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Characterization of *MMP-9* gene from grass carp (*Ctenopharyngodon idella*): An *Aeromonas hydrophila*-inducible factor in grass carp immune system

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ABSTRACT

Matrix metalloproteinase-9 (MMP-9) belongs to a family of zinc-dependent endopeptidases and is associated with vital inflammatory processes. Here, we isolated and characterized MMP-9 cDNA from grass carp (*Ctenopharyngodon idella*) (designated as CiMMP-9). The cDNA was 2880 bp long and encoded a putative protein of 675 amino acids, with a predicted molecular mass of 75.816 kDa and an isoelectric point (pI) of 5.25. CiMMP-9 contained all three classical MMP-9 family signatures. The mRNA of CiMMP-9 was constitutively expressed in all tested tissues of untreated grass carp, with the highest expression levels in the blood, trunk kidney, head kidney and spleen. CiMMP9 transcript was present in unfertilized eggs, which suggests that CiMMP9 transcription is maternally inherited. Fluorescent real-time quantitative RT-PCR was used to examine the expression of the CiMMP-9 gene in *C. idella* after being challenged with *Aeromonas hydrophila*. A clear time-dependent expression pattern of CiMMP-9 was found after the bacterial challenge, and mRNA expression reached a maximum level at 7 days post challenge. This indicates that MMP-9 is inducible and is involved in immune responses, thus suggesting that CiMMP-9 plays an important role in *A. hydrophila*-related diseases and in early embryonic development stages in *C. idella*.

1. Introduction

Matrixmetalloproteinases (MMPs) constitute a multigene family of over 25 secreted and cell surface enzymes that process or degrade numerous pericellular substrates [1]. MMPs also cleave intracellular substrates and have been found within cells in the nucleus, mitochondria and in various vesicular and cytoplasmic compartments. Intracellular substrate proteolysis by MMPs is involved in innate immune defense and apoptosis, and affects oncogenesis and the pathology of cardiac, neurological, protein conformational and autoimmune diseases [2]. In terms of structure, a typical MMP consists of a propeptide, a catalytic domain, a Zn^{2+}-binding domain, a linker or hinge region of variable length and a hemopexin domain, which contributes to substrate specificity and to interactions with endogenous inhibitors and cargo receptors [3]. Based on their substrate specificities and sequence characteristics, MMPs can be divided into four main classes: collagenases, gelatinases, stromelysins and membrane-type MMPs [4]. Only the two gelatinases MMP-9 and MMP-2 have all three 58-amino-acid fibronectin-like type II modules inserted in their catalytic domains [5]. The biggest MMP is MMP-9, which has a molecular weight (latent/active) of 76/67 kDa.

Because MMPs can degrade ECM molecules, their main function has been presumed to be remodeling of the ECM. They are thought to play important roles during embryonic development, as ECM remodeling is a critical component of tissue growth and morphogenesi [6]. Moreovre, the MMPs contribute to not only ECM remodeling but also to many other cellular functions. MMP activities are required in cell migration, cellular behavior and the release of active molecules that bind to the ECM suggesting that MMPs play important roles in the developmental process [7]. The expression of fugu *MMP-9* mRNA is also involved in the bone development in the caudal fin. Homozygous mice with a null mutation in the MMP-9 gene exhibit an abnormal pattern of skeletal growth plate vascularization and ossification [8]. Biological studies of MMP-9 have tuned the field from being primarily cancer-oriented towards vascular and inflammatory research. The recognition that MMP-9 is

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induced by many pro-inflammatory cytokines, whereas its inhibitors are increased by anti-inflammatory cytokines, has generated interest to target MMP-9 in acute lethal conditions, such as bacterial meningitis, sepsis and endotoxin shock, and in acute exacerbations of chronic diseases [9].

MMP-2 is synthesized constitutively by mesenchymal cells and is activated by membrane-type MMPs (MT-MMPs), whereas MMP-9 is produced mainly by inflammatory cells, such as monocytes/macrophages, neutrophils and eosinophils, and is activated by other MMPs [10]. Teleost MMP-2/9 genes have been cloned from carp [11], channel catfish [12], puffer fish [7], Japanese flounder [13] and zebra fish [14]. Although gelatinases, MMP-2 and MMP-9, shared some substrate specificities, these enzymes are known to be synthesized by different cell types [15]. In the fugu embryos, spatial and temporal expression profiles differ between fugu gelatinases [15]. Recent studies found MMP-9 to be up regulated by *Neoheterobothrium hirame* infection in Japanese flounder, and identified MMP-9 as an infection marker for future studies [16]. To evaluate their biological activities, MMP-9 genes from a few teleost species were studied and showed similar functions to their mammalian counterparts. The prominent expression of *MMP-9* in the head kidney and other immune competent organs confirmed its contribution to the innate immune response in the gilt-head (sea) bream [17] and other vertebrates [18]. Due to its conserved sequence homology and structure, MMP-9 also plays an important role in both cell migration and tissue remodeling during inflammation in teleost [19]. Some results also suggest an active role for MMP-9 in both the initial and the resolution phases of inflammation in lower vertebrates [19].

