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UP-REGULATING EXPRESSIONS OF TOLL-LIKE RECEPTOR 3 AND MX GENES IN GILLS BY GRASS CARP REOVIRUS IN RARE MINNOW, GOBIOCYPRIS RARUS

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Abstract: Exposure to the external aqueous environment enhances the susceptibility to infectious diseases in fish gills. To understand the mechanism of local innate immunity against infectious grass carp reovirus (GCRV) in the gills, we identified the partial cDNA sequence of *Mx*, an antiviral effector molecule, in rare minnow *Gobiocypris rarus*. Real-time quantitative RT-PCR (qRT-PCR) was employed to quantitate mRNA levels of a pattern recognition receptor, *Toll-like receptor* 3 (*TLR*3), and type I interferons indicator molecule *Mx*. The artificial infection experiments revealed that *TLR*3 and *Mx* mRNA expressions were simultaneously significant up-regulation at 12h postinjection ($p < 0.05$), the transcription level of *TLR*3 recovered at 48h postinjection ($p > 0.05$), and the *Mx* mRNA expression kept at high transcription level till moribund ($p < 0.05$). These results highlighted the importance of the gills as a tissue capable of mounting a local immune response, and the interferon pathway was activated to GCRV infection.

Key words: GCRV; Gills; *Gobiocypris rarus*; *Mx*; *TLR*3

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Grass carp hemorrhagic disease causes catastrophic losses in grass carp culture in China, and the double-stranded RNA (dsRNA) virus grass carp reovirus (GCRV) has been identified as the etiological agent. GCRV mainly infects fingerlings and yearlings of the grass carp *Ctenopharyngodon idellus* and of the black carp *Mylopharyngodon piceus*[1]. The small-sized rare minnow *Gobiocypris rarus* is also susceptible to experimental infection[2]. We investigated the immune defense mechanism of the host to GCRV using the rare minnow as a model.

Mammalian Toll-like receptors (TLRs) recognize components of invading microbes and trigger the first line of innate immune response that is mediated by transcriptional induction of a large number of genes. *Toll-like receptor* 3 (*TLR*3) is thought to be a signature molecule of cellular response to viral infection[3], because it responds to dsRNA, a common byproduct of viral replication[4], which may originate from single stranded RNA (ssRNA) or dsRNA viruses[5,6]. Viral dsRNA induces dendritic cell maturation through *TLR*3[5]. The recognition of *TLR*3 induces the production of pro-inflammatory cytokines, type I interferons (interferon α and β) which are key molecules for antiviral response[7—9], and that encode intracellular viral stress-inducible proteins[4].

One of the most important mechanisms of antiviral defence is the production of interferons (IFN). Type I IFN, produces an antiviral state in the surrounding cells, mediated by the production of IFN-induced pro-

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teins that act as intracellular mediators, which include the 2′5′-oligoadenylate synthetase, the *Mx* proteins, etc. In higher vertebrates, *Mx* proteins have been shown to inhibit the replication of several viruses with very different behaviours[10].

Piscine *Mx* is an IFN-induced protein[11] with antiviral activity against a number of RNA viruses[12], and has been induced through different mechanisms both *in vivo* and *in vitro*[10].

Gills are vulnerable to infection because of exposure to an aquatic environment containing pathogens during respiration. The innate immune response in gills may play an important role in host defense against various pathogens, and must be activated rapidly after pathogen recognition. But the expressions of *TLR*3 as well as *Mx* have not been clarified in GCRV infected gills.

In the current work, we focused on the natures of the signaling pathway activated by GCRV in gills. *Mx* partial sequence has been cloned in rare minnow. The real-time quantitative RT-PCR (qRT-PCR) has developed to estimate mRNA expression levels of *TLR*3 and *Mx* after infection with GCRV in gills. The study served the studies of antiviral immune responses in gills.

