Article

Grass Carp Prx 3 Elevates Host Antioxidant Activity and Induces Autophagy to Inhibit Grass Carp Reovirus (GCRV) Replication

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Abstract: Peroxiredoxins are a family of antioxidant proteins that protect cells from oxidative damage caused by reactive oxygen species (ROS). Herein, the peroxiredoxin 3 gene from grass carp (Ctenopharyngodon idellus), named CiPrx3, was cloned and analyzed. The full-length cDNA of CiPrx3 is 1068 bp long, with a 753 bp open reading frame (ORF) that contains a thioredoxin-2 domain, two peroxiredoxin signature motifs, and two highly conserved cysteine residues. CiPrx3 was ubiquitously expressed in all the tested tissues, while its expression level was altered significantly after exposure to grass carp reovirus (GCRV) and pathogen-associated molecular patterns (PAMPs). CiPrx3 was localized in the mitochondria of transfected cells and concentrated in the nucleus after poly (I:C) treatment. Transformation of CiPrx3 into Escherichia coli enhanced host resistance to H2O2 and heavy metals. Purified recombinant CiPrx3 proteins could protect DNA against oxidative damage. Overexpression of CiPrx3 in fish cells reduced intracellular ROS, increased cell viability, and decreased cell apoptosis caused by H2O2 stimulation and GCRV infection. Further study indicated that CiPrx3 induced autophagy to inhibit GCRV replication in fish cells. Collectively, these results imply that grass carp Prx3 elevates host antioxidant activity and induces autophagy to inhibit GCRV replication.

Keywords: grass carp; peroxiredoxin 3; grass carp reovirus; virus replication; ROS; antioxidant; autophagy

1. Introduction

Peroxiredoxins (Prxs), also termed thioredoxin peroxidase (TPx), are a family of antioxidant enzymes. By scavenging reactive oxygen species (ROS), Prxs protect cells from oxidative damage [1]. Prxs are ubiquitously conserved in a wide variety of living organisms ranging from prokaryotes to eukaryotes [2–5]. The Prxs family can be classified into six subtypes based on their structure and catalytic domains: the typical 2-Cys type (Prx1, Prx2, Prx3, and Prx4), the atypical 2-Cys type (Prx5), and the 1-Cys type (Prx6) [6,7]. The first member of the Prxs family was discovered in the yeast Saccharomyces cerevisiae as an enzyme with a molecular weight of 25 kDa that protects cellular components from reactive sulfur species rather than ROS [8]. Moreover, Prx family members were also reported to be involved in a variety of physiological processes, including cell growth, differentiation, embryonic development, immune response, apoptosis, lipid droplet-related metabolism, and ROS balance [9].

Peroxiredoxin 3 (Prx3) is a member of the typical 2-Cys peroxiredoxin family and is located exclusively in mitochondria. Previous studies have suggested that Prx3 is involved in maintaining the redox balance in mitochondria and acts as a key regulator of apoptosis [10,11]. For example, Prx3 levels establish a redox set point that is essential for proper
mitochondrial structure, function, and cell cycle progression of mesothelioma cells [12].
Overexpression of Prx3 improves glucose tolerance in mice by reducing mitochondrial 
\( \text{H}_2\text{O}_2 \) [13]. Prx3 is an indispensable ROS scavenger that protects tumor cells against 
oxidative damage and subsequent apoptosis [14]. To date, the Prx3 gene has been cloned 
from several fish species, including medaka [15], rock bream [16], common carp [17], and 
big-belly seahorses [5]. However, the functions of Prx3 in teleost fish, especially the function 
in host immune response and defense against pathogens, are still unknown. Understanding 
the particular function of Prx3 in teleost fish will benefit fish disease control and prevention, 
as well as disease-resistant fish breeding programs.

Grass carp (Ctenopharyngodon idellus), a traditional aquaculture species in China with 
a history spanning over 60 years, occupies more than 21% of total freshwater aquaculture 
production. Grass carp is the most highly consumed freshwater fish in China, and its 
production has added up to approximately 5.50 million tons in recent years [18]. However, 
there are often disease outbreaks that induce huge economic losses to grass carp aquaculture. 
In particular, grass carp reovirus (GCRV) induced hemorrhagic disease is one of the most 
serious diseases that requires attention [19]. Nevertheless, no effective drugs or vaccines 
against GCRV have been found until now. Therefore, identification of grass carp genes 
involved in host immune response is important for antiviral drug development or fish 
breeding programs.

In this study, the Prx3 gene was cloned from grass carp. The gene structure, expression 
profile, and localization pattern were analyzed. Moreover, the functions of Prx3 during 
antioxidant and GCRV infection were also investigated. Our results provide important 
information for further understanding the function of Prx3 in teleost fish.

2. Materials and Methods

2.1. Ethics Statement

All animal experiments complied with the ARRIVE guidelines and followed the Guide 
for the Care and Use of Laboratory Animals (State Scientific and Technological Commission 
of China, 2017). The protocol was approved by the Ethics Committee of the Institute of 
Hydrobiology, Chinese Academy of Sciences (CAS) (protocol code: IHB2020-0810, approval 
date: 10 August 2020). All surgery was performed under MS-222 anesthesia, and all efforts 
were made to minimize suffering.

2.2. Cell and Antibodies

Grass carp ovary (GCO) cells were cultured in M199 medium (HyClone, Logan, UT, USA) 
supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL 
streptomycin under humidified conditions with 5% CO\textsubscript{2} at 28 °C. Mouse anti-β-actin 
antibody was purchased from Proteintech (Proteintech, Wuhan, China). Mouse anti-
p62 antibody was purchased from Beyotime (Beyotime, Shanghai, China). Rabbit anti-
LC3B antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and HRP-
conjugated goat anti-mouse IgG were purchased from Sigma (Sigma, Saint Louis, MS, 
USA). Rabbit anti-NS80 and Rabbit anti-VP5 antibodies were prepared in our laboratory. 
Briefly, the complete ORF sequence of NS80 and VP5 was amplified and ligated into the 
pET-32a expression vector. The resulting plasmids (pET-32a-NS80 and pET-32a-VP5) were 
transformed into E. coli BL21 and cultured in 400 mL LB medium until an OD\textsubscript{600} of 0.6 
was reached. The cells were then induced with IPTG for 4 h at 30 °C in order to express 
the fusion protein. The fusion protein was purified using BeyoGold™ His-tag Purification 
Resin, mixed with an equal volume of Freund’s adjuvant (Sigma, Saint Louis, MS, USA), 
and thereafter used to immunize the rabbit. Anti-NS80 or anti-VP5 serum was collected 
after immunizing the rabbit three times.

