro-Luc, or pIFN-Ipro-Luc, and pRL-TK Renilla plasmid. In normal condition, CiCYP1A had no impact on the promoter activity of *IRF3* (Fig. 4A) and even increased the promoter activity of *IRF7* (Fig. 4B), but potently suppresses the promoter activity of *IFN-I* (Fig. 4C). After poly(I:C) infection, compared to the control, CiCYP1A significantly suppressed the promoter activity of *IRF3*, *IRF7*, *and IFN-I*, especially for *IFN-I*.

3.6. CiCYP1A suppressed the expression levels of IFN-I

In order to further elucidate the roles of *CiCYP1A* in the *IFN-I* signaling pathway, plasmids pCMV-HA-CYP1A were transfected into CO cells. The over-expression of CiCYP1A was confirmed by Western blot (Fig. 5A and C). Besides, other cells were sampled for qRT-PCR to detected the expression levels of *IRF3*, *IRF7*, *and IFN-I*. As showed in Fig. 5B, the expression levels of *IFN-I* were significantly decreased by CiCYP1A after poly(I:C) infection, while the expression levels of *IRF3* and *IRF7* were too low to detect. Besides, in order to verify the function of CiCYP1A on *IFN-I* expression, the expression levels of *IRF3, IRF7*, *and IFN-I* were measured after GCRV infection, the results showed that the expression levels of *IRF3* and *IFN-I* but not *IRF7* were suppressed by CiCYP1A.

4. Discussion

In mammal, CYP1A subfamily has two members: CYP1A1 and CYP1A2 [38] and the function of two enzymes are different, CYP1A1 metabolizes the exceptionally hydrophobic PAHs and polyhalogenated aromatic hydrocarbons, while CYP1A2 mainly handles hydrophilic amines [9]. In fish, only one CYP1A in *Danio rerio*, *Oryzias latipes*, *Salmon salar*, *Tachysurus fulvidraco*, *Channa punctate*, *Anguilla japonica* and so on was reported for now, while *Oncorhynchus mykiss* has two members with 98% similarity in protein [39], which indicating the CYP1A gene in fish is somewhat different from that of mammals. In the study, grass carp *CiCYP1A* was identified and functionally characterised. CiCYP1A codes of 524 amino acids and consists a conserved low complexity region (aa 44–55) and a transmembrane region (TM, aa 15–27), which is also existence in *Cyprinus carpio*, *Tachysurus fulvidraco*, and *Homo sapiens* CYP1A2 but absent in the *Danio rerio*, *Oryzias latipes*, *Salmon salar*, *Channa punctate*, *Anguilla japonica*, and *Oncorhynchus mykiss*. This TM region may play an important functions and needs further study.

Mammalian CYP1A1 is mainly expressed in human extrahepatic tissues including the intestine, lung, placenta, lymphocytes, and is expressed at very low levels in the liver, while, CYP1A2 is highly expressed in the liver [40–42]. Tissue distribution analysis showed that *CiCYP1A* was mainly expressed in the liver and almost ten times as that of the intestine, which was the second highest tissue (Fig. 2A). In this regard, CiCYP1A is more like a homologue of CYP1A2 in mammals. In fish, CYP1A was usually used as a biomarker of pollution in freshwater environments. In the liver, CYP1A was induced by contaminants like crude oil in several species of fish such as *Oncorhynchus mykiss*, *Salmo salar*, *Sebastes schlegeli*, and other fishes [43–46]. In this study, the expression levels of *CiCYP1A* in the liver and spleen were rapidly induced on the first day after GCRV infection, but in the gill and intestine, *CiCYP1A* was almost suppressed in the GCRV-infected days (Fig. 2B–E), which indicating *CiCYP1A* was involved in GCRV-induced immunity.

In human immune cells, inhibiting CYP1A increases the expression levels of the stem cell factor receptor (c-Kit) and interleukin (IL)-22, revealing CYP1A activity is relevant for immune modulation [22]. In fish, IFN-I plays crucial roles in immune response against viruses [47], thus, it is necessary to explore the roles of *CYP1A* in *IFN-I* signaling pathway. In the study, the promoter activity of *IFN-I* signaling pathway genes, including *IRF3, IRF7*, *and IFN-I*, was significantly suppressed after poly(I:C) infection (Fig. 4A–C). Interestingly, even in the normal condition, *CYP1A* still showed potent inhibition on the promoter activity of *IFN-I* (Fig. 4C). Further, the mRNA expression levels of *IFN-I* were suppressed by overexpressed CYP1A after poly(I:C) infection. Besides, the mRNA expression levels of *IRF3* and *IFN-I* but not *IRF7* were suppressed by overexpressed CiCYP1A, indicating that CiCYP1A suppressed the expression levels of *IFN-I* by *IRF3* not *IRF7* after GCRV infection (Fig. 5B and F).

There has been reported that CYPs family members are commonly localised in the endoplasmic reticulum or at other locations including the outer nuclear membrane and mitochondrial and plasma membrane [48,49]. In the study, the subcellular localisation of the CiCYP1A was investigated. In CO and CIK cells, compared to the control group, CiCYP1A was distributed in the whole cytoplasm (Fig. 3). Besides, in CIK and 293T cells, the further results showed that CiCYP1A was located in more than ER and MT (Fig. S3), which was different from that of mammal.

In conclusion, *CiCYP1A* was identified from grass carp. The expression profiles showed that *CiCYP1A* was constitutively expressed in all detected tissues of healthy fish and could be modulated by GCRV infection. Subcellular localisation analysis indicated CiCYP1A was located in the cytoplasm. Besides, CiCYP1A potently suppressed the expression of *IFN-I*, which may provide new insights into the gene function of *CiCYP1A* in immunity. But how CiCYP1A regulates *IFN-I* signaling pathway is still unclear and needs further study.

Acknowledgments

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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.2019.10.022.

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