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Molecular Cloning, Tissue Distribution, and Hypoxia and Ammonia Stress Response of the p38 MAPK of Black Seabream (*Acanthopagrus schlegelii*)

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Key words
Ammonia stress, Black seabream, Hypoxia stress, Molecular cloning, p38 MAPK

ABSTRACT

P38 mitogen activated protein kinase (MAPK) has important effects in inflammation and immune regulation. Understanding the molecular characteristics of p38 MAPK would elucidate the environmental stress resistance of cultured fishes. The full-length 2642 bp cDNA of black seabream (*Acanthopagrus schlegelii*) included a 1083 bp open reading frame that encoded a 360-residue protein. The protein contained a Thr-Gly-Tyr (TGY) double phosphorylation site, an Ala-Thr-Arg-Trp (ATRW) substrate binding site, and a key functional ERK docking (ED) site. Sequence analysis revealed that the p38 MAPK protein of black seabream shared high sequence homology (86–94%) with those of other marine fishes. Quantitative real time-polymerase chain reaction revealed that *p38 MAPK* was expressed in the heart, brain, spleen, gill, head kidney, muscles, kidneys, intestines, and liver, and the expression was highest and lowest in the spleen and brain, respectively. The splenic expression of *p38 MAPK* increased significantly after 6 h of ammonia stress ($P < 0.05$), while its expression in the gills and liver increased significantly after 24 h of ammonia stress ($P < 0.05$). *P38 MAPK* expression increased significantly in the spleen, head kidney, and gills following 12 h of hypoxia stress ($P < 0.05$); however, the expression decreased after 12 h of returning to normoxia. A p38 MAPK was identified and characterized in black seabream, which was highly conserved and expressed in various tissues. Ammonia and hypoxia stress tests revealed that the p38 MAPK of black seabream has important roles in environmental stress responses.

INTRODUCTION

Black seabream farming has been increasing in the coastal areas of China in recent years owing to its delicious flesh, high nutritional value, and rapid growth. Ammonia is an important pollutant in the intensive aquaculture environments in the coastal areas of China. According to the study of Murthy *et al*. (2001), ammonia toxicity causes oxidative stress in fishes and affects their growth. The high breeding density and high temperature of intensive aquaculture practices often cause the oxygen levels in the water to fall below normal. The resulting hypoxia causes shortness of breath, oxidative stress, subsequent head floating, and can eventually be fatal (Raaij *et al*., 1996). Previous studies have demonstrated that the stress caused by low oxygen and high ammonia levels can damage the immune and antioxidant functions of black seabream and promote inflammation (Shi *et al*., 2019, 2020). However, the mechanism underlying the damage caused by ammonia and hypoxia stress remains poorly understood to date.

Mitogen-activated protein kinases (MAPK) are a class of intracellular serine/threonine protein kinases, which have important effects in cellular responses to extracellular stimuli (Ono and Han, 2000). The MAPK superfamily comprises four subfamilies, namely, the extracellular regulated kinase (ERK), big MAPK1 (BMK1), C-Jun N-terminal kinase (JNK), and activated protein kinase (SPAK), and p38 MAPK subfamilies, which are involved in different signaling pathways (Garrington and Johnson, 1999; Mahtani *et al*., 2001). Of these, the p38

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MAPK signaling pathway affects a variety of intracellular responses and plays a significant role in the generation of inflammation and response to environmental stress (Raingeaud *et al*., 1995; Regan and Cohen, 2009; Huang *et al*., 2011). It has been demonstrated that the activation of the p38 MAPK signaling pathway contributes to the development of inflammation (Herlaar and Brown, 1999) and plays a role in immune regulation by acting on toll-like receptors (Li *et al*., 2013; Yee and Hamerman, 2013). The production and release of various inflammatory factors, including, pro-inflammatory factors, chemokines, growth factors, cyclooxygenase-2, interleukin (IL)-10, and other factors, depend on the regulation of p38 MAPK, which is in turn activated by various inflammatory factors, such as tumor necrosis factor (TNF)-α, IL-1, and IL-31(Waetzig *et al*., 2002). The expression of the *p38 MAPK* gene can also be induced by heat and cold stress as well as microbial infections (Mizoguchi *et al*., 1996). A variety of algae can alleviate inflammation and stress by downregulating the expression of the *p38 MAPK* gene (Nakamura *et al*., 2006; Kim *et al*., 2011; Sanjeewa *et al*., 2017). These findings suggest that the stress and inflammation response of black seabream could be in connection with the expression of the *p38 MAPK* gene.

To date, the majority of studies on p38 MAPK have primarily focused on yeasts, mammals (Wilsbacher *et al*., 1999), and fishes (Hansen and Jørgensen, 2007; Cai *et al*., 2011; Zhang *et al*., 2019); however, there is a scarcity of studies on the complete sequence and functions of the *p38 MAPK* gene of black seabream. Therefore, the full-length *p38 MAPK* gene of black seabream was cloned and subjected to functional analysis, following which the changes in gene expression under ammonia and hypoxia stress were also investigated. The results obtained here provide valuable insights for understanding the mechanism underlying the immunity and stress response of black seabream via the p38 MAPK pathway.

