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Original Research Article

Cloning, expression pattern, and potential role of *MAPK*p38 and *NF-κB*p65 in response to lipopolysaccharide in yellow catfish (*Pelteobagrus fulvidraco*)

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ABSTRACT

Mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NF-κB) pathways are considered to be two crucial intracellular signaling cascades in pro-inflammatory responses. In this study, we reported the coding sequences (CDS) of *MAPK*p38 and *NF-κB*p65 from yellow catfish. We also investigated the gene structure, expression patterns and functional role in yellow catfish. The CDS of *MAPK*p38 is 1,086 bp encoding 361 amino acids (AA). The MAPKp38 protein has a long highly conserved serine/threonine protein kinases catalytic domain. The *NF-κB*p65 CDS is 1,794 bp, and the gene encodes 597 AA, with a Rel homology domain (RHD) which consists of a RHD-DNA-binding domain and an Ig-like, plexins, transcription factors (IPT) domain. Moreover, MAPKp38 and NF-κBp65 protein of bony fish and other vertebrates have a single clade. Quantitative real-time PCR analysis revealed the presence of the *MAPK*p38 and *NF-κB*p65 transcript in 12 tissues of healthy yellow catfish. The highest expression levels of *MAPK*p38 and *NF-κB*p65 were detected in the heart and liver, respectively. Upon stimulation with an intraperitoneal injection of lipopolysaccharide (LPS), the expression levels of *MAPK*p38 and *NF-κB*p65 were up-regulated in the intestine. These results indicated that *MAPK*p38 and *NF-κB*p65 play important roles in mediating the response protection against LPS in yellow catfish.

1. Introduction

Innate immunity in fish is the first line of defense against invading pathogens, which provides crucial signals for activation of adaptive immune response (Akira et al., 2001). These innate immune responses to pathogens are triggered by the recognition of specific structures of invading pathogens, or so-called pathogen-associated molecular patterns (Miyake, 2007). Upon recognition, pattern recognition receptors trigger a series of signaling events that result in the expression of type I interferons (IFN-I), IFN-stimulated genes, and inflammatory cytokines, which play a vital role in the protection of a host suffering from microbial infection (Sun and Ding, 2006).

Lipopolysaccharide (LPS), an important component of the outer membrane of gram-negative bacteria, is a part of resident intestinal flora, and can cause tissue injury (Liang et al., 2007; Wu et al., 2013). Mammalian Toll-like receptor 4 (*TLR4*) recognizes LPS, then transmits to nuclear factor kappa B (*NF-κB*) and mitogen-activated protein kinases (*MAPK*) through two signaling pathways — namely, the myeloid differentiation primary response gene 88 (*MyD88*)-dependent pathway and the TIR-containing adapter inducing IFN-β (TRIF)-dependent pathway, resulting in production of IFN-γ and pro-inflammatory cytokines (Kawai and Akira, 2009).

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The NF-κB is a homodimeric or heterodimeric complex formed by the Rel homology domain (RHD)-containing proteins, which plays a central role in inducing the production of inflammatory cytokines such as IL-1β, IL-6, and IL-8 after LPS stimulation (Hoffmann et al., 2006). In non-stimulated cells, NF-κB is retained in the cytosol by inhibitory molecules (inhibitors of NF-κB, IκB) (Baldwin, 1996). Upon LPS stimulation, this p65/p50 dimer dissociated from IκB and translocated to the nucleus resulting in combining with the promoter and enhancer region of related genes, thus further induce the gene expression of inflammatory cytokines (Baldwin, 1996; Hoffmann et al., 2006). Besides NF-κB signaling pathway, the MAPK pathway is considered to be one of the crucial intracellular signaling cascades in pro-inflammatory responses (Zhang et al., 2015). The MAPK are a diverse group of intracellular serine/threonine kinases, including ERK, JNK, and p38, which have important functions in cellular response to cytokines in the immune system. In particular, the MAPKp38 pathway is necessary for the secretion of pro-inflammatory cytokines after LPS stimulation (Mercau et al., 2014). The activity of NF-κB is also positively regulated by MAPKp38 by impinging on the p65 subunit in a direct or indirect manner. Previous study indicated that 4 serine residues of p65 could be phosphorylated via stress-activated kinase 1 (MSK1), which located at the downstream of p38a (Kefaloyianni et al., 2006). Collectively, LPS-induced inflammatory process is mediated by NF-κBp65 and MAPKp38 signaling pathways.

Yellow catfish *Pelteobagrus fulvidraco*, an omnivorous freshwater fish, is regarded as a good candidate for fresh water culture in China and East Asia for its delicious meat and high market value (Dan et al., 2013). The *NF-κB*p65 and *MAPK*p38 mRNA expression levels were up-regulated by LPS exposure (Jiang et al., 2015). However, as the important signaling molecules in the TLR pathway, *NF-κB*p65 and *MAPK*p38 in yellow catfish have been rarely studied. Thus, we cloned the coding sequence (CDS) of *MAPK*p38 and *NF-κB*p65, and then examined their tissues expression pattern. Additionally, yellow catfish were induced intestinal inflammatory response by intraperitoneally injected LPS to verify the potential functions of these two genes in the immune system via analyzing their expression levels.