2.3. GCRV Challenge and PAMP Stimulation

Healthy three-month-old grass carp with an average weight and length of 3–5 g and 
5–8 cm were used in the study. All of the fish were obtained from a full-sib family bred
by the Guanqiao Experimental Station, Institute of Hydrobiology, CAS. Before further processing, the fish were fed commercial feed (Tong Wei, Chengdu, China) twice a day and acclimatized in aerated fresh water at 28 °C for one week. Grass carp were used for further study after no abnormal symptoms were observed.

In accordance with our previous description, we conducted a virus challenge experiment [20]. Briefly, approximately 100 grass carp were intraperitoneally injected with GCRV in a volume of 200 µL (GCRV subtype II, 2.97 × 10^3 RNA copies/µL). At 0–6 days post-infection (dpi), five fish individuals were collected, and spleen and liver samples were removed for analysis. Then, RNA was prepared to analyze the response of CiPrx3 after GCRV infection. Moreover, five uninfected fish were selected, and samples from the skin, gill, spleen, muscle, brain, liver, intestine, heart, head kidney, and middle kidney were obtained. RNA from these tissues was prepared in order to analyze the tissue distribution of CiPrx3.

In addition, 150 grass carp were collected for pathogen-associated molecular patterns (PAMPs) stimulation. Fish were divided into three groups (50 per group) and then intraperitoneally injected with 200 µL PBS, 200 µL lipopolysaccharide (LPS) (0.5 mg/mL, dissolved in PBS), or 200 µL poly (I:C) (1 mg/mL, dissolved in PBS). At 3, 6, 12, 24, and 48 h post-injection (hpi), five fish individuals from each group were collected, and spleen and liver samples were removed for analysis. RNA from these tissues was prepared in order to analyze the response of CiPrx3 after PAMP stimulation.

2.4. cDNA Cloning

Total RNA was isolated from the tissues of healthy grass carp using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was measured using the Qubit RNA assay kit (Life Technologies, California, USA), and integrity was assessed with the RNA nano 6000 assay kit (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNA was synthesized by using the ReverTra Ace kit (Toyobo, Osaka, Japan) and oligo (dT)-adaptor as primers. Specific primers (Table S1) were designed according to the cDNA sequences of zebrafish Prx3 (NM_001013460.3) and the deduced cDNA sequences of grass carp Prx3 to amplify the ORF sequence of CiPrx3. Moreover, the 5' and 3' untranslated regions (UTRs) of CiPrx3 were obtained by rapid amplification of cDNA ends (RACE) using the 5' and 3' full RACE Kit (TaKaRa, Kusatsu, Japan). PCR products were inserted into pMD18-T vectors (Takara, Kusatsu, Japan) and transformed into E. coli DH5α for sequencing by a commercial company (TsingKe, Beijing, China). Finally, the full-length cDNA of CiPrx3 was obtained by assembling the ORF sequence and 5’ and 3’ UTR sequences using DNAMAN software.

2.5. Sequence Analysis

BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (accessed on 15 January 2022) was used to search for CiPrx3 homologues in the National Center for Biotechnology Information (NCBI). The Sequence Manipulation Suite (SMS) (http://www.bio-sof.net/sms) (accessed on 23 January 2022) [21] was used to analyze the nucleotide and predicted amino acid sequences of CiPrx3. Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de) (accessed on 23 January 2022) [22] software was used to predict the functional domains of the amino acid sequence of CiPrx3. ClustalW2.1 (http://www.clustal.org/clustal2) (accessed on 23 January 2022) [23] was used to perform multiple sequence alignments of Prx3 among different species. MEGA 7.0 software (http://www.megasoftware.net/index.html) (accessed on 23 January 2022) [24] was used to construct the Maximum Likelihood (ML) phylogenetic tree on the basis of amino acid sequences, and the bootstrap values of the branches were obtained by testing the tree 10,000 times.

2.6. Gene Expression Analysis

Total RNA was isolated from ten tissues of five healthy grass carp and reverse transcribed to obtain cDNA as described above. RNA concentration and integrity were also determined as described previously. cDNA from the same tissues was mixed and served as
the template for real-time quantitative PCR (RT-qPCR) analysis of the expression level of CiPrx3 in the different tissues. RT-qPCR was performed using a fluorescence quantitative PCR instrument (Bio-Rad, Hercules, CA, USA). Each reaction mixture contained 0.8 µL forward and reverse primers (for each primer), 1 µL cDNA template, 10 µL 2 × SYBR qPCR Master Mix (Vazyme, Nanjing, China), and 7.4 µL ddH2O. Three replicates were included for each sample, and β-actin was used as an internal control to normalize the gene expression. The program was as follows: 95 °C for 10 s; 40 cycles of 95 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s; and melt curve construction. The $2^{-\Delta\Delta Ct}$ method was employed in order to calculate the relative expression levels [25]. The specific primers for RT-qPCR are listed in Table S1.

Moreover, total RNA was isolated from liver and spleen tissues of grass carp collected on different days (0–6 days) post GCRV infection or at different time points (3, 6, 12, 24, and 48 h) after PAMP stimulation, and then reverse transcribed to obtain cDNA as described above. The cDNA from the same tissues was mixed and served as the template for RT-qPCR analysis of the response of CiPrx3 after GCRV infection and PAMP stimulation. The program, reaction mixture, and primers for RT-qPCR were the same as above.