1 Materials and Methods

1.1 Virus strain, test animals, experimental infection procedure and sample collection The GCRV-991 strain used in this experiment was kindly donated by Professor Tiaoyi Xiao in Hunan Agricultural University. The virus titer was determined by the end-point dilution method[13]. Briefly, 500 μL per well of the GCRV stock in triplicate repeated ten-fold serial dilutions (10^{-3}—10^{-8}) were used to infect monolayer CIK cells (*Ctenopharyngodon idellus* kidney cells) in a 24-well plate. Following a 60min absorption period at 25℃, the unabsorbed virus was removed and the 0.6mL of 0.7% low melt point agarose (mixed 1.4% sterilized agarose and equal volume Medium 199) was added. After solidification of agarose (—0.5h), 1 mL of Medium 199 containing 20mM Hepes was supplemented and incubated under 25℃. Five days later, the virus plaques were counted for determination of the virus titer and expressed by PFU/mL. The virus titer was about 2×10^{8} PFU/mL *in vitro* plaque assay.

Young adult rare minnows with a fresh weight of 2—3g were obtained from a laboratory-breeding stock and acclimatized to new laboratory conditions for one week in a quarantine area. They were maintained in 25 L aerated aquariums (two animals per liter) at 28℃ and fed once a day with commercial diet (feed composition: protein 32%, starch 63%, fat 3%, additive 2%).

In GCRV challenge experiments, each control or challenged sample was intraperitoneally injected with 10μL of PBS per gram body weight or GCRV suspended in PBS (2×10^{8} PFU/mL), respectively. Three individuals were randomly killed and fresh gill tissue was sampled at 0, 6, 12, 24, 36, 48, 72, 96, 120, and 144h (moribund fish) postinjection, respectively. The samples were immediately homogenized in TRIZOL® LS reagent (Invitrogen) and total RNA were isolated according to the manufacturer's instruction. The extracted total RNA were incubated with RNase-free DNase I (Roche) to remove contaminated genomic DNA, followed by phenol/chloroform extraction and ethanol precipitation. Reverse transcription was performed with SuperScript™ III Reverse Transcriptase (Invitrogen) and random hexamer primers.

1.2 Cloning of partial sequence of the rare minnow Mx For the initial experiments, GCRV were introduced by intraperitoneal injection. After 24h postinjection, the animals were killed and gills were gathered. cDNA was made as above.

To check IFN pathway activated by GCRV in gills, we cloned type I IFN specific indicator molecule *Mx* partial sequence. The degenerate primers for cloning *Mx* cDNA from rare minnow were designed, based on the published sequences of the *Mx* gene from grass carp *Ctenopharyngon idellus* (Accession No., **AY395698**), common carp *Carassius auratus* (Accession no., **AY303812**), and *Danio rerio* (Accession no., **NM182942**). PCR was carried out in an ABI 9700 Thermal Cycler Instrument in a 25μL reaction volume containing 1μL of cDNA reversely transcribed from gills infected GCRV, 2.5μL of 10×buffer, 2.5μL of Mg^{2+} (25mmol/L), 1μL of dNTPs (10mmol/L), 1μL of Taq polymerase (1U/μL), 1μL of each primer

(10mmol/L) SMFc and SMRb (Tab. 1), and 15μL of nuclease-free water. The cycling profile was a denaturing step of 94℃ for 5min followed by 35 cycles of 94℃ 30s, 60℃ 30s and 72℃ 1min, and 5min at 72℃ for the final extension. The PCR product was separated on an agarose gel, purified using the Axygen gel purification kit (Axygen) and ligated into pMD-18 T vector (TaKaRa). The ligation reaction was then transformed into competent Top10 cells (Invitrogen). After screening by PCR, three positive colonies were sequenced with an ABI 3730 DNA sequencer (Applied Biosystems). A 989 bp sequence was obtained. By BLASTX searching in the GenBank database, the sequence was significantly homologous to known fish *Mx* sequences.