The grass carp (*Ctenopharyngodon idella*) is one of the most important fish species farmed in China [20]. Despite their favorable growth traits, farmed grass carp are rather susceptible to various diseases. Outbreaks of diseases associated with bacteria such as *Aeromonas hydrophil*a have caused high fish mortality, resulting in reduced production and considerable economic losses [21]. Immune responses in fish can be experimentally stimulated by injection of bacteria [22]. Therefore, an immunological challenge model using *A. hydrophila* has a great potential to improve our understanding of immune or regulatory effects.

In the present study, the full-length cDNA of grass carp *MMP-9* was cloned and characterized, and the tissue-specific and embryogenetic expression patterns were analyzed by qRT-PCR. Transcriptional analysis was carried out to test the immune responses of MMP-9 after an experimental challenge of *C. idella* using *A. hydrophila*. The qRT-PCR results indicated that MMP-9 is functional during the embryo-genesis of *C. idella*. In addition, the expression patterns of *MMP-9* in *C. idella* exposed to a bacterial challenge suggest that MMP-9 has potential roles in innate immune responses.

2. Materials and methods

2.1. Animal treatment and RNA extraction

Grass carp with an average weight of 50 g were cultured individually in the Wujiang National Farm of Chinese Four Family Carps, Jiangsu Province, China. Animals were raised at 28 °C in aerated 400 L tanks for one week before the experiment and fed twice daily (in the morning and late in the afternoon) at a ratio of 5% of the total biomass. Embryos and fries were obtained from the Wujiang National Farm of Chinese Four Family Carps, and reared in a hatching trough with constant pool water flow at 21 ± 1 °C. On day 3 post hatching, the fries could swim steadily and were fed with freshwater rotifers captured from the pool. For full-length cDNA cloning and expression pattern analysis, the brain, muscle, trunk kidney, liver, head kidney, skin, spleen, heart, gill, intestine and fin were dissected from three unchallenged fish. Blood samples (approximately 1–2 mL/fish) were taken from the caudal fin using a 2 mL syringe. Samples were immediately centrifuged at 3000 rpm at 4 °C for 10 min to separate blood cells. Total RNA was isolated by using the RNAiso Plus kit (TaKaRa, Japan) and stored at −80 °C after incubation with RNase-free gDNA Eraser (TaKaRa). The RNA concentration and purity was determined by measuring the absorbance at 260 nm and 280 nm with the Nanodrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, USA).

2.2. Full-length cDNA cloning of MMP-9

Full-length cDNA of MMP-9 was obtained through reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The first cDNA was synthesized from the isolated RNA using the High Fidelity PrimeScript RT-PCR Kit (TaKaRa). Primers (Table 1) were based on conserved regions of this gene from other fish species. The PCR program was: 1 cycle of 94 °C for 3 min; 31 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; 1 cycle of 72 °C for 10 min. The PCR product was ligated into a pGEM-T easy vector (Promega, Madison, USA), transformed into competent *E. coli* DH5α cells, plated on an LB-agar Petri dish and incubated overnight at 37 °C. Positive clones containing the insert with the expected size were identified by colony PCR. Three of the positive clones were picked and sequenced on an ABI PRISM 3730 Automated Sequencer, using BigDye terminator v3.1 (Applied Biosystems, USA).

Based on the sequences of the conserved region we obtained from *C. idella*, 5′- and 3′- RACE PCR were performed to define the putative 5′- and 3′- ends of MMP-9, respectively. The gene-specific primers (Table 1) were designed according to the conserved region sequences. RACE and RACE-PCR were conducted with the SMART RACE cDNA Amplification Kit and Advantage 2 PCR Kit (Clontech, USA). The conditions for the PCR were five cycles of 94 °C for 30 s; 70 °C for 1 min; 72 °C for 3 min, followed by 31 cycles (94 °C for 1 min; 68 °C for 1 min; 72 °C for 3 min) and 72 °C for 10 min after the last cycle. PCR products were cloned and sequenced as described above.