2. Materials and methods

All animal experimental procedures and sample collections were performed in accordance with the guidelines of Institutional Animal Care and Use Committee of the Sichuan Agricultural University under permit NO. DKY-B20140302.

2.1. Animals and immune challenge experiment in vivo

Yellow catfish were obtained from Tongwei fisheries (Sichuan, China) and maintained in tanks (200 cm × 150 cm × 100 cm) in a recirculation system. They were kept at 28 ± 2 °C on a 12D:12L photoperiod and fed a commercial diet (containing 400 g/kg crude protein and 70 g/kg crude fat) twice a day to visual satiation. The water pH and dissolved oxygen were 7.8 ± 0.3 and 5.5 ± 0.3 mg/L, respectively. Fish were acclimatized for 2 weeks prior to the experiment. After the acclimatization period, 6 healthy fish weighing approximately 60 g were collected randomly and anesthetized in tricaine methane sulfonate (MS-222) at 120 mg/L (Zhou et al., 2015). The brain, gills, heart, spleen, head-kidney, mid-kidney, stomach, intestine, liver, pancreas, muscle, and skin were quickly removed, pooled, frozen in liquid nitrogen, and stored at −80 °C until further mRNA expression profile analysis. Thirty-six fish (average body weight 78.12 ± 0.26 g) from acclimatized fish were randomly divided into 2 groups with 3 replicates per group and 6 fish per replicate (18 fish for each group). One group was intraperitoneally injected with LPS (*Escherichia coli* O111:B4, L2630, Sigma; 4 mg/kg body weight) for 48 h, and the other group was injected with PBS as a control. At the end of trial, the fish intestines were sampled and frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2. RNA isolation and synthesis of cDNA

Total RNA was isolated from each tissue by using Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. The RNA purity of each sample was determined by calculating the OD260:OD280 ratio by a NanoDrop 1000 spectrophotometer (Hach, America). The RNA integrity was assessed by inspection of the 28S and 18S ribosomal RNA bands in a 1% agarose gel. First-strand cDNA was obtained from the total RNA by using a PrimeScript reverse transcription kit with gDNA Eraser (TaKaRa) according to the manufacturer's instructions. The reverse transcription products (cDNA) were stored at −80 °C for cDNA cloning, sequencing, and relative quantification by PCR.

2.3. Cloning of the sequences and molecular characterization analysis

All specific primers in this study for cloning were designed via Primer Premier 5.0 software according to the conserved regions of published nucleotide sequence and synthesized by the Huada Gene Company (Beijing, China) as shown in Table 1. Intestine cDNA was used as the template to amplify cDNA fragments. The PCR conditions were as follows: one cycle of 95 °C for 3 min; 35 cycles of 95 °C for 30 s, optimal temperature for 30 s, 72 °C for 1 min; and one cycle of 72 °C for 10 min. After verification with 1% agarose gel electrophoresis, the purified PCR products were ligated into the pMD19-T vector (TaKaRa) and positive clones were sequenced bidirectionally by Sangon Corporation (Shanghai, China).

The CDS of *MAPK*p38 and *NF-κB*p65 genes were edited and assembled with Editseq and Seqman in the DNAStar software. The homology of genes in vertebrates and multiple alignments of amino acid (AA) sequences were performed with ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/). The protein domains were predicted by the conserved domain search tool from NCBI (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cg). The neighbor-joining method was used to construct a phylogenetic tree using MEGA 5.0. Moreover, the three-dimensional structure of MAPKp38 protein was predicted

Table 1
Primer sequences used in the present study.

Name	Sequence (5′-3′)	Annealing temperature, °C	Products, bp
Genes cloning			
*MAPK*p38-AF1	ATGTCCCACAAGGAGAGACC	57.0	367
*MAPK*p38-AR1	GCTTCTGGCACTTGACGATA		
*MAPK*p38-AF2	ATCTGTATGCTCGGCGTATG	58.2	976
*MAPK*p38-AF2	TCAGGACTCCATCTCATCTCC		
*NF-κB*p65-AF1	ATGGCTGAAAGGTTCAACTGG	54.7	554
*NF-κB*p65-AR1	CTGTTGTCGTAGATGGGCTGAG		
*NF-κB*p65-AF2	AAAGCAACGATTCCACCAAA	59.0	766
*NF-κB*p65-AR2	GTCCGCTTCCTCTTCTCCAT		
*NF-κB*p65-AF3	CACCGCCCTACTGCGATACAAA	59.0	1,052
*NF-κB*p65-AF3	CTAAGTGGGATGCCCGGACAG		
Quantitative real-time PCR			
*MAPK*p38-QF	TCTGTATGCTCGGCGTATGAC	58.2	85
*MAPK*p38-QR	CGTGGATGATGGACTGGAAA		
*NF-κB*p65-QF	AGAGCAACGATTCCACCAA	58.2	137
*NF-κB*p65-QR	GCAGTCTTTTCCCACCAGC		
β-actin-QF	CCTAAAGCCAACAGGGAAAA	59.0	186
β-actin-QF	ATGGGGCAGAGCATAACC		

based on the known three-dimensional structure of human MAPKp38 (PDB: 1r39.1.A) by the AA homology modeling on (www.expasy.org/swissmod/SWISSMODEL.html). Finally, the results of the protein secondary structure prediction and the homology modeling were compared.