1.3 Expression analyses of TLR3 **and** Mx **in gills** By qRT-PCR, we determined the expressions of the immune recognition receptor *TLR*3, an original gene of IFN pathway, and effector molecule *Mx*, an indicator gene of IFN. The house-keeping gene β-*actin* was used as the internal standard.

*TLR*3 and β-*actin* genes of rare minnow were previously cloned by us, and the GenBank accession numbers were **DQ885908** and **EF370390** respectively (not published).

The forward primer for *TLR*3 was SGTF41 and the reverse was SGTR16a (Tab. 1). The forward *Mx* primer and the reverse *Mx* primer were SMF52a and SMR53a (Tab. 1), respectively. The β-*actin* primer sequences were forward SGAF86 and reverse SGAR87 (Tab. 1). The qRT-PCR was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each reaction consisted of 1μL cDNA, 8μL nuclease-free water, 10μL SYBR Green PCR master mix (Toyobo), and 0.5μL each primer set (5μmol/L). The PCR cycling conditions were 95℃ for 2min, 40 cycles consisting of 95℃ for 25s, 60℃ for 30s, and 72℃ for 60s, followed by dissociation curve analysis to verify the amplification of a single product. Reactions were run in triplicate. The threshold cycle (CT) value for each sample was determined using the automatic setting on the ABI Sequence Detection System. The CT values were exported into a Microsoft Excel Sheet for subsequent data analyses.

Tab. 1 Oligonucleotide primers used in these experiments

Primer name	Sequence (5′→3′)	Amplicon length (nt) and primer information
*TLR*3		
SGTF41 (forward)	CACTTCACCAGGTAATGGAGGAC	117
SGTR16a (reverse)	AAGGTTTCAACATGCCACGAC	qRT-PCR
Mx		
SMFc (forward)	TGACACGCTGTCCTCTKGTA	989
SMRb (reverse)	GKTTTCCTCCGTCTTWAWGG	Gene cloning
Mx		
SMF52a (forward)	GACACGCTGTCCTCTGGTAT	103
SMR53a (reverse)	CAGTTTCTTTGTTTGGCTCTG	qRT-PCR
β-*actin*		
SGAF86 (forward)	GATGATGAAATTGCCGCACTG	135
SGAR87 (reverse)	ACCAACCATGACACCCTGATGT	qRT-PCR

Note: K = G/T; W = A/T

The differences in the CT values of target gene to the corresponding internal control β-*actin* gene, ΔCT (CT_{gene} - CT_{actin}), were calculated. The relative expression level of target gene to β-*actin* was described using the equation $2^{-\Delta CT}$, and the value represented an 1/n-fold difference relative to β-*actin* gene. The $2^{-\Delta CT}$ was multiplied by 1000 in order to simplify the presentation of the data. The data obtained from qRT-PCR analyses were subjected to one-way analysis of variance (one-way ANOVA) followed by an unpaired, two-tailed t-test. *P* values less than 0.05 were considered statistically significant.

1.4 Nucleotide sequence accession number The sequence of *Mx* was deposited in GenBank under accession number **EF095273**.