2.7. Fluorescence Observation

The complete open reading frame (ORF) of CiPrx3 was amplified and inserted into plasmids pEGFP-N3. Obtained plasmids (pEGFP-CiPrx3) were confirmed by DNA sequencing. GCO cells grown on coverslips in 6-well plates were transfected with pEGFP-CiPrx3 using Lipofectamine™ 3000, according to the manufacturer’s instructions. The transfected cells were stimulated by LPS or poly (I:C) or not stimulated. After 24 h, cells were fixed with 4% paraformaldehyde and then stained with Hoechst 33342. Finally, the cells were mounted with 50% glycerol and observed under an UltraVIEW VOX confocal system (PerkinElmer, Fremont, CA, USA). Moreover, pDsRed2-Mito and pEGFP-CiPrx3 were cotransfected into GCO cells to determine the precise localization of CiPrx3. The transfected cells were fixed and stained as described above and observed under the same confocal system.

2.8. Protein Expression and Purification

The complete ORF sequence of CiPrx3 was amplified and then ligated into the pEASY-Blunt expression vector (TransGen, Beijing, China). The resulting plasmid (pEASY-CiPrx3) was transformed into the E. coli BL21 (DE3) strain, and the bacterium was induced for 10 h with 1 mM IPTG at 20 °C to express the fusion protein. Then, cells were collected and the fusion protein was purified with NI-NTA resin according to the manufacturer’s protocols, as described previously [26]. The concentration of the purified protein was determined by the BCA Protein Assay Kit (Novagen, Hilden, Germany). Moreover, Western blotting was performed in order to confirm the correctness of the purified fusion protein. An anti-His tag antibody (Proteintech, Wuhan, China) was used as the primary antibody at a 1:1000 dilution, followed by HRP-conjugated goat anti-rabbit IgG (Tiangen, Beijing, China) at a 1:5000 dilution as the secondary antibody. Finally, the immunoblot signals were detected using an HRP-DAB Detection Kit (Tiangen, Beijing, China).

2.9. Mixed-Function Oxidase Assay

The mixed-function oxidase (MFO) assay was carried out as described previously [26] in order to determine the degree of DNA breakage caused by the ROS generated from the thiol/Fe$^{3+}$/O$_2^-$ MFO system and to assess whether CiPrx3 could protect the DNA from breakage. A total reaction volume of 50 µL mixtures (10 µM FeCl$_3$, 10 mM DTT, and different concentrations of purified rCiPrx3 fusion protein) was incubated at 37 °C for 1 h. Then, 1 µg of pcDNA3.1 supercoiled DNA was added to each reaction mixture and incubated further at 37 °C for another 1 h. Finally, the reaction mixtures were analyzed using a 1% agarose gel, stained with ethidium bromide, and then visualized under UV light.
2.10. Antioxidant Activity and Heavy Metal-Resistant Ability Assay

*E. coli* BL21 cells were transformed with pEASY-CiPrx3 or pEASY-Blunt and then cultured in 5 mL LB with ampicillin at 37 °C (220 rpm) until the OD$_{600}$ reached approximately 0.6. Then, the cultures were induced with IPTG for 3 h. The agarose plates were coated with 200 µL *E. coli* BL21 cells containing pEASY-CiPrx3 or pEASY-Blunt. Sterile filter papers (diameter: 6 mm) were soaked with heavy metal ions (Copper, Zinc, Chromium, and Ferrum) at a concentration of 1 mol/L or soaked with H$_2$O$_2$ at different concentrations (30%, 20%, 15%, 10%, 6%, and 3%) and then placed on agarose plates which were cultured overnight. The inhibition zone diameters between the two *E. coli* BL21 strains were measured and compared.

Moreover, we also analyzed the antioxidant activity of CiPrx3 in fish cells. The ORF sequence of CiPrx3 was amplified and fused with the HA tag and then inserted into pcDNA3.1 vector to obtain plasmid pcDNA3.1-CiPrx3. GCO cells were transfected with pcDNA3.1 or pcDNA3.1-CiPrx3 and then treated with H$_2$O$_2$ or infected with GCRV. Cells were harvested at different time points after treatment/infection. Cell viability was examined by CCK-8 assay. Cell apoptosis and intracellular ROS were detected by flow cytometry with an ANNEXIN V-FITC/PI Apoptosis Assay kit (ZomanBio, Beijing, China) and a reactive oxygen species assay kit (Beyotime, Shanghai, China).

2.11. Anti-Viral Effect Analysis

In order to analyze the role of CiPrx3 during GCRV infection, GCO cells were transfected with pcDNA3.1 or pcDNA3.1-CiPrx3 and then infected with GCRV at a multiplicity of infection (MOI) of 1. Cells were harvested at different time points (12, 24, and 36 h) post-infection. The relative mRNA expression levels of genes encoding the GCRV nonstructural protein NS80 or structural proteins VP5 and VP7 were determined by RT-qPCR. Western blotting was also performed to examine the relative protein expression levels of NS80 and VP5. Rabbit anti-NS80 and Rabbit anti-VP5 antibodies were used as the primary antibodies at a 1:1000 dilution, followed by HRP-conjugated goat anti-rabbit IgG at a 1:5000 dilution as the secondary antibody.

2.12. Autophagy Level Detection

Plasmid pEGFP-LC3B, which contained the autophagy report gene LC3B, was constructed as described previously [27]. pEGFP-LC3B was cotransfected with pcDNA3.1 or pcDNA3.1-CiPrx3 into GCO cells and collected at 24 h post-transfection. Cells were subjected to fluorescence observation in order to examine the GFP-LC3B puncta in transfected cells. Furthermore, GCO cells were transfected with pcDNA3.1 or pcDNA3.1-CiPrx3 and then infected with 1 MOI of GCRV. Cells were harvested at different time points post-infection, and protein expression levels of LC3-II/LC3-I and autophagy substrate P62 were determined by Western blotting. Rabbit anti-LC3B antibodies and mouse anti-p62 antibodies were used as the primary antibodies, followed by HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG as the secondary antibody.