2.4. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) analysis were conducted to determine the mRNA abundance by using the Bio-Rad CFX96 Detection System (Bio-Rad, Hercules, CA, USA) as described in our previous study. Beta-actin was used as an internal control gene to correct for RNA loading variation, and the primers were synthesized according to Chen et al. (2015). The qPCR reaction system was performed using 5 μL SYBR Premix Ex Taq II, 3.2 μL RNase free dH_2O, 1 μL cDNA template, 0.4 μL follow and reverse primers. Gene-specific primers and annealing temperature used in quantitative analysis of gene expression are listed in Table 1. The PCR thermo cycling conditions were initiated with a denaturation step of 95 °C for one min and followed 40 cycles of amplification (95 °C for 5 s and annealing at a different temperature for 30 s). A melting curve analysis was generated following each amplification reaction assay to check the specificity and purity of all PCR products. The gene relative expression levels of *MAPK*p38 and *NF-κB*p65 were normalized to β-actin by using the $2^{-\Delta\Delta CT}$ method.

2.5. Statistical analyses

All statistical analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA) software. All data were presented as means ± standard error (SE). Tissue mRNA relative expression levels were subjected to a one-way analysis of variance with tissues as the factors. Differences in mRNA expression pattern between the tissues were determined using Duncan's multiple range tests at a $P < 0.05$ level of significance. In the immune challenge assay, the differences between two groups were assessed by using a *T*-test.

3. Results

3.1. cDNA cloning and sequence analysis

In the present study, we obtained a 1,794-bp full-length CDS of *NF-κB*p65 (GenBank accession NO. MK645606) and a 1,086-bp full length CDS of *MAPK*p38 (GenBank accession NO. MK645604), and the genes encode 597 and 361 AA, respectively. As shown in Fig. 1, MAPKp38 protein was induced the weight of 41.6 kDa, and its isoelectric point was 5.40. The analysis of protein domains revealed that MAPKp38 has a long highly conserved serine/threonine protein kinases catalytic domain. Moreover, considering the predicted AA sequence, there was a conserved motif in MAPKp38 protein sequenced threonine-glycine-tyrosine (T-G-Y). As the result of this study, AA sequence of yellow catfish MAPKp38 has a quite highly identity (92.0% to 98.1%) to other fishes and over 80% to amphibian, birds, and mammals.

Regarding the deduced protein sequence, yellow catfish *NF-κBp65* encoded 597 AA and weighed 66.4 kDa in size, with a RHD. In addition, its isoelectric point was 6.24. As shown in Fig. 2, The RHD contains 2 structural domains: a RHD-DNA-binding domain and an Ig-like, plexins, transcription factors (IPT) domain. Moreover, yellow catfish NF-κBp65 has an 11-AA nuclear localization sequence (NLS) motif sequenced "RLMEKRKRTEG". The consistency of multiple alignments only in N-terminal domain sequences showed 15% to 20% higher than alignment in entire CDS, and entire AA sequence of yellow catfish NF-κBp65 showed 72.27% identity to *Danio rerio* and shared approximate 47% to mammals.

3.2. Homology modeling

In order to better understand the detailed structures of yellow catfish MAPKp38, blast search and a Swiss-model analysis were used for homology modeling and estimate its three-dimensional structure. The three-dimensional structure of yellow catfish was established via human MAPKp38 (1r39.1.A) with 85.56% identity. The results showed that yellow catfish MAPKp38 protein has 12 α-helices and 10 β-sheets (Fig. 3A). As shown in Fig. 3A, some differences were also found in the exact amount of AA, e.g., there were 6 AA β-sheets at positions 8 to 13 in the N-terminal region of human MAPKp38 but only 7 AA β-sheets at corresponding positions (8 to 14 AA) for yellow catfish. However, in the N-terminal red marked AA sequence fragment of MAPKp38, there were both 7 β-sheets and the same α-helix at 63 to 78 AA of yellow catfish and human. At C-terminal, they both had the same α-helices contained 13 AA. Thus, the results revealed a possibility that a 2-layer sandwich might be formed by N-terminal 111-AA fragment and C-terminal α-helices in yellow catfish MAPKp38 like human proteins (Fig. 3B, domain 1). The middle domain of yellow catfish and human MAPKp38 has 4 same long-chain α-helices (at 125-144 AA, 204-219 AA, 229-240 AA, and 280-289 AA), 2 β-sheets and some short chain α-helices. Accordingly, the middle domain of yellow catfish MAPKp38 also formed α-helices and β-sheets, which had been proved by the Swiss-model analysis (Fig. 3B, domain 2).