2.3. Sequence analysis

The open reading frame (ORF) of MMP-9 cDNA was determined using the program ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Nucleotide and amino acid sequence identity and the prediction of conserved domains in the peptide were

Table 1
Primers used in this study.

Gene	Sense (5′–3′)	Antisense (5′–3′)	Application
MMP-9	ACCCCAAGTGAGAAAACAAC	AGTAAAAGTCCCCAAAACAG	Cloning
		GGAAGTGTATGACTGTCCCAGGAAGATG	5′ RACE
	GTCTGTTTCTGGTGGCTGCTC		3′ RACE 1st
	TCGAGGAATTAACTTTG		3′ RACE 2nd
	ACTTGGAGTTGTGGCTTTTC	AGGGCTCTGTCATCAGGTTA	qRT-PCR
β-actin	CCTTCTTGGGTATGGAATCTTG	AGAGTATTTACGCTCAGGTGGG	qRT-PCR

performed using the BLAST program (GenBank, NCBI). The putative amino acid sequence of the MMP-9 protein was analyzed for the presence of signal peptides using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) [23]. Multiple sequence alignments were performed using the CLUSTALW 1.8 program [24]. Needle program (http://www.ebi.ac.uk/Tools/emboss/align/) was used to calculate identities among different HSP60 genes of different species. A phylogenetic tree was constructed, based on the deduced full-length amino acid sequences alignment by the neighbor-joining (NJ) algorithm and Maximum Likelihood method embedded in Mega 5.0 [25]. The reliability of the estimated tree was evaluated by the bootstrap method with 1000 pseudo-replications. The accession numbers of the 25 sequences used in this analysis are listed in Fig. 2.

2.4. Tissue and embryo-genetic expression analysis

MMP-9 mRNA content in tissues was examined in the samples from three unchallenged fish to illustrate the spatial expression patterns of MMP-9 in grass carp. Various tissues were tested, including blood, brain, muscle, trunk kidney, liver, head kidney, skin, spleen, heart, gill, intestine and fin. Total RNA was extracted as described above.

To study the embryo-genetic expression profiles, embryos and early larvae from different development stages (10 specimens from each stage) were collected and stored in liquid nitrogen. These stages included unfertilized eggs, 0 h post-fertilization, embryos at 16-cell stage, morula stage, gastrula stage, eye sac-appearance stage, caudal fin- appearance stage, muscular effect stage, heart