2.13. Statistical Analysis

All experimental data were analyzed by one-way variance (ANOVA) through SPSS (version 16.0; IBM Corporation, Armonk, NY, USA). Differences were considered significant and extremely significant at $p \leq 0.05$ and $p \leq 0.01$, respectively. $p \leq 0.05$ and $p \leq 0.01$ were denoted by * and **, respectively.

3. Results

3.1. Cloning and Characterization of Grass Carp Peroxiredoxin 3

The full-length cDNA of the grass carp peroxiredoxin 3 gene (CiPrx3) was obtained by PCR and RACE. The full-length cDNA of CiPrx3 is 1068 long, with a 753 bp ORE, a 13 bp 5' UTR, and a 302 bp 3' UTR (Figure S1A). Structural prediction of the deduced amino acid sequence of CiPrx3 was carried out by SMART software. The results showed that the
CiPrx3 protein sequence contained a thioredoxin-2 domain, two peroxiredoxin signature motifs (FYPLDFTFVCPTEI and GEVCFA), and two highly conserved cysteine residues (Cys103 and Cys224) (Figure S1B).

Multiple alignments of the deduced amino acid sequence of CiPrx3, with its homologs in other fishes and mammals, indicated that Prx3 proteins were highly conserved in both fish and mammals, especially the two peroxiredoxin signature motifs (FYPLDFTFVCPTEI and GEVCFA) (Figure 1A). Moreover, pairwise sequence alignment revealed that CiPrx3 shares 70.4–97.6% protein sequence identity with its homologs from other species, in which CiPrx3 showed the most similar identity to that of Mylopharyngodon piceus (97.6% sequence similarity), followed by Cyprinus carpio and Danio rerio (93.2% and 92.4% sequence similarity, respectively) (Figure 1B).

Figure 1. Multiple sequence alignment of CiPrx3 with its homologues from other species. (A) Multiple sequence analysis of CiPrx3 by ClustalW. The numbers of amino acids are listed on the left side of alignments. The black shade represents 100% identity, while dark gray represents 80% identity. Prx3 signature motifs FYPLDFTFVCPTEI and GEVCFA are marked with red lines. (B) Identity and similarity of CiPrx3 with its counterparts from other species. The percent identity is shown in the upper triangle, and the percent similarity is shown in the lower triangle. GenBank accession numbers for the protein sequences are as follows. Mylopharyngodon piceus Prx3 (ALD62539.1); Trematomus bernacchii Prx3 (APG79659.1); Oplegnathus fasciatus Prx3 (AJC98155.1); Miichthys miiuy Prx3 (AGT56738.1); Cyprinus carpio Prx3 (ALG02339.1); Homo sapiens Prx3 (AAH08435.1); Mus musculus Prx3 (EDL01849.1); Danio rerio Prx3 (AAH92846.1); Oryzias melastigma Prx3 (AE51070.1); Xenopus laevis Prx3 (NP 001086130.1).
In order to determine the molecular evolutionary relationship of CiPrx3, a phylogenetic tree was constructed by using Prx protein sequences from various vertebrate species. As shown in Figure 2, the phylogenetic tree could be divided into three branches, which correspond to the typical 2-Cys Prxs, atypical 2-Cys Prxs, and 1-Cys Prxs. CiPrx3 was located in the branch of typical 2-Cys Prxs and closely related to that of C. carpio and M. piceus (Figure 2).

Figure 2. Maximum Likelihood (ML) phylogenetic tree analyses of CiPrx3 with other Prx proteins from other species. The confidence in each node was assessed by 10,000 bootstraps.
3.2. Expression Patterns of CiPrx3

RT-qPCR was carried out to study the tissue distribution of CiPrx3 in healthy grass carp. Using the middle kidney as a baseline, mRNA expression levels of CiPrx3 in other tissues were expressed as fold changes relative to the middle kidney expression level. As shown in Figure 3A, CiPrx3 was ubiquitously expressed in all ten tested tissues, while the expression level differed among these different tissues. Relatively high expression levels of CiPrx3 were detected in the liver (9.25-fold), muscle (8.45-fold), intestine (6.22-fold), and gill (5.91-fold), whereas relatively low expression levels were detected in the middle kidney (1.00-fold) and head kidney (0.70-fold).

![Graph showing tissue distribution of CiPrx3 expression](image)

Figure 3. Expression patterns of CiPrx3. (A) Tissue distribution of CiPrx3. The determined tissues included SK: skin; S: spleen; B: brain; G: gill; L: liver; HK: head kidney; H: heart; MK: middle kidney; M: muscle; and I: intestine (n = 5). The relative expression is the ratio of gene expression in different tissues relative to that in the middle kidney. (B,C) Expression level of CiPrx3 in the liver (B) and spleen (C) after GCRV infection. The relative expression is the ratio of gene expression after exposure to GCRV (1, 2, 3, 4, 5, and 6 days) to that in the control group (0 day) in the same tissue. (D,E) Expression pattern of CiPrx3 in the liver (D) and spleen (E) after PAMP stimulation. The relative expression was calculated as the ratio of the gene expression level in the LPS- and poly (I:C)-treated groups relative to that in the PBS-treated group at the same time point. The β-actin was used as an internal control. All data are given in terms of relative mRNA expression as the mean ± SD. Asterisks represent significant differences (* = p ≤ 0.05, ** = p ≤ 0.01).
The total RNA from the liver and spleen of grass carp at 0–6 days post GCRV infection was reverse transcribed, then used for RT-qPCR analysis to determine the response of CiPrx3. In the liver, the mRNA expression level of CiPrx3 was increased at 1–5 dpi, reached the peak level at 5 dpi (21.75-fold), then declined to the baseline level at 6 dpi (0.98-fold) (Figure 3B). The mRNA expression pattern of CiPrx3 in the spleen was different from that observed in the liver. Specifically, the CiPrx3 expression level was unchanged at 1 dpi (1.00-fold), and decreased at 2 dpi (0.62-fold) and 3 dpi (0.61-fold), followed by an increase at 4–6 dpi (1.72–1.82-fold) (Figure 3C).

