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Genomic structure of grass carp *Mx2* and the association of its polymorphisms with susceptibility/resistance to grass carp reovirus

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ABSTRACT

Mx (myxovirus-resistant) proteins are induced by interferon and inhibit viral replication in many vertebrates. In the present study, the organization of grass carp (*Ctenopharyngodon idella*) *Mx2* (*CiMx2*) gene sequence was elucidated and its single nucleotide polymorphisms (SNPs) were investigated to explore their association with susceptibility/resistance to grass carp reovirus (GCRV). The *CiMx2* genomic sequence is composed of 8108 bp, containing 12 exons and 11 introns. Exon size ranges from 29 to 648 bp, and intron size varies from 89 to 1925 bp. Five SNPs were discovered in the complete sequence of *CiMx2* genomic sequence and four of them were found to be located in introns. The 7 C/T locus is a non-synonymous mutation. The genotype and allele distribution were examined by PCR-RFLP in susceptible and resistant fish. The results indicate that genotypes at the 1191 C/A and 1205 G/A loci are significantly associated with the resistance of grass carp to GCRV ($P < 0.05$). To further verify the correlation, an additional infection experiment was carried out. The mortality in CC genotype individuals (0%) at 1191 C/A locus was significantly lower than that in AA (61.11%) and AC (71.17%) genotypes ($P < 0.05$). At 1205 G/A site, no AA genotype individual was found; the mortality in AG genotype group was 53.06%, which was significantly lower than that in GG genotype group (90.48%) ($P < 0.05$). The results demonstrate that genotype 1191 AC, 1191 AA and 1205 GG individuals are susceptible to GCRV, while 1191 CC and 1205 AG are resistant. The 7 C/T and 528 C/T loci are in high linkage disequilibrium, however, no significant association was found between the haplotype and resistance to GCRV ($P > 0.05$). These results provide potential markers for further investigation of selective breeding of resistant grass carp to GCRV.

1. Introduction

The type I interferon (IFN) system plays a critical role in limiting the spread of viral infection. In the type I IFN response, IFN-induced antiviral proteins include double-stranded RNA-activated protein kinase (PKR), 2′,5′-oligoadenylate synthetase (OAS), inducible nitric oxide synthase (iNOS), RNA-specific adenosine deaminase (ADAR) and myxovirus-resistant protein (Mx) amongst others (Samuel, 2001). The Mx protein, as a sensitive and more stable marker of the IFN response, has received special attention (Wu and Chi, 2007). Mx proteins have been found either in nuclear or cytoplasmic location (Lee and Vidal, 2002). The Mx protein contains a conserved GTP-binding domain and a dynamin family signature in the N-terminus, an effector region, enclosing the central interactive domain (CID) and the leucine zipper (LZ) motif, in the C-terminus (Haller et al., 2007). A unique property of some Mx GTPases is their antiviral activity against a wide range of viruses by blocking an early stage in their life cycle, soon after their entry into the host cells and before genome amplification (Kochs et al., 2005; Haller et al., 2009).

Mx proteins have been detected in a large number of animal species (Lindenmann, 1962; Staeheli et al., 1989; Fernandez-Trujillo et al., 2011). The first Mx was found in mice, and its resistance to orthomyxovirus influenza A was demonstrated (Lindenmann, 1962). Later on, *Mx* genes were discovered in many other species (Staeheli et al., 1989; Fernandez-Trujillo et al., 2011). The first *Mx* gene in fish was reported in perch (*Perca fluviatilis*) (Staeheli et al., 1989). Subsequently, piscine *Mx* genes have been identified in rainbow trout (*Oncorhynchus mykiss*) (Trobridge and Leong, 1995), Atlantic salmon (*Salmo salar*) (Robertsen et al., 1997), Japanese flounder (*Paralichthys olivaceus*) (Lee et al., 2000), fugu (*Takifugu rubripes*) (Yap et al., 2003), channel catfish (*Ictalurus punctatus*) (Plant and Thune, 2004), orange-spotted grouper (*Epinephelus coioides*) (Chen et al., 2006), rare minnow (*Gobiocypris rarus*) (Su et al., 2009b) and grass carp (*Ctenopharyngodon idella*) (accession No. AY395698). However, the complete *Mx* genomic sequences in fish are only available in pufferfish (Yap et al., 2003), catfish (Plant and Thune, 2008), gilthead seabream (Fernandez-Trujillo et al., 2011) and senegalese sole (accession No. EU717076).

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Table 1
Primers used for genomic structure exploration and SNP detection in *CiMx2*.