2 Results

2.1 Identification of Mx gene

The PCR product amplified by the degenerate primers was 989bp (Fig. 1). A BLASTX search of the GenBank protein database demonstrated that the rare minnow gene shared high sequence similarity with known fish *Mx* proteins, especially to *Ctenopharyngodon idella Mx* (accession no., **AAQ95584**). The deduced amino acid sequence contained a DYNc domain that mediates vesicle trafficking. DYNc participates in the endocytic uptake of receptors, associated ligands, and plasma membrane following an exocytic event[14].

```
1    T  R  C  P  L  V  L  K  L  K  K  I  S  K  D  K  N  W  H  Q
1    TGACACGCTGTCCTCTGGTATTGAAACTGAAGAAAATTTCAAAGGACAAAAATTGGCATC
21   W  H  G  L  L  S  Y  Q  S  Q  T  K  K  L  K  D  P  S  E  I
61   AGTGGCATGGATTGCTGTCATATCAGAGCCAAACAAAGAAACTGAAAGACCCATCAGAAA
41   E  D  A  V  L  N  A  Q  T  V  L  A  G  K  G  E  G  I  S  H
121  TAGAAGATGCTGTCTTAAATGCTCAGACAGTATTGGCTGGAAAGGGAGAAGGGATCAGTC
61   E  M  I  T  L  E  I  Q  S  S  D  V  P  D  L  T  L  I  D  L
181  ATGAAATGATCACTCTGGAGATCCAGTCCAGTGATGTCCCTGACCTCACTCTCATTGATC
81   P  G  I  A  R  V  A  T  G  N  Q  P  K  D  I  E  K  Q  I  K
241  TGCCAGGCATTGCTAGAGTTGCCACTGGCAACCAGCCAAAAGACATCGAGAAACAAATAA
101  D  L  I  E  K  F  I  K  R  Q  E  T  I  S  L  V  V  V  P  A
301  AAGATCTAATTGAAAAGTTCATTAAAAGACAAGAAACCATCAGCTTGGTTGTGGTGCCTG
121  N  I  D  I  A  T  T  E  A  L  Q  M  A  S  K  V  D  S  T  G
361  CAAACATTGACATCGCCACCACTGAGGCACTGCAGATGGCATCCAAAGTAGATTCAACCG
141  Q  R  T  L  G  I  L  T  K  P  D  L  V  D  K  G  M  E  D  I
421  GACAAAGGACTCTGGGTATTCTGACTAAACCAGATTTAGTGGACAAAGGCATGGAGGATA
161  V  V  R  T  V  N  N  Q  V  I  Q  L  K  K  G  Y  M  I  V  K
481  TAGTGGTCAGAACAGTCAATAATCAAGTGATACAACTGAAGAAGGGCTACATGATCGTGA
181  C  R  G  Q  Q  D  I  N  E  K  L  D  L  V  K  A  L  E  K  E
541  AGTGCAGAGGCCAGCAAGACATCAATGAGAAGCTTGATCTGGTCAAAGCGTTGGAAAAAG
201  R  H  F  F  D  E  H  S  H  F  R  S  L  L  E  E  G  K  A  T
601  AAAGACATTTTTTTGACGAACATTCTCATTTCAGGTCTCTTCTTGAAGAAGGAAAAGCTA
221  I  P  L  L  A  E  R  L  T  K  E  L  V  E  H  I  T  K  T  L
661  CAATACCCCTTCTTGCAGAAAGACTCACAAAAGAATTGGTCGAACACATTACTAAAACAC
241  P  Q  L  Q  K  Q  L  E  M  K  L  E  K  T  T  E  D  L  R  A
721  TACCACAGTTGCAGAAACAACTTGAGATGAAATTAGAGAAGACGACTGAGGATCTTAGAG
261  L  G  D  G  V  P  T  D  E  Q  E  K  I  N  F  F  I  T  K  I
781  CACTGGGAGATGGAGTTCCTACTGATGAACAAGAGAAGATCAATTTTTTTATCACGAAAA
281  R  Q  F  N  D  A  I  E  G  V  K  R  A  E  E  D  L  K  N  S
841  TTCGCCAGTTCAATGATGCCATTGAAGGAGTAAAGAGGGCAGAAGAAGATCTAAAAAACT
301  D  K  R  V  F  T  K  I  R  E  E  F  G  N  W  K  L  V  L  D
901  CAGACAAAAGGGTCTTTACCAAAATCAGGGAGGAATTTGGAAACTGGAAACTCGTCCTGG
321  A  K  S  I  K  T  E  E  T
961  ATGCCAAATCCATTAAGACGGAGGAAACC
```

Fig. 1　Partial nucleotide and predicted amino acid sequences of *Mx* in rare minnow

The forward primer for qRT-PCR is under line, and the reverse primer is under wavy line. The predicted DYNc domain is shaded in protein sequence