3.3. Phylogenetic analyses

To verify the evolutionary relationships of identity of MAPKp38 and NF-κBp65 sequences, we use full-length predicted AA of yellow catfish and dozens of other vertebrates, including fishes, amphibians, birds, and mammals to construct phylogenetic trees using the MEGA5.0 software by the neighbor-joining method (Fig. 4). As shown in Fig. 4A, MAPKp38 protein of bony fish and other vertebrates have a single clade. Moreover, yellow catfish has a closer evolutionary relationship to *Salmo salar* than *D. rerio*. The phylogenetic tree of NF-κBp65 was pictured in Fig. 4B. Notably, bony fish and other vertebrates have a single clade. Yellow catfish has a closer evolutionary relationship to *Cypriniformes* than *Perciformes*.

3.4. Tissue expression

According to the known sequences in this study, we further determined the abundance of target gene transcripts in different tissues of 6 fish by qPCR using primers located inside coding regions. The tissue mRNA distributions are shown in Fig. 5. The *MAPK*p38 and *NF-κB*p65 mRNA were detected in all tissues analyzed, with the highest levels in the heart (1.90 fold) and liver (2.54 fold), respectively.

3.5. Gene expression analysis of post LPS challenge

The LPS injection was used to investigate the immune response and role of *MAPK*p38 and *NF-κB*p65 *in vivo*. The intestine was chosen as representative tissues. As shown in Fig. 6, mRNA expression levels of *MAPK*p38 and *NF-κB*p65 were significantly up-regulated in the intestine after LPS challenge