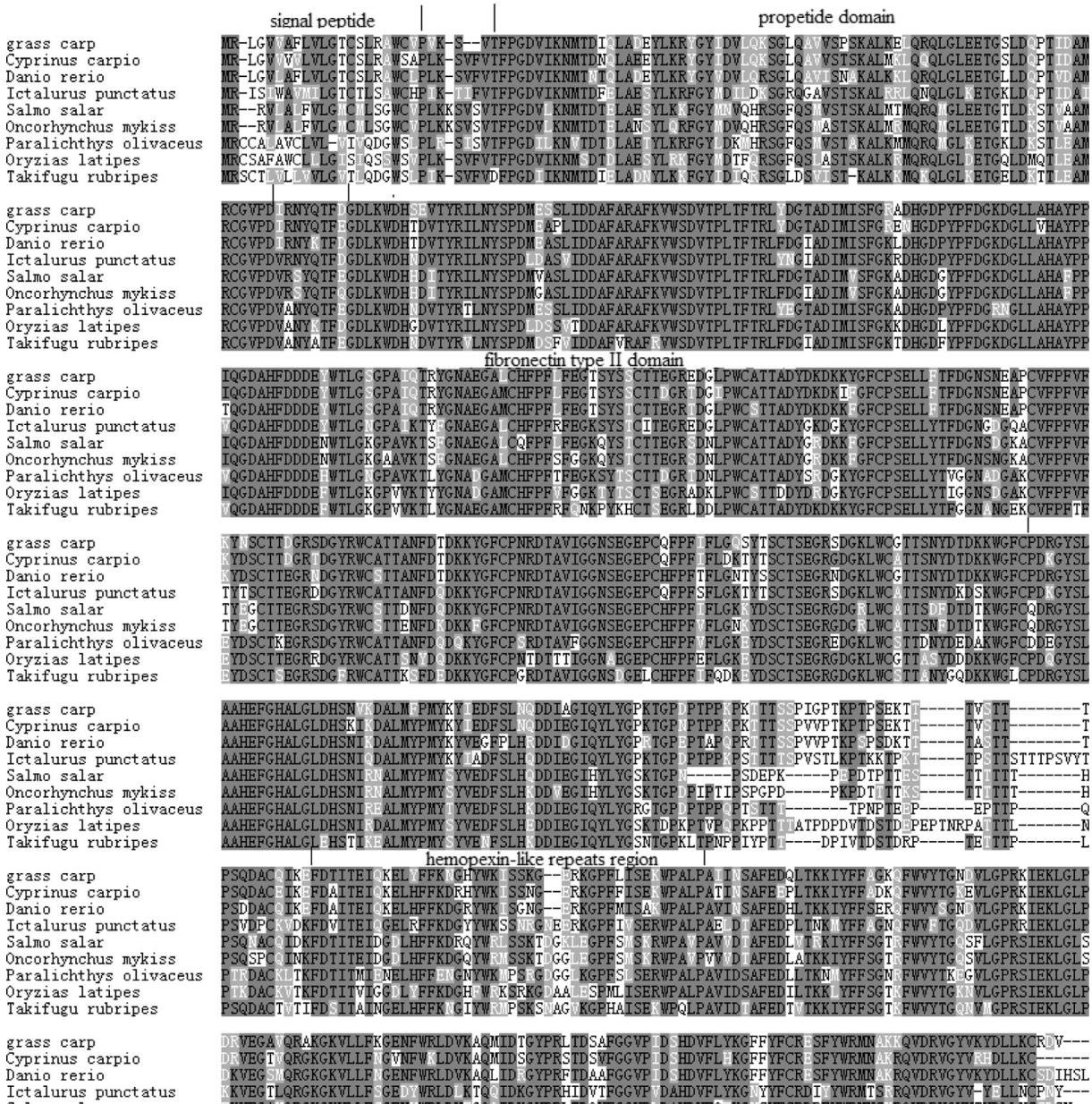

Fig. 1. Multiple sequence alignment of MMP-9 proteins. Analysis was performed by ClustalW (1.81), using representatives of MMP-9 from different teleost species. Signal peptide, propetide domain, fibronectin type II domain and hemopexin-like repeats region are marked with a single line.