2.2 Quantifications of TLR3 mRNA expressions after virus challenge

The infected fish appeared obvious symptoms from 72h postinjection and died off from 144h postinjection.

qRT-PCR was developed to determine the *TLR*3 expression profile after viral challenge, with β-*actin* as internal control. A dissociation curve showing a single peak at the melting temperature expected for that amplicon suggested specific amplification. The temporal expressions of the *TLR*3 gene after viral challenge were shown in Fig. 2. The mRNA expressions in control and blank groups had no significant difference in gills ($p > 0.05$) (data not shown). During the first 24h after viral challenge, the expression of *TLR*3 mRNA was up-regulated gradually. At 12h postinjection, the expression was significant difference from control ($p < 0.05$). The summit was at 24h postinjection. At 48h postinjection, the transcript recovered to normal level ($p > 0.05$).

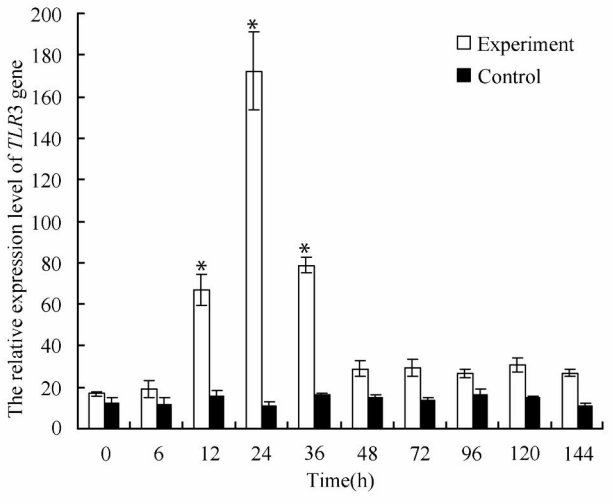

Fig. 2 The temporal expression of *TLR*3 in gills

The controls were injected with PBS; the experiments suffered from GCRV infection. Each column represented the level of *TLR*3 mRNA relative to β-*actin*, expressed as the mean ± SE of triplicate qRT-PCR assays ($2^{-\Delta CT} \times 1000$). Error bars indicated standard error. Asterisk indicated the significant difference between experiment group and control group ($p < 0.05$)

2.3 Mx transcriptional regulation after inoculation with GCRV

To examine expression profile, qRT-PCR was employed to test the time-dependent expression pattern in gill tissue of rare minnows challenged by GCRV at the time point 0, 6, 12, 24, 36, 48, 72, 96, 120 and 144h postinjection. An unpaired, two-tailed t-test showed no significant difference in *Mx* gene transcription among blank, control and challenged group within 6h postinjection. The dissociation curve displayed unique peak.

After infected by GCRV, the *Mx* mRNA transcripts were sharply elevated at 12h postinjection ($p < 0.05$). The peak of up regulation was observed at 24h postinjection (11.13-fold increased relative to control). As time progressed, the *Mx* mRNA expressions kept high levels till moribund (Fig. 3). Statistically significant differences were observed from 12 to 144h postinjection ($p < 0.05$).

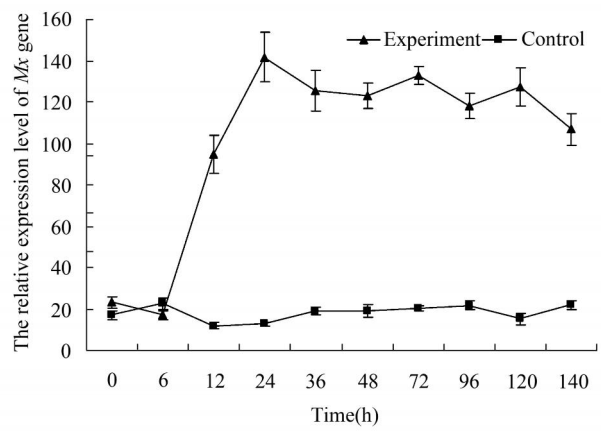

Fig. 3 The relative expression of *Mx* transcripts in gills by qRT-PCR

The control groups were injected with PBS; the experiment groups were challenged by GCRV. Error bars indicated standard error

3 Discussion

Gills are not the immune organ, but it frequently contacts various pathogens in the process of respiration. How does it defend pathogens?