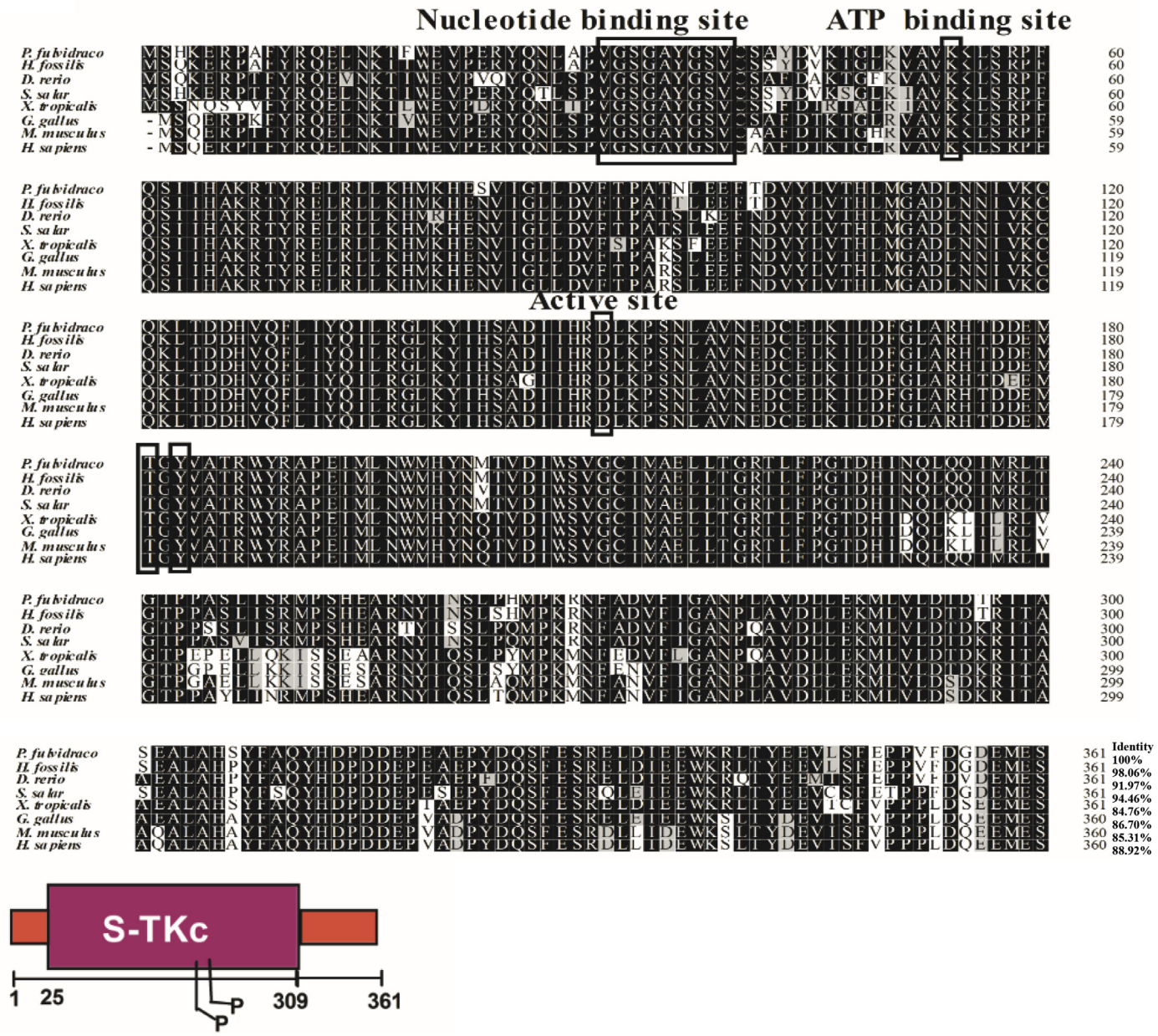

Fig. 1. Amino acid sequences alignment of MAPKp38 proteins from yellow catfish and 7 other species from GenBank. S-TKc = Serine/Threonine protein kinases catalytic. The sequences and their accession numbers are as follows: *Pelteobagrus fulvidraco*, MK645604; *Heteropneustes fossilis*, AMA21978.1; *Danio rerio*, BAB11807.1; *Salmo salar*, NP_001117170.1; *Xenopus tropicalis*, NP_001080300.1; *Gallus gallus*, XP_001232616.1; *Mus musculus*, NM_011951.3; *Homo sapiens*, NP_001306.1. The domain sites and name were marked.

compared to the control treatment (9.35 and 7.04-fold, respectively).

4. Discussion

In the present study, we firstly characterized the CDS sequences of *MAPK*p38 and *NF-κB*p65, tissue distribution, and mRNA expression in responses to LPS injection in yellow catfish. The *MAPK*p38, one of the *MAPK* family members, was ubiquitously expressed throughout zebrafish embryogenesis (Krens et al., 2006). It has been proved to be the most vital molecule of MAPK family in response to inflammation. In the present study, *MAPK*p*38* was highly expressed in most tissues with relatively lower expression in the kidney of yellow catfish (Fig. 5A). As an important protein involved in multiple physiological processes, yellow catfish MAPKp38 showed highly conserved AA sequence and domain as the multiple alignment analysis (Fig. 1). The activation of MAPKp38 linked to phosphorylation by the dual-specificity MAP2K (MAPK kinases) in MAPK cascade signal pathway (Derijard et al., 1995). In the present study, a T-G-Y region exists in 181 to 183 AA of yellow catfish MAPKp38 (Fig. 1), which could be recognized by MAP2K (Wood et al., 2009). Combined this motif with the same structure named 2-layer sandwich and orthogonal bundle in yellow catfish MAPKp38 like human (Fig. 3), yellow catfish MAPKp38 might be activated by MAPK cascade signal pathway and transmit inflammation signal.

In the present study, we also isolated the yellow catfish *NF-κB*p65 gene. The *NF-κB*p65 gene encoded a 597 AA peptide which has an N-terminal 273 AA RHD (Fig. 2). The consistency of multiple