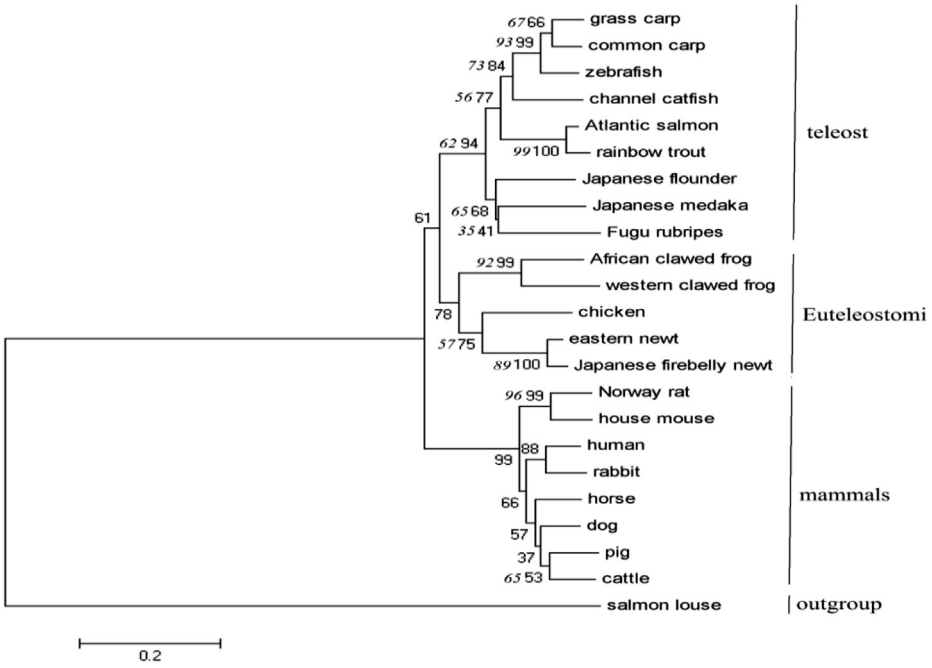

Fig. 2. Phylogenetic analysis of MMP-9. Unrooted phylogenetic tree of MMP-9 from vertebrates, using the salmon louse MMP-9 from invertebrates as out-group. The tree was constructed by using the Neighbor-Joining (NJ) and Maximum Likelihood methods from a gapped alignment. The values on the tree nodes are NJ percentage bootstrap values (regular font) and ML bootstrap values (*italics*). The protein sequences used for phylogenetic analysis were as follows: Grass carp (GenBank ID. ADU34085.1), common carp (BAB39390.1), zebra fish (NP_998288.1), channel catfish (ABO86718), Atlantic salmon (NP_001133929), rainbow trout (NP_001117842), Japanese flounder (BAB68366), Japanese medaka (NP_001098350), Fugu rubripes (NP_001032959), African clawed frog (NP_001079972), Western clawed frog (NP_001006843), chicken (NP_989998), eastern newt (AAX14805),Japanese firebelly newt (BAA11523), Norway rat (EDL96479), house mouse (AAX90605), human (NP_004985.2), rabbit (NP_001075672), horse (NP_001104772), dog (NP_001003219), pig (NP_001033093), cattle (DAA23127), salmon louse (ADD38666.1).

beating stage, mental stages and 1, 2, 3, 4, 5, 6, 7, 10 and 15 days post hatching [26]. Total RNA was extracted as described above.

Quantitative real-time PCR was performed in this section. The specific primers for *MMP-9* (Table 1) were designed according to the full-length sequences obtained. The *C. idella* β-actin (Table 1) was identical to previous reports [27].

2.5. Detection of the expression patterns induced by A. hydrophila

For the bacterial challenge, 12 fish as challenge group were intrapleurally injected with formalin-killed *A. hydrophila* S2 (Aquatic Pathogen Collection Centre of Ministry of Agriculture, China), is a Gram negative bacteria, at a dose of 7.0×10^6 cells suspended in 100 μL PBS per fish; 12 fish were similarly injected with 100 μL sterile PBS per fish as control group [28]. 3 fish were sampled at 4 h, 1 day, 3 days and 7 days post injection from each group, respectively. Blood, brain, muscle, trunk kidney, liver, head kidney, skin, spleen, heart, gill, intestine and fin were collected from each fish and were used to isolate total RNA. To detect the expression changes of *MMP-9* after bacterial challenge RNA detection was performed by real-time PCR with the same primers as mentioned above.

2.6. Real-time PCR

The real-time quantitative RT-PCR was performed on a 7500 Fast Real-Time PCR System (ABI). About 1 μg RNA from each sample was reverse-transcribed using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa). The first-strand cDNA was subsequently used as the template for PCR. Beta-actin served as an referencefor cDNA normalization [29]. The qRT-PCR mixture consisted of 50 ng of cDNA sample, 7 μL nuclease-free water, 10 μL of 2 × SYBR Premix Ex taq™ (TaKaRa), 0.4 μL of ROX Reference Dye, and 0.8 μL of each gene-specific primer (10 μM). The PCR cycling condition were 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 34 s followed by dissociation curve analysis at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s to verify the amplification of a single product. The threshold cycle (CT) value was determined on the 7500 v2.0 Software and the data were exported into a Microsoft Excel Sheet for subsequent analysis. Here the relative expression ratios of target genes in the treated groups versus that in the control group were calculated by the $2^{-\triangle\triangle CT}$ method [30]. One-way ANOVA tests were performed using SPSS 17.0 to determine significant differences. Each experiment was repeated in triplicates.

2.7. Statistical analysis

Data from the qRT-PCR was expressed as means ± SE. Differences among groups were analyzed by a one-way ANOVA with post-hoc Dunnett's T3 test. Comparison of the fold change of gene expression in different tissues after challenge with *A. hydrophila* of grass carp was analyzed with Student's *t*-test. Significance was accepted at the level of $P < 0.05$.

3. Results

3.1. Cloning and molecular characterization of MMP-9

The full-length cDNA fragment was obtained by overlapping three cDNA fragments: a conserved region, 5′- RACE and 3′- RACE. The full-length MMP-9 cDNA consisted of 2880 nucleotides (nt) with a 2025 nt open reading frame (ORF) encoding 675 amino acids (aa). The polyadenylation signal (AATAAA) was located at 16-21 nt upstream of the poly-A tail of the MMP-9 sequence. The MMP-9

cDNA sequence and deduced amino acid sequences have been submitted to GenBank with the assigned accession number HQ153831.