Following GCRV infection in rare minnow, Wang and his colleagues discovered aggregated GCRV particles in the endothelial cell cytoplasm in gills blood vessel under electron microscope, which indicated the gills are one of the main organs infected by GCRV and this organ may take an important part in the spread and infection of GCRV[15].

DsRNA is a virus-specific signature and a ligand for *TLR*3[16]. The occurrence of dsRNA-induced signaling and functional events closely correlates with the *TLR*3 mRNA profile[17].

To further investigate the immune mechanism of GCRV infection in gills, we artificially infected rare minnow with GCRV, and the mRNA expression profiles of immune recognition receptor *TLR*3 and antiviral effector molecule *Mx* were analyzed in this study.

*TLR*3, an antiviral innate immune receptor, recognizes dsRNA, preferably viral origin and induces type I IFN production, which causes maturation of phagocytes and subsequent release of chemical mediators from phagocytes against some viral infections[18,19]. Viral infection or stimulation of *TLR*3 triggers signaling cascades, leading to activation of the transcription factors *IRF*-3 and *NF*-κ*B*, which collaborate to induce transcription of type I IFN genes[20].

Gills constitutively expressed *TLR*3 at low level and *TLR*3 was up-regulated upon exposure to dsRNA. In accordance with the expression of *TLR*3, GCRV stimulation induced the activation of *Mx* transcription, an antiviral effector molecule and indicator of type I IFN induction[21—24].

These findings implicated involvements of *TLR*3, and type I IFN signaling in the pathogenesis of GCRV, especially in early stage, which were associated with up-regulation of *TLR*3 and *Mx* genes. The innate immune responses were activated in the host defense against GCRV invasion, and they were responsible for the rapid clearance of viruses from the local infection.

The time-course experiments with the GCRV-991 strain revealed that *TLR*3 was augmented simultaneously compared to *Mx*. The observations supposed the configuration of *TLR*3 transformed after stimulation by GCRV and activated the signal pathway, meanwhile, its expression was enhanced to induce more strong immune reaction.

These data demonstrated that GCRV injection potently stimulated gills to up-regulate a number of different immune molecules, including immune recognition receptor and antiviral effector molecule. Gills are a potential port of entry for infectious agents. Inducible expressions of *TLR*3 and *Mx* in gills might represent a critical protection mechanism against virus invasion.

Taken together, our findings provided evidence that GCRV initiated a battery of rapid innate immune responses that were important for antiviral defense in gills. These results highlighted the importance of the gills, not only as a target of pathogens but also as a tissue capable of mounting immune responses.

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草鱼呼肠孤病毒上调稀有鮈鲫鳃中 TLR3和 Mx基因的表达

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摘要: 鳃暴露在水环境中,增加了对疾病的易感性。为了研究稀有鮈鲫人工感染草鱼呼肠孤病毒过程中鳃部先天性免疫反应机制,我们克隆了抗病毒效应分子 *Mx*基因的部分序列,用适时荧光定量 PCR检测双链 RNA的模式识别受体 (*Toll-like receptor* 3, *TLR*3)及 I型干扰素指示基因 *Mx*的表达。*TLR*3和 *Mx*基因的表达在注射病毒后 12h显著升高 ($p < 0.05$), *TLR*3的表达水平在注射后 48h恢复到正常水平 ($p > 0.05$),而 *Mx*的高水平表达一直持续到实验结束 ($p < 0.05$)。结果表明在 GCRV感染中,鳃能发生局部免疫反应,其干扰素途径被激活。

关键词: 草鱼呼肠孤病毒;鳃;稀有鮈鲫; *Mx*; *TLR*3