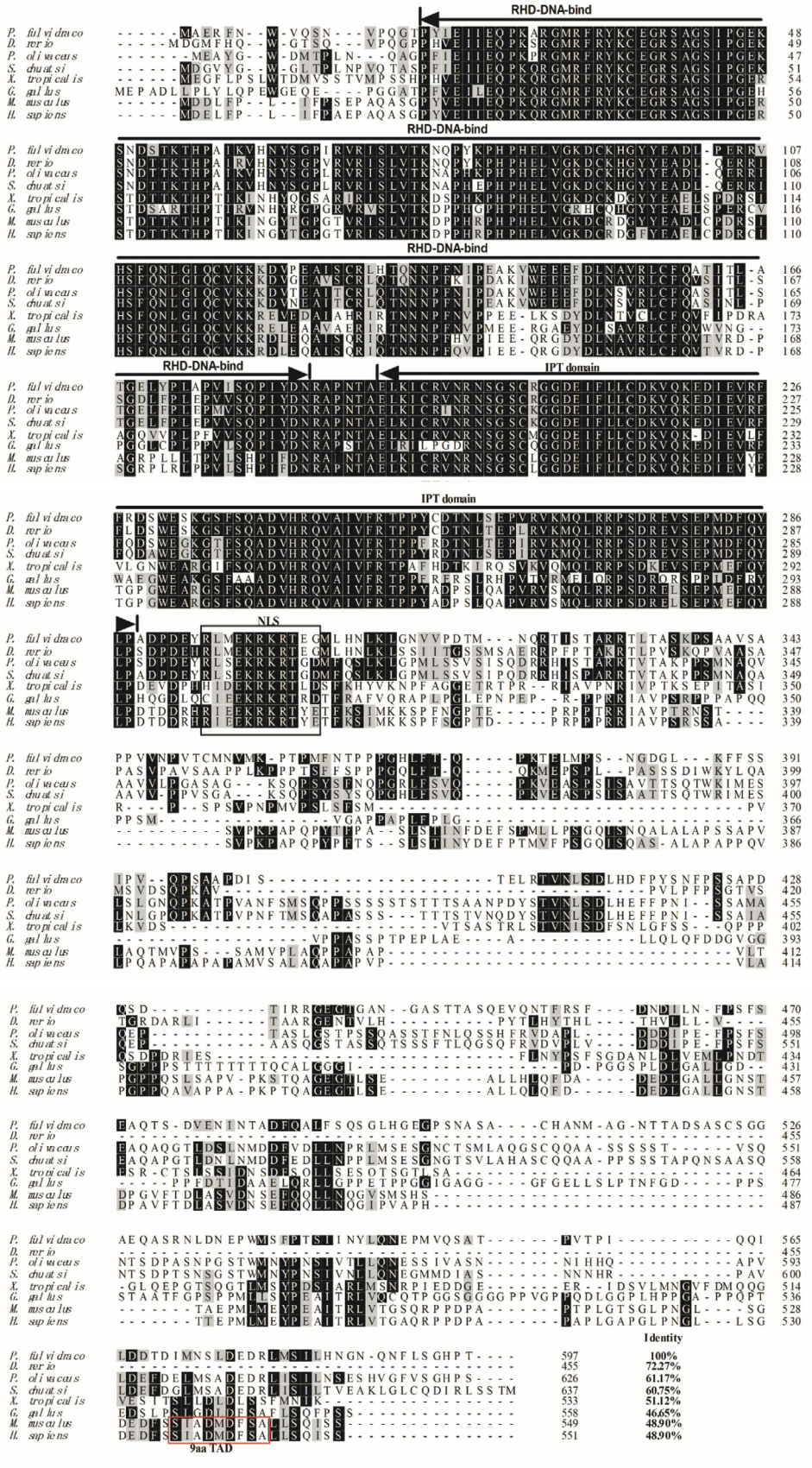

Fig. 2. Amino acids sequences alignment of NF-κBp65 proteins from yellow catfish and 7 other species from GenBank. RHD = Rel homology domain; IPT = transcription factors; NLS = nuclear localization sequence. The sequences and their accession numbers are as follows: *Pelteobagrus fulvidraco*, MK645606; *Danio rerio NF*, NP_001001839.2; *Paralichthys olivaceus*, ADM86237.1; *Siniperca chuatsi*, ABW84004.1; *Xenopus tropicalis*, NP_001001211.1; *Gallus gallus*, NP_990460.1; *Mus musculus*, NP_033071.1; *Homo sapiens*, NP_068810.3. The domain sites and name were marked.

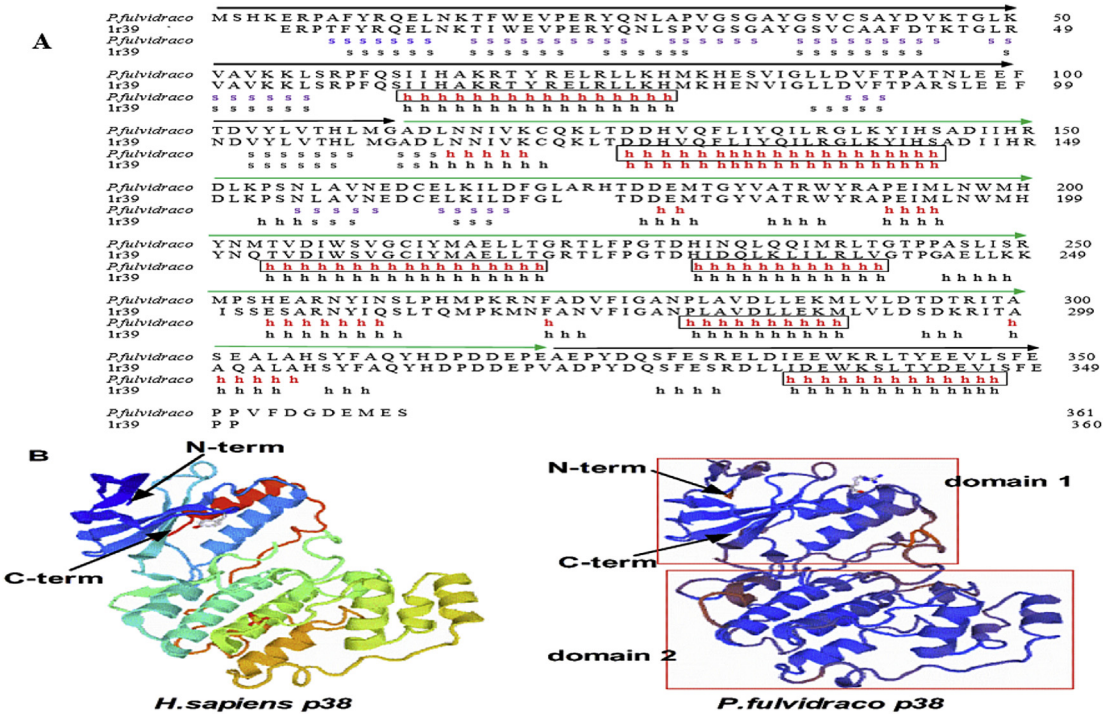

Fig. 3. Homology modeling of MAPKp38 protein (A) of yellow catfish based on the crystal structure of human p38 domain (1r39: 4-352 amino acid). Amino acid sequence alignments: "s" indicates the amino acid residues responsible for formation of the "bend"; "h" indicates the amino acid residues responsible for formation of the "α-helix"; "black arrow line" indicates the amino acid residues responsible for formation of the "domain 1"; "green arrow line" indicates the amino acid residues responsible for formation of the "domain 2"; "rectangular box" indicates the same "α-helix" of MAPKp38 between human and yellow catfish. Diagram compares the relative position of the amino acid residues of yellow catfish *MAPK*p38 with that of the human ortholog. The three-dimensional structures of *MAPK*p38 (B) of human and yellow catfish. Protein domain was constructed by comparative protein modeling program SWISS-MODEL. The coiled structure is "α-helix"; the zigzag lamellar structure with arrows is β-sheet.