Moreover, the expression of CiPrx3 was analyzed after PAMP stimulation. As shown in Figure 3D,E, CiPrx3 showed different response patterns in the liver and spleen after LPS and poly (I:C) stimulation. In the liver, LPS stimulation induced the downregulation of CiPrx3 at 3 hpi (0.67-fold), and then upregulated CiPrx3 at 6 hpi (2.58-fold), 12 hpi (2.71-fold), and 24 hpi (1.84-fold). The expression level did not change at 48 hpi (1.12-fold). Poly (I:C) stimulation resulted in dramatic upregulation of CiPrx3 expression levels at 12 hpi (19.90-fold), whereas its expression level was decreased or not changed at other time points (Figure 3D). In the spleen, the CiPrx3 expression level increased at 3 hpi (3.92-fold), 6 hpi (2.35-fold), and 48 hpi (1.55-fold) and showed no significant change at 12 hpi (1.00-fold) and 24 hpi (1.09-fold) after LPS stimulation. Interestingly, the CiPrx3 expression level was increased at all examined time points (1.93–4.67-fold) after poly (I:C) stimulation (Figure 3E).

3.3. Subcellular Localization of CiPrx3 Proteins

In order to investigate the subcellular localization of the CiPrx3 protein, GCO cells were transfected with pEGFP-CiPrx3 and subjected to fluorescence observation at 24 h post-transfection. pEGFP-N3 was transfected at the same time as a negative control. As shown in Figure 4A, green fluorescence of the CiPrx3-EGFP fusion protein was found to be distributed only in the cytoplasm of transfected cells, while naked EGFP was distributed uniformly in whole cells. Moreover, pDsRed2-Mito, a mitochondria-specific marker vector, was co-transfected with pEGFP-CiPrx3 to determine the precise localization of CiPrx3. Figure 4B shows that the green fluorescence of CiPrx3-EGFP was colocalized with the red fluorescence of mitochondria, suggesting CiPrx3-EGFP was localized in the mitochondria. In addition, the effects of PAMP stimulation on the subcellular localization of the CiPrx3 protein were investigated. As shown in Figure 4C, subcellular localization patterns of CiPrx3 protein were not changed after LPS stimulation, whereas CiPrx3 protein seemed to concentrate in the nucleus after poly (I:C) stimulation, suggesting the nuclear translocation of CiPrx3 proteins.

3.4. The Antioxidant Activity of CiPrx3

E. coli BL21 strains were transformed with plasmids pEASY-CiPrx3 or pEASY-Blunt, cultured in agarose plates, and then stimulated by different concentrations of H2O2 to test the antioxidant activity of CiPrx3. As shown in Figure 5, the growth of the pEASY-Blunt transformed strain was significantly inhibited when compared with the pEASY-CiPrx3 transformed strain. The inhibition zone diameter of the pEASY-CiPrx3-transformed strain was significantly smaller than that of the control group under each concentration of H2O2 except for the 3% H2O2 (Figure 5A,B). To be specific, the diameter values of the pEASY-CiPrx3 transformed strain were 1.00-, 0.87-, 0.85-, 0.71-, 0.54-, and 0.50-fold to that of control group under each concentration of H2O2 (Figure 5C). Furthermore, GCO cells were transfected with pcDNA3.1-CiPrx3 or pcDNA3.1 and then treated with H2O2 (0.4 mM) and collected at different time points (1 h, 3 h, 6 h) to detect the intracellular ROS by flow cytometry. The overexpression of CiPrx3 in transfected cells was confirmed by Western blotting using an anti-HA antibody (Figure 5D). As shown in Figure 5E,F, the fluorescence intensity of ROS in pcDNA3.1-CiPrx3 transfected cells was significantly lower than that of pcDNA3.1 transfected cells in all of the examined time points.
be distributed only in the cytoplasm of transfected cells, while naked EGFP was distributed uniformly in whole cells. Moreover, pDsRed2-Mito, a mitochondria-specific marker vector, was co-transfected with pEGFP-CiPrx3 to determine the precise localization of CiPrx3. Figure 4B shows that the green fluorescence of CiPrx3-EGFP was colocalized with the red fluorescence of mitochondria, suggesting CiPrx3-EGFP was localized in the mitochondria. In addition, the effects of PAMP stimulation on the subcellular localization of the CiPrx3 protein were investigated. As shown in Figure 4C, subcellular localization patterns of CiPrx3 protein were not changed after LPS stimulation, whereas CiPrx3 protein seemed to concentrate in the nucleus after poly (I:C) stimulation, suggesting the nuclear translocation of CiPrx3 proteins.

Figure 4. Subcellular localization of CiPrx3 in GCO cells. (A) Subcellular localization patterns of CiPrx3 protein in normal cells. (B) CiPrx3-EGFP colocalized with mitochondria. (C) Subcellular localization patterns of CiPrx3 protein under PAMP stimulation. Red arrows indicate the nuclear translocation of the CiPrx3 fusion protein induced by poly (I:C) stimulation. Scale bar = 20 μm.
Figure 5. The antioxidant activity of CiPrx3. (A, B) E. coli BL21 strains were transformed with pEASY-Blunt or pEASY-CiPrx3 and cultured in agarose plates treated with different concentrations of H$_2$O$_2$. Scale bar = 1 cm. (C) Inhibition zone diameter of E. coli after H$_2$O$_2$ stimulation. (D) Confirmation of the overexpression of CiPrx3 in GCO cells by Western blotting. GCO cells were transfected with pcDNA3.1-CiPrx3 or pcDNA3.1 and harvested at 24 h for Western blotting by using an anti-HA antibody. (E) Calculation of relative fluorescence intensity of ROS in pcDNA3.1-CiPrx3 or pcDNA3.1 transfected cells. (F) CiPrx3 reduced the intracellular ROS caused by H$_2$O$_2$ stimulation. GCO cells were transfected with pcDNA3.1-CiPrx3 or pcDNA3.1 and then treated with H$_2$O$_2$ (0.4 mM) and collected at different time points (1 h, 3 h, and 6 h) to detect the intracellular ROS by flow cytometry. Data were shown as the mean ± SD (n = 3). Asterisks represent significant differences (** = p ≤ 0.01).
Moreover, it was proposed that virus infection could cause the accumulation of ROS in cells and thus induce cell damage and apoptosis [28]. Therefore, we investigated the antioxidant effect of CiPrx3 during virus infection. GCO cells were transfected with pcDNA3.1-Prx3 or pcDNA3.1, and then infected with GCRV. Cells were collected at different time points. Flow cytometry indicated that the intracellular ROS in pcDNA3.1-CiPrx3 transfected cells were remarkably lower than those in the control group, especially in the 24 hpi (Figure 6A,B). Interestingly, we observed that the mRNA expression levels of two typical antioxidant genes, nuclear factor erythroid 2-related factor 2 (NRF2) and heme oxygenase-1 (HO-1), were decreased in the pcDNA3.1-Prx3 transfected cells when compared with the control cells (Figure 6C,D). Similar trends were also observed for other members of the Prxs family (Figure S3). Collectively, these results suggest the antioxidant activity of CiPrx3.