Analysis of the protein sequence indicated that MMP-9 has a predicted signal peptide of 20 amino acids (aa). The deduced protein had a calculated molecular weight of 75.816 kDa and a theoretical isoelectric point of 5.25. Blastp analysis showed that this gene had the highest similarity to the *Cyprinus carpio* MMP-9 (Identity = 602/674 (89%), E-value = 0.0), indicating that it could be a *C. idella* orthologue of MMP-9 (named CiMMP-9). The CiMMP-9 protein has three repeats of the fibronectin-type II domain inserted in the catalytic domain.

3.2. Alignment and phylogenetic analysis

A BLAST analysis revealed significant sequence similarity between CiMMP-9 and MMP-9s from other organisms. CiMMP-9 shared 90.1% identity with MMP-9 of the common carp (*C. carpio*, AB057407) and 82.2% with MMP-9 of zebra fish (*Danio rerio*, BC160656). A multiple polypeptide sequence alignment revealed a high degree of identity of MMP-9 and is shown in Fig. 1.

A phylogenetic tree was constructed by neighbor-joining and maximum likelihood methods, using salmon louse (*Lepeophtheirus salmonis,* ADD38666) MMP-9 as out-group to the vertebrate MMP-9 family. As shown in Fig. 2, the MMP-9s from euteleostomi were clustered together with MMP-9s from teleost, and CiMMP-9 was grouped with other teleost MMP-9s.

3.3. Tissue-specific and induced expression patterns of MMP-9

Various tissues and organs sampled from adult animals were processed in order to perform a relative analysis of the *MMP-9* mRNA expression. As shown in Fig. 3, *MMP-9* was detected in all the tissues sampled. *MMP-9* mRNA was abundantly expressed in the blood, although a large amount was also found in the trunk kidney, head kidney and spleen tissue.

To examine the in vivo transcriptional responses of MMP-9, fish were challenged with bacteria (*A. hydrophila*). Expression profiles were determined in the blood, brain, muscle, trunk kidney, liver, head kidney, skin, spleen, heart, gill, intestine and fin, using quantitative real-time RT-PCR. After being challenged with *A. hydrophila*, *MMP-9* expression was rapidly up-regulated (Fig. 4) in most tissues except the brain and intestine. Interestingly, transcription of *MMP-9* was gradually down-regulated in most tissues up to 3 days after *A. hydrophila* challenge (Fig. 5). In most tissues *MMP-9* reached the highest expression level at day 7.

3.4. Embryo-genetic expression patterns of MMP-9

The expression patterns of *MMP-9* during embryogenesis from eggs to early larvae were analyzed by quantitative real-time PCR. As shown in Fig. 5, high *MMP-9* expression levels were detected in early embryos at 3, 4 and 10 days post fertilization.

4. Discussion

Here, we describe the identification and analysis of an MMP-9 gene from the grass carp, *C. idella*. MMP-9 is ubiquitous among all living organisms and highly conserved at the amino acid sequence level, such as in the propeptide domain (A), the catalytic domain (E) and the C-terminal hemopexin-like domain (I) [31]. MMP-9 has three repeats of a fibronectin-type II domain inserted in the catalytic domain, which has been shown to be involved in binding to denatured collagen or gelatin Refs. [32,33]. Murphy and Knäuper recently reviewed the role of the hemopexin-like domains in relation to the substrate specificities and activities of various MMPs [34]. The hemopexin-like domain is important for interactions with tissue inhibitors of metalloproteinases (TIMPs) [35]. However, the alignment of the hemopexin-like domains shows that MMP-2 and MMP-9 fall into two different clusters. The hemopexin-like domain of MMP-9 diverges at a higher hierarchical level, implicating that it is somewhat different from that of MMP-2, even though both are involved in TIMP binding [35].

In our phylogenetic analysis, CiMMP-9 clustered with MMP-9 sequences of other fish species. Based on the molecular characterization, pairwise multiple alignment and phylogenetic results, we could confirm that the newly identified CiMMP-9 is a member of the MMP-9 family. The high similarity in crucial domains between CiMMP-9 and MMP-9 from other vertebrates suggests that these proteins may perform similar regulatory functions.