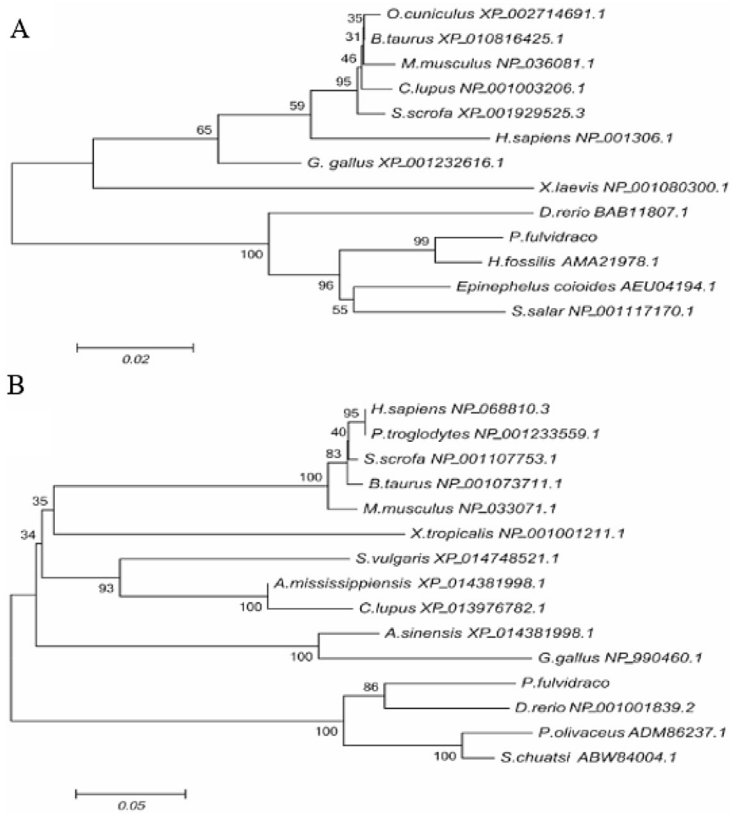

Fig. 4. Phylogenic analysis of the *MAPK*p38 (A) and *NF-κB*p65 (B) with equal-length proteins of others species. The trees were constructed by the neighbor-joining method based on Poisson correction model with 1,000 bootstrap replicates. The numbers at the branches indicate bootstrap values. The bar indicates the genetic distance.