Primer name	Sequence (5′-3′)	Position (bp)	Fragment size (bp)	Application
MXF383	ACATCACATAGGCGTCGCTG	1–1151	1151	5′ UTR and exon 1
MXR384	CTTTCCTGAGCTCTGGTCACCT			
MXF312	GGTCTGAACCAGCACTATGAA	1020–2337	1318	Exon 1, 2, 3 and intron 1, 2
MXR313	CATGACTGATCCCTTCTCCC			
MXF314	CTGGAACGGGAGAAGGGAT	2311–3046	736	Exon 3, 4, 5 and intron 3, 4
MXR315	TCGGCACTTTACGATCATGTAG			
MXF320	AGAAGGGCTACATGATCGTAAAG	3018–3633	616	Exon 5, 6 and intron 5
MXR321	TCAACCAATTCTTTTGTGAGTCT			
MXF324	GACTCACAAAAGAATTGGTTGAAC	3612–6641	3030	Exon 6, 7, 8, 9 and intron 6, 7, 8
MXR354	AGGTTCCTCCAGCTCCTCAAT			
MXF355	ACCCTCAGAGATGAGGTAGAAGAGTAT	6519–7082	564	Exon 9, 10 and intron 9
MXR335	ATGAACGGCCTTTGCCCA			
MXF352	GGAGAGGATTGTCTACTCCCAA	6990–7658	669	Exon 10, 11 and intron 10
MXR353	AGCACTCTTCAGGCGCTC			
MXF385	GAATGCAATGCTTGCCATGA	7541–8014	474	Exon 11 and 3′ UTR
MXR386b	CAATCTAAATCTTTTGCCAAAACAG			

The association of single nucleotide polymorphisms (SNP) of the *Mx* gene with antiviral activity is widely studied in mammals and birds (Hijikata et al., 2000; Nakatsu et al., 2004; Sulandari et al., 2009; Sironi et al., 2010). RFLP analysis of the *Mx* gene was employed to select susceptible or resistant strains to infectious hematopoietic necrosis virus (IHNV) in rainbow trout (Trobridge et al., 2000). However, there have been no studies of SNPs in fish *Mx* genes until the current study.

Grass carp is an important freshwater species in China, suffering from grass carp reovirus (GCRV), a double stranded RNA (dsRNA) virus (Jiang et al., 2009; Su et al., 2009a). GCRV causes severe hemorrhagic disease in juvenile grass carp (Su et al., 2009c). The main measures to prevent the disease are drugs and vaccines, and the effects are not ideal. Selection for disease resistance breeding may be an alternative method to solve the problem.

In the present study, we employed the unique grass carp *Mx* cDNA sequence available in GenBank (accession No. AY395698) to amplify the corresponding grass carp *Mx* genomic sequence. This cDNA and deduced amino acid sequences are the closest homologous to the corresponding sequences of crucian carp *Carassius auratus Mx2* gene (Zhang et al., 2004) (Supplementary material 1). We detected the full length gene sequence, identified many SNPs and tested their associations with resistance to GCRV. The results could be the potential markers for selective breeding of resistant grass carp to GCRV.

2. Materials and methods

2.1. Exploration of genome structure of CiMx2

There is no available genomic sequence information on *CiMx* gene. Based on the available *CiMx2* cDNA sequence (accession No. AY395698), some primers were designed by primer premier 5.0 to amplify the genomic sequence gradually. Eight pairs of primers worked well, and eight overlapping fragments were amplified and sequenced with the automated sequencer ABI 3730 (Applied Biosystem), which covered the full-length DNA sequence (Table 1). To assure only a single *Mx* gene was amplified, the eight pairs of primers were across used to set up PCR.

2.2. Grass carp, virus challenge and sample collection

200 grass carp, averaging 10 cm in body length, were collected from three fish farms (Hengkou, Ankang and Zhouzhi fish farms in Shaanxi, China), where no hemorrhagic disease of grass carp was found in recent years. The fries of the three farms were obtained from the same fish hatchery. One week prior to infection, fish were transferred to the quarantine facilities with aerated freshwater at 28 °C for adaptation. Grass carp were divided into four groups (50 animals in each group) for the injection experiments. The conditions were same among the tanks and the fish were randomly distributed into different tanks. Three groups were maintained in three aquariums and intraperitoneally injected with 100 μl of GCRV (097strain, 3.63×10^7 TCID50/ml), suspended in PBS, per gram body weight. The fourth group was injected with PBS as control. All the fish were observed every 6 h for any mortality and to collect samples until the termination of the experiment at 240 h post-challenge. Grass carp that died in the first 72 h post-challenge were classified as susceptible individuals for their high sensitivity to GCRV and symptoms of hemorrhagic disease of grass carp (such as congestive muscle, operculum, fin base, intestine, air bladder), while the animals that survived over 240 h post-challenge were considered as resistant group, and the rest were discarded for their ambiguity to GCRV infection. GCRV was detected by RT-PCR in muscle tissue (Su et al., 2010). Approximate one gram caudal fin was cut and kept at −80 °C until DNA isolation. About 100 mg caudal fin was homogenized, and DNA was extracted with traditional phenol–chloroform method and was stored at −20 °C.

2.3. Identification and analysis of polymorphic loci

The whole genomic sequence of *CiMx2* gene was scanned with eight pairs of gene specific primers (Table 1). The amplicons are from 474 bp to 3030 bp. PCR reaction was performed in a Peltier Thermal Cycler (Bio-Rad) in 25 μl reaction volume containing 100 ng of DNA template. The PCR products, from 10 susceptible individuals and 10 resistant individuals, were purified and sequenced. The polymorphic loci were detected from the sequence alignment of different individuals using Vector NTI Suite 11.0 (Invitrogen). One single nucleotide polymorphism was detected in the second exon, and four in introns (Fig. 1).

Transcription factor binding sites (TFBSs), which interact with transcription factors to regulate transcription, are likely to be located in various region of genes, including untranslated region (UTR), exon, intron, even outside the gene. The putative binding sites for transcription factors of *CiMx2* were screened by TFSEARCH analysis (http://www.cbrc.jp/research/db/TFSEARCH.html).