![](image)

**Figure 6.** CiPrx3 reduced the ROS caused by GCRV infection. (A) Flow cytometry assayed the intracellular ROS caused by GCRV infection. GCO cells were transfected with pcDNA3.1-CiPrx3 or pcDNA3.1 and then infected with GCRV and collected at different time points (6 h, 12 h, and 24 h) to detect the intracellular ROS by flow cytometry. (B) The calculated relative fluorescence intensity of GCO cells. (C,D) The mRNA expression levels of nuclear factor erythroid 2-related factor 2 (NRF2) and heme oxygenase-1 (HO-1). Data were shown as the mean ± SD (n = 3). Asterisks represent significant differences (* = p ≤ 0.05, ** = p ≤ 0.01).

### 3.5. The Heavy Metal-Resistant and DNA Protection Ability of CiPrx3

The role of CiPrx3 after exposure to different kinds of heavy metals was investigated. As shown in Figure 7A, the inhibition zone diameters of the pEASY-CiPrx3-transformed strains were markedly smaller than those of the control group under heavy metal ion Cu, Cr, Zn, and Fe treatment (Figure 7A). Specifically, the inhibition zone diameters of the pEASY-CiPrx3-transformed group were 0.81-fold, 0.80-fold, 0.78-fold, and 0.85-fold to those of the pEASY-Blunt-transformed group, respectively (Figure 7B). Collectively, these results indicate that CiPrx3 enhanced host resistance to heavy metals.
Figure 7. The heavy metal-resistant and DNA protection ability of CiPrx3. (A) The heavy metal-resistant ability of CiPrx3. E. coli BL21 strains were transformed with the pEASY-CiPrx3 vector or pEASY-Blunt and then treated with different kinds of heavy metal ions. Scale bar = 1 cm. (B) Inhibition zone diameter of E. coli after heavy metal stimulation. Data were shown as the mean ± SD (n = 3). Asterisks represent significant differences (** = p ≤ 0.01). (C) Determination of the DNA protection ability of CiPrx3 by MFO assay; 1: pcDNA3.1 without incubation; 2: pcDNA3.1 + FeCl₃ (10 µM); 3: pcDNA3.1 + DTT (10 mM); 4: pcDNA3.1 + MFO mix (10 µM FeCl₃ + 10 mM DTT); 5: pcDNA3.1 + MFO mix + 5 ng of rCiPrx3; 6: pcDNA3.1 + MFO mix + 50 ng of rCiPrx3; 7: pcDNA3.1 + MFO mix + 500 ng of rCiPrx3; 8: pcDNA3.1 + MFO mix + 1 µg of rCiPrx3; 9: pcDNA3.1 + MFO mix + 2 µg of rCiPrx3; 10: pcDNA3.1 + MFO mix + 4 µg of rCiPrx3. OC DNA: open circular plasmid DNA; CCC: covalently closed circular DNA.

The recombinant CiPrx3 protein was purified and confirmed by Western blotting (Figure S2). Then, an MFO assay was carried out in order to investigate whether the CiPrx3 protein could prevent DNA damage caused by the ROS generated from an MFO system. As shown in Figure 7C, only FeCl₃ could cause damage to the supercoiled DNA (Figure 7C, line 2), and the DNA damage was more serious when both DTT and FeCl₃ existed (Figure 7C, line 4). Nevertheless, DNA damage was inhibited when the rCiPrx3 protein existed, and the inhibitory effects were dose-dependent (Figure 7C, lanes 5–10), suggesting the DNA protection ability of CiPrx3.

3.6. The Anti-Apoptosis Ability of CiPrx3

In order to further investigate the function of CiPrx3 during apoptosis, cells were transfected with pcDNA3.1-CiPrx3 or pcDNA3.1 for overexpression and then treated with different concentrations of H₂O₂. The CCK-8 and flow cytometry assay indicated that the cell viability of the pcDNA3.1-CiPrx3 transfected group was significantly higher than (Figure 8A), while the cell apoptosis rate was significantly lower than those of the pcDNA3.1 transfected group (Figure 8B,C). Moreover, the transfected cells were infected with GCRV and then harvested at 12 and 24 hpi in order to investigate expression patterns of apoptosis-related genes. Results revealed that both apoptosis-related genes, Caspase 3 and p53, showed significantly lower expression levels in pcDNA3.1-CiPrx3 transfected cells when compared with the pcDNA3.1 transfected cells (Figure 8D,E). These results suggest the anti-apoptosis ability of CiPrx3.
Figure 8. The anti-apoptosis ability of CiPrx3. (A) Cell viability of GCO cells after being treated with different concentrations of H2O2. (B) Flow cytometry assay of cell apoptosis of GCO cells after being treated with different concentrations of H2O2. GCO cells were transfected with pcDNA3.1-CiPrx3 or pcDNA3.1 and stimulated by different concentrations of H2O2. Cells were collected to detect cell apoptosis by flow cytometry. (C) Calculated cell apoptosis rate of GCO cells after being treated with different concentrations of H2O2. (D, E) The relative mRNA expression level of Caspase 3 and p53.