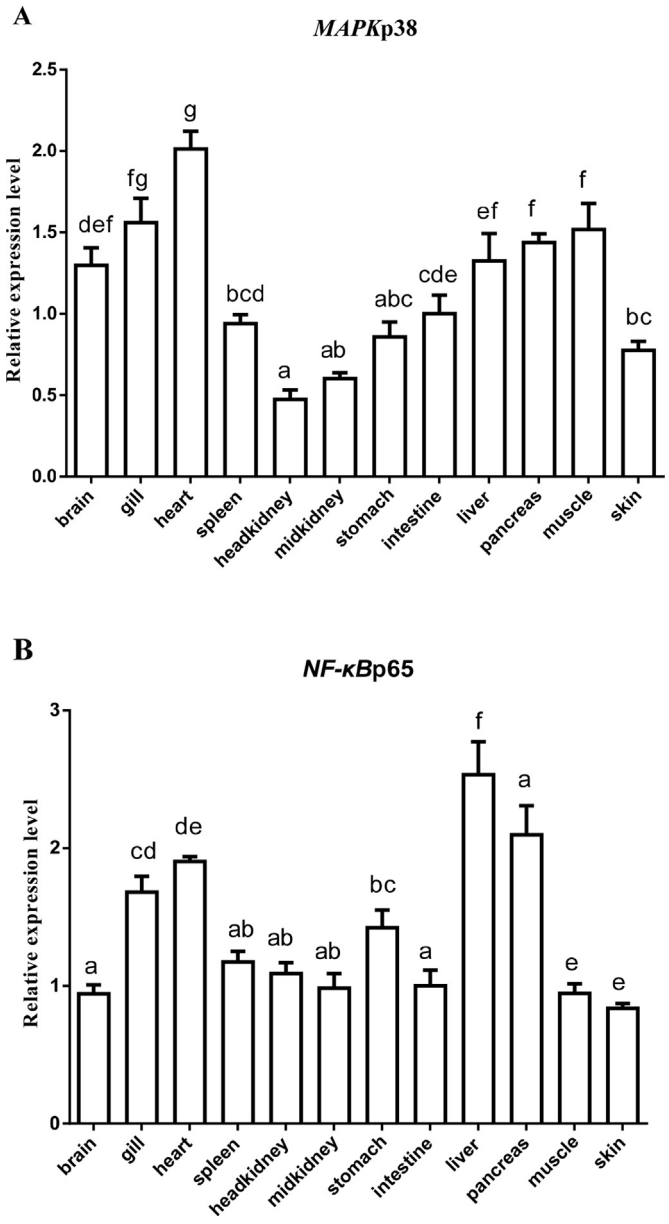

Fig. 5. Tissue distributions of *MAPK*p38 (A) and *NF-κB*p65 (B) transcripts in yellow catfish were analyzed by qPCR. Bar graphs represent the mean expression levels ($n = 6$) of target transcripts in different tissues normalized to β-actin gene transcript. In each target transcript, the bars with the same letter are not significantly different as evaluated using the Duncan's multiple comparison. The threshold cycles (CT values) of the reference sample were 24.6 and 25.3 for *MAPK*p38 and *NF-κB*p65, respectively.

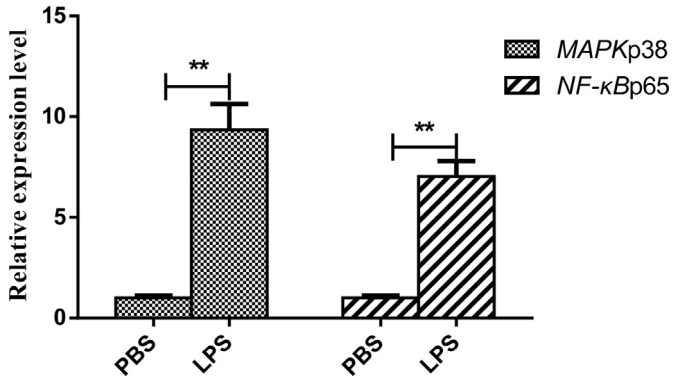

Fig. 6. Transcriptional regulation of *MAPK*p38, and *NF-κB*p65 in intestine post LPS (4 mg/kg body weight) challenge compared to the control (PBS). PBS = phosphate-buffered saline; LPS = lipopolysaccharide. The data were showed as means ± SE ($n = 6$). Two asterisks (**) indicate a significant difference by the LSD test ($P < 0.01$) between the two groups in the same gene.

alignments only in N-terminal RHD sequences showed 15% to 20% higher than alignment in entire CDS (Fig. 2). Thus, the unconserved C-terminal side might be owed to distant gene evolutionary relationship of *NF-κB*p65 and might make its new functions in different animals. A NLS motif had been located behind of RHD in yellow catfish. The *NF-κB*p65 is the catalytic subunit of *NF-κB* involved in response to infection, inflammation and stress (Zhong et al., 1998). Upon stress, the IκB, which was bound with 2 structures of RHD domain of p65 in cytoplasm, was phosphorylated and exposed NLS motif to enter its nucleus and work on its target genes (Vermeulen et al., 2003; Wang et al., 2016). The present study indicated the RHD in yellow catfish *NF-κB*p65 consist of 2 structural domains: RHD-DNA-binding domain and IPT domain (Fig. 2), which might separately form a mainly beta sandwich like human. Thus, the AA sequences characters implied the intranuclear transcription regulation function of yellow catfish NF-κBp65.

To assess the basal mRNA distribution of *MAPK*p38 and *NF-κB*p65, we performed the qPCR assays in a variety of tissues. Combined the results of tissue distribution with sequence analysis, the potential important functions of *MAPK*p38 and *NF-κB*p65 genes in immune response were partly indicated. Thus, we investigated the expression change of these 2 genes in response to LPS in yellow catfish. The LPS acted as a powerful stimulators of innate immunity in diverse eukaryotic species, such as adult mice (Fukushima et al., 2015) and human (Zhou et al., 2013). Previous studies indicate LPS challenges regulate immune genes expression (Liu et al., 2016) and increase *MyD*88 expression level in the intestine of yellow catfish (Yu et al., 2018). The present result showed that the mRNA abundance of yellow catfish *MAPK*p38 and *NF-κB*p65 in intestine were up-regulated after LPS stimulation (Fig. 6), suggesting that these genes were sensitive to LPS-induced immune response. In the Jian carp intestine, LPS could be recognized by TLR4 and activate the expressions of *MAPK*p38 and *NF-κB*p65 resulting in the production of inflammatory cytokines (Jiang et al., 2015). However, the pattern recognition molecules in yellow catfish should be further confirmed upon LPS injection.

In conclusion, we cloned full-length CDS of *MAPK*p38 and *NF-κB*p65 of yellow catfish. The present data provided evidence that these immune response genes in yellow catfish are structurally conserved and resemble those of other vertebrate orthologs. Moreover, LPS stimulation could activate the transcription of these two genes, which suggests their functions in immune response and inflammatory response in yellow catfish.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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