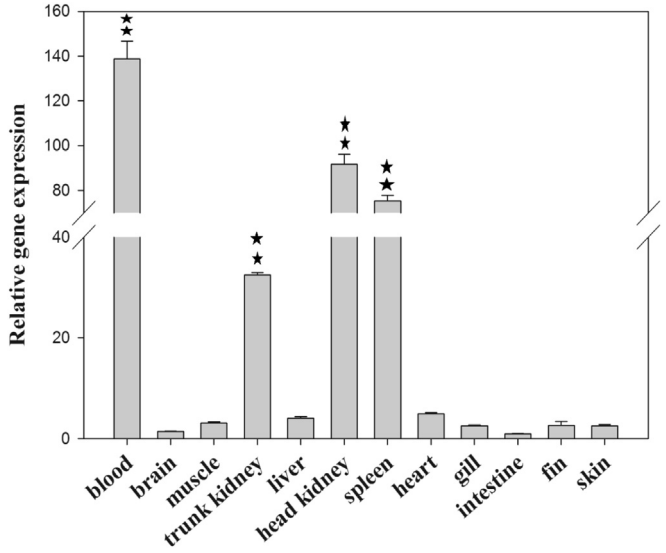

Fig. 3. Tissue expression profile of the *CiMMP-9* gene in *C. idella*. Relative mRNA levels obtained by qRT-PCR were calculated based on the standard curve and normalized to the *β-actin* mRNA level. Data are presented as mean ± SE; $^{**}P < 0.01$.

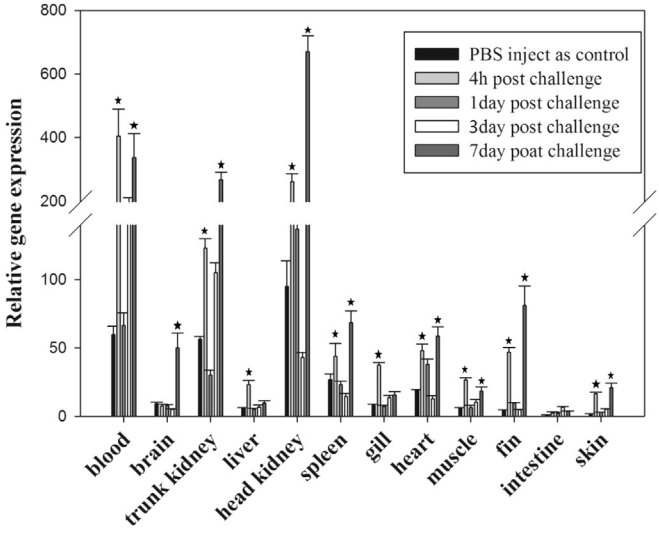

Fig. 4. Expression profile of *CiMMP-9* after challenge of *C. idella* with *A. hydrophila*. Control samples were injected with PBS. The relative expression of the transcript from qRT-PCR was calculated based on the standard curve and normalized to the *β-actin* mRNA level. Data are presented as mean ± SE; $^{*}P < 0.05$.

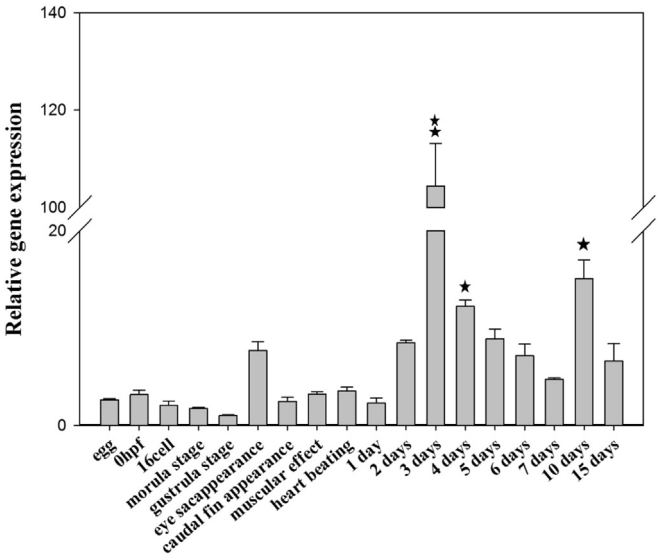

Fig. 5. Expression profile of *CiMMP-9* during different early development stages. The relative expression of the gene transcript from qRT-PCR analysis was calculated ssbased on the standard curve and normalized to the *β-actin* mRNA level. Data are presented as mean ± SE; $^{\star}P < 0.05$, $^{\star\star}P < 0.01$.