GCO cells were transfected with pcDNA3.1-CiPrx3 or pcDNA3.1 and then infected with GCRV. Cells were harvested at different time points post-infection and the expression of apoptosis-related genes caspase 3 and p53 was detected by RT-qPCR. Data were shown as the mean ± SD (n = 3). Asterisks represent significant differences (* = p ≤ 0.05, ** = p ≤ 0.01).

3.7. CiPrx3 Inhibits GCRV Replication

In order to further investigate the role of CiPrx3 during virus infection; GCO cells were transfected with pcDNA3.1-CiPrx3 or pcDNA3.1 and then infected with GCRV, and cells were harvested at different time points. The mRNA and protein expression levels of
viral non-structural protein NS80 and structural protein VP5 and VP7 in two groups were
examined. RT-qPCR showed that mRNA expression levels of all three genes in pcDNA3.1-
CiPrx3 transfected cells were significantly lower than those in pcDNA3.1 transfected cells
(Figure 9A–C). Moreover, WB analysis also revealed similar trends, in which protein
expression levels of both NS80 and VP5 in pcDNA3.1-CiPrx3 transfected cells were markedly
lower than those of the controls (Figure 9D–F). These results indicated that CiPrx3 inhibits
GCRV replication.

![Figure 9](image)

Figure 9. The anti-viral effect of CiPrx3. (A–C) Relative mRNA expression level of VP5 (A), VP7 (B),
and NS80 (C). GCO cells were transfected with pcDNA3.1-CiPrx3 or pcDNA3.1 and then infected with
GCRV. Cells were harvested at different time points post-infection for RT-qPCR analysis. (D) Western
blotting analysis of the anti-viral effect of CiPrx3. GCO cells were transfected with pcDNA3.1-CiPrx3
or pcDNA3.1 and then infected with GCRV. Cells were harvested at different time points post-
infection for WB analysis. (E,F) Calculated protein expression levels of VP5 (E) and NS80 (F) at
different time points post-infection. Data were shown as the mean ± SD (n = 3). Asterisks represent
significant differences (* = p ≤ 0.05, ** = p ≤ 0.01).

3.8. CiPrx3 Promotes Autophagy to Inhibit GCRV Replication

Some studies have reported that peroxiredoxins activate autophagy while it is impor-
tant during host defense against pathogen infection [27,29,30]. Therefore, we investi-
gate whether CiPrx3 could induce autophagy and subsequently defend against GCRV infection.
GCO cells were co-transfected with autophagy report plasmids GFP-LC3B and pcDNA3.1-CiPrx3
or pcDNA3.1 for fluorescence observation. As shown in Figure 10A, the fluorescence
of GFP-LC3 was distributed in the whole cells in pcDNA3.1 transfected cells, whereas
pcDNA3.1-CiPrx3 induced the accumulation of GFP-LC3 puncta in the cytoplasm, suggest-
ing the induction of autophagy. Moreover, WB analysis showed that pcDNA3.1-CiPrx3
significantly enhanced LC3-II expression and increased the ratio of LC3-II/LC3-I, but
decreased the level of autophagy substrate P62 (Figure 10B–D). Altogether, these results
indicated that CiPrx3 promotes autophagy.
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Figure 10. Overexpression of CiPrx3 enhanced autophagy. (A) CiPrx3 induced the accumulation of GFP-LC3B puncta in transfected cells. GCO cells were co-transfected with autophagy report plasmids GFP-LC3B and pcDNA3.1-CiPrx3 or pcDNA3.1 for fluorescence observation. Scale bar = 20 μm. (B) CiPrx3 enhanced autophagy after GCRV infection. GCO cells were transfected with pcDNA3.1 or pcDNA3.1-CiPrx3 and then infected with GCRV; cells were harvested at different time points post-infection and protein expression levels of LC3-II/LC3-I and P62 were detected by Western blotting. (C,D) The calculated protein expression levels of LC3-II/LC3-I (C) and P62 (D) at different time points post GCRV infection. Data were shown as the mean ± SD (n = 3). Asterisks represent significant differences (** = p ≤ 0.01).

In order to confirm whether the inhibition of GCRV replication by CiPrx3 is dependent on autophagy, cells were treated with 3-MA, a classic autophagy inhibitor, followed by plasmids transfection and GCRV infection. Cells were harvested at different time points after infection. As shown in Figure 11A,B, as expected, overexpression of CiPrx3 promoted autophagy, whereas the enhanced autophagy level induced by CiPrx3 was blocked by 3-MA, and appeared as a decreased LC3-II/LC3-I ratio and an increased level of P62 (Figure 11A,B). Moreover, after being treated with 3-MA, both the mRNA and protein expression levels of NS80 and VP5 were increased when compared with untreated cells (Figure 11C–F). Collectively, these results implied that CiPrx3 promotes autophagy to inhibit GCRV replication.
Figure 11. CiPrx3 enhanced autophagy to defend against GCRV. (A) The enhanced autophagy level induced by CiPrx3 was blocked by 3-MA. Cells were transfected with pcDNA3.1 or pcDNA3.1-CiPrx3 and treated with 3-MA, then were infected with GCRV and harvested at 24 h post-infection. Protein expression levels of LC3-II/LC3-I and P62 were detected by Western blotting. (B) The calculated protein expression levels of LC3-II/LC3-I and P62 at 24 h post-infection. Data were shown as the mean ± SD (n = 3). Asterisks represent significant differences (* = p ≤ 0.05, ** = p ≤ 0.01).

4. Discussion

ROS, such as hydrogen peroxide (H_2O_2), hydroxyl radicals (HO*), and superoxida
tion (O_2*−), are mainly produced by mitochondria as a result of an aerobic respiration process [31]. Normal cellular functions require low levels of ROS, which are involved in intracellular signal transduction [32], regulation of gene expression [33], host defense against pathogenic infections [34], and so on. Nevertheless, the mass accumulation of ROS might lead to nonspecific damage to proteins, lipids, and nucleic acids, and is associated with various diseases, such as cancers, cardiovascular diseases, and neurological diseases [35]. The balance between the production and elimination of ROS is therefore one of the most important topics in cell biology and physiology [36]. Peroxiredoxins have been identified in various organisms ranging from prokaryotes to eukaryotes, playing an important role in protecting organisms against oxidative stress [37]. In fish, reports on peroxiredoxins mainly focused on Prx1 and Prx2 [26,38], while information about Prx3 is limited. Herein, the Prx3

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gene from grass carp was cloned, and its role in antioxidant activity and GCRV replication was analyzed.

Sequence analysis and multiple alignments revealed that Prx3 proteins were highly conserved in both fish and mammals, especially the two peroxiredoxin signature motifs (FYPLDFTFCPEI and GEVCFA) and the two highly conserved cysteines (Cys103 and Cys224), suggesting the important role of Prx3 during evolution. The two conserved Cys residues are required for their catalytic function, indicating that Prx3 could act as a reductant to reduce ROS production during cell metabolism and oxidative stress [7]. CiPrx3 showed the most similar identity to Prx3 from M. piceus, C. carpio, and D. rerio. Moreover, the phylogenetic tree implied that CiPrx3 was clustered into the same clade as the Prx3 class in teleost fish. All of the results suggest that CiPrx3 belongs to the typical 2-Cys Prx subfamily.

In all examined tissues, CiPrx3 was constitutively expressed with different expression levels. Relatively higher expression levels were found in the liver and muscle, which are important organs for metabolic processes and energy production [39,40]. Mitochondria are considered energy factories of cells and should therefore be abundant in the liver and muscle for energy production [41]. A large number of mitochondria in the liver and muscle may cause the accumulation of ROS [42–44]. Therefore, the higher expression level of CiPrx3 in the liver and muscle may be beneficial for reducing the accumulation of ROS. Highly expressed levels of Prx3 in liver or muscle tissues were also observed in other fish, such as medaka [15], gilthead sea bream [45], rock bream [16], and large-belly seahorses [5], implying the conserved role of Prx3.

The antioxidative activity of CiPrx3 was determined by transforming the prokaryotic vector pEASY-CiPrx3 into E. coli BL21 strains or transfecting the eukaryotic vector pcDNA3.1-CiPrx3 into GCO cells. Both were then treated with H\(_2\)O\(_2\). The stains on cells with pcDNA3.1-CiPrx3 presented significant growth activity, cell viability, and antiapoptotic activity compared with controls, suggesting the antioxidative activity of CiPrx3 [14,46,47]. Moreover, MFO assays showed that the rCiPrx3 protein could inhibit DNA damage in a dose-dependent manner, further indicating the antioxidative activity of CiPrx3. Similar results of Prxs-mediated inhibition of DNA damage caused by the MFO system were also reported in other fish species [5,48]. Interestingly, we also found that the E. coli BL21 strains with CiPrx3 also showed resistance to heavy metal toxicity, which illustrated the important role of CiPrx3 not only in the host response to oxidative stress, but also in the response to heavy metal toxicity.

It was reported that GCRV infection could cause the accumulation of ROS in cells [49]. Overexpression of CiPrx3 in fish cells remarkably reduced intracellular ROS caused by GCRV infection, further suggesting the antioxidative activity of CiPrx3. Surprisingly, after overexpression of CiPrx3 in GCO cells, we observed reduced mRNA expression levels of other antioxidant genes, such as NRF2, HO-1, and other members of Prxs family. The clarity of abundant ROS in organisms requires the cooperation of many enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and peroxiredoxins (Prxs) [35]. NRF2 is a transcription factor that activates the oxidative stress defense system by inducing antioxidant and detoxifying enzymes to protect cells from oxidative damage [50]. HO-1 and peroxiredoxins are the target genes that are regulated by NRF2 [51,52]. Thus, it could be speculated that overexpression of CiPrx3 reduced intracellular ROS and resulted in decreased levels of NRF2, as well as its target genes, such as HO-1 and peroxiredoxins.

Regarding the peroxiredoxin family, Prx1 and Prx2 were reported to be involved in the fish immune response [1,53], while it is still unclear whether Prx3 participates in the immune response. The expression of CiPrx3 was significantly altered after exposure to GCRV and PAMPs, indicating that CiPrx3 may be involved in the immune response. In order to reveal the specific role of CiPrx3 during virus replication, GCO cells were transfected with pcDNA3.1 and pcDNA3.1-CiPrx3 for overexpression, and then infected with GCRV. Both RT-qPCR and Western blotting results indicated that CiPrx3 inhibits GCRV
replication. Interestingly, we also observed the induction of autophagy by CiPrx3 along with the inhibition of GCRV replication, prompting us to speculate whether the inhibition dependent on autophagy. Therefore, cells were treated with autophagy inhibitor 3-MA, followed by transfection and GCRV infection. As expected, the enhanced autophagy level induced by CiPrx3 was blocked by 3-MA. Meanwhile, we also observed decreased mRNA and protein expression levels of viral genes after being treated with 3-MA. Therefore, these results indicated that the inhibition of GCRV replication by CiPrx3 is dependent on autophagy.

5. Conclusions

In conclusion, peroxiredoxin 3, a member of the typical 2-Cys Prxs subfamily, was cloned from grass carp. CiPrx3 was localized in the mitochondria of transfected cells and concentrated in the nucleus after poly (I:C) treatment. CiPrx3 enhanced the resistance of Escherichia coli to H$_2$O$_2$ and heavy metals, and reduced intracellular ROS in fish cells after H$_2$O$_2$ treatment and GCRV infection. Purified recombinant CiPrx3 protein could protect DNA against oxidative damage. Furthermore, CiPrx3 could induce autophagy and subsequently inhibit GCRV replication in fish cells. Our study provides more information for gaining further understanding of the immune function of Prx3 in teleost fish.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox11101952/s1, Figure S1: Sequence analysis of CiPrx3; Figure S2: Prokaryotic expression and Western blotting analysis of CiPrx3; Figure S3: The effect of CiPrx3 overexpression on mRNA expression of other members from Prxs family; Table S1: Primer sequences used in the study.

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