The results of qRT-PCR showed that under normal conditions, grass carp *MMP-9* mRNA was expressed at high levels in the blood, trunk kidney, head kidney and spleen. The MMP gene family appears to be induced by LPS in macrophages of trout [36], and a widescreen transcriptomic analysis of carp metalloproteinases detected a large amount of *MMP-9* mRNA mainly in hematopoietic organs, HK and spleen [11,37]. The innate immune system is the main defense of invertebrates and a fundamental defense mechanism of fishes [38]. The major difference to other vertebrates is that fishes lack bone marrow and lymph nodes and use the kidney as a major lymphoid organ instead [39]. In our study, high expression of CiTIMP-9 was found in immune competent organs, such as the spleen, head kidney, trunk kidney and blood. Although both MMP-2 and MMP-9 belong to the gelatinase family [5], they have different functions [40]. The MMP-2 has low expression levels in kidney and blood [41] and *MMP-9* has high levels, so we suggest that MMP-2 and MMP-9 also have different functions in *C. idella.*

After infection with *A. hydrophila*, the temporal expression of CiMMP-9 mRNA in most tissues was significantly up-regulated at 4 h post injection ($P < 0.05$), reduced to low levels at days 1 and 4 post injection ($P > 0.05$), and reached a maximum level at day 7 post injection, thus indicating that the gene was regulated by bacterial infection. MMP-9 activity might also be a marker for early diagnosis of adenocarcinoma [42]. Studies have suggested that an uncontrolled increase of MMP-9 activity is causally linked to severe symptoms of inflammatory diseases [43]. Interestingly, we found in our study that the expression levels of *MMP-9* on days 1 and 3 were reduced. We assume that the use of formalin-killed *A. hydrophila* allows for the transitory expression of a full range of protective antigens, although there might be other reasons. After a bacterial infection, MMP and TIMP expression profiles point to the prevalence of MMP-inhibition by endogenous inhibitors. This is similar to the situation found in human sepsis, where increased TIMP-1 expression levels lead to a diminished MMP-9/TIMP-1 ratio in plasma [44]. Another reason might be that MMP inhibition was induced by endogenous inhibitors. During wound healing, MMP-9 has been suggested to be involved in keratinocyte migration and granulation tissue remodeling [45]. A number of MMPs have also been identified as major contributors to wound healing, which is a complex process that includes cell proliferation, migration, matrix synthesis, contraction, wound site (WS) growth factor production, and cell-matrix signaling [46]. In this case, high expression of *MMP-9* after 7 days could can be interpreted as required for tissue repair. Interestingly, high *MMP-9* expression levels appeared only after 4 h in liver and gill tissues and after 7 days in brain whereas no significant changes were observed at these time-points in the intestine. We therefore suggest that MMP-9 is only involved in the early immune response in the liver and gills. The antibacterial ability of intestinal bacteria may protect host fish to some extent against pathogenic bacteria [47]. We used intrapleural injection to avoid direct stimulation of the intestine. Therefore, we hypothesize that the intestine did not receive sufficient stimulation in this study. Taken together, our analysis of MMP-9 expression in *C. idella* suggests its potential involvement in a wide range of immune-related processes.

In addition to its involvement in immune functions, MMP-9 appears to be an important factor during the embryonic development. MMP-9 deficiency greatly affects the vascularization and ossification of the growth plates [8]. MMP-9 seems to be expressed in specific organs in a precise temporal sequence during development [48]. Bone development requires the recruitment of osteoclast precursors from the surrounding mesenchyme, thereby allowing the key events of bone growth such as marrow cavity formation, capillary invasion, and matrix remodeling. MMP-9 is critical for early bone development [49]. In puffer fish MMP-9 in the marginal blood vessels degrades matrix proteins for migrating osteoclasts and consequently contributes to bone development in the pectoral fin [7]. According to the study described above, MMP-9 is primarily involved in skeletal growth and angiogenesis. The expression of Ci*MMP-9* from the early developmental stages onwards suggests that it is involved in skeletal growth and angiogenesis. Has high expression in the 3 dpf grass carp hatching larvae, we speculate CiMMP-9 in the marginal blood vessels degrades the matrix proteins for the migrating the osteoclasts and consequently contributes to bone development in the pectoral fin. The data of our expression studies also support the suggestion that MMP-9 could have an essential role in the development of *C. idella*.

In conclusion, an *MMP-9* gene was cloned from the grass carp, CiMMP-9 is constitutively expressed in various tissues, suggesting that it might be involved in a multifunctional role in healthy fish. Moreover, the expression of *CiMMP-9* is modulated by bacterial *A. hydrophila* infection, and CiMMP-9 displays a maternal expression during embryogenesis, suggesting its importance in both immune and developmental processes. This study is an initial step for further investigation the antibacterial activity of CiMMP-9 and to explore the importance of CiMMP-9 at early larval stages of grass carp development.

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