MATERIALS AND METHODS

Fish sampling

The samples of black seabream, with a mean body weight of 33.82 g, were kept in fishing nets in offshore rafts (2.5 × 2.5 × 2.5 m) at a water temperature of 24–33°C, dissolved oxygen (DO) level of 7.2 mg L^{-1}, pH range of 7.6–8.0, and under a dark/light cycle of 12 h/12 h. A commercial diet was used twice a day at 6:30 a.m. and 17:30 p.m. to feed fishes, for 2 weeks. After 2 weeks, six black seabream were randomly selected, and their heart, brain, spleen, gills, head kidney, muscle, kidney, intestine, and liver tissues were collected for cloning and analyzing the tissue distribution of *p38 MAPK*.

Molecular cloning of p38 MAPK

The total RNA was extracted according to the manufacturer's protocol and the quality of the total RNA was assessed. A 1 µg aliquot of total RNA was subsequently reverse transcribed into cDNA using a PrimeScript RT Reagent Kit. A part of the cDNA sequence of *p38 MAPK* was obtained from the transcriptome of black seabream, which was obtained in our laboratory by high-throughput DNA sequencing. The 5′-rapid amplification of cDNA ends (RACE) and 3′-RACE were used to obtain the full-length cDNA of *p38 MAPK*, according to the method described by Zhang *et al*. (2016). The primers used for cloning the cDNA of *p38 MAPK* with RACE are enlisted in Table I.

Table I. Primers used for cloning and analyzing *p38 MAPK* expression.

Application	Sequence (5' → 3')
RACE	F1 AAGGCGATATGGGAAGTGC
	R1 TCTCGGCTCTCAAAGCTCTG
5'- RACE	GSP-51 CGGAGTCGTGCAGCCTGCCTTAAAC
	GSP-52 TTGTTCCCCTCTCCGCTTGCTTTCA
3'- RACE	GSP-31 TTCATCGGTGCCAACCCACAAGC
	GSP-32 TGGACACAGACAAACGGATAACAGCAGC
p38 MAPK	F -AGACGATATGGGAAGTCCCG
	R TGGCGTGGATGATGGACTG
β-actin	F ACAGTGCCCATCTATGAAGGCT
	R GGCTGTGGTGGTGAAGGAGTAG

Sequence and phylogenetic analyses

The full-length nucleotide and amino acid sequences of the cDNA of the cloned gene were analyzed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for determining whether the cloned gene was indeed the target gene. The sequence homologues of the p38 MAPK protein of black seabream were determined, and the cDNA sequences, open reading frame (ORF), and predicted sequence of the encoded protein were analyzed using the DNAMAN software. A multiple sequence alignment of the p38 MAPK protein sequences of black seabream and other fishes was subsequently constructed. A phylogenetic tree was constructed using the Neighbor-Joining method in MEGA7.0 software with 1000 bootstrap replicates for ensuring accuracy.

Ammonia stress tests

A total of 24 black seabream were selected for the ammonia stress experiments following adaptation and 24 h of fasting. For the ammonia stress test, NH_4Cl was slowly added until the concentration of ammonia in the bucket was 35 mg L^{-1}, as described in the study by Shi *et*

al. (2020). The liver, gill, spleen, and head kidney were collected before ammonia stress (0 h) and at 6 h, 12 h, and 24 h after stress from six fishes selected randomly at each time point, and subsequently stored at -80°C in a refrigerator for further analyses.

Hypoxia stress tests

Following adaptation and 24 h of fasting, 24 black seabream were randomly selected for the hypoxia stress experiments. Based on literature reports (Shi *et al*., 2020), the DO in the water was reduced from 7.12 mg L^{-1} to 2.5 mg L^{-1} within 30 min by introducing nitrogen into the bucket, and the DO was maintained at 2.5 mg L^{-1} for 12 h by adjusting the amount of injected air and flushed nitrogen. The nitrogen charge was ceased after 12 h of hypoxia stress. The level of DO in the water was gradually increased to normal after approximately 35 min, and normoxia was maintained for 12 h. The liver, gills, spleen, and head kidneys were collected before hypoxia stress (0 h), after 6 h and 12 h of hypoxia stress, and after 12 h of recovery under normoxia, from six black seabream selected randomly at each time point, and the tissues were stored at -80°C in a refrigerator.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The primers used for qRT-PCR are enlisted in the Table I. The primers were designed based on the nucleotide sequence using the Primer 5.0 software (PREMIER Biosoft International, San Francisco, CA, USA). The qPCR analyses were performed using a Bio-Rad CFX Connect System (Bio-Rad, Hercules, CA, USA). The system and cycling conditions used for qPCR have been previously described in the study by Zhang *et al*. (2019). The relative gene expression levels were normalized to those of avian β-actin using the $2^{-\Delta\Delta Ct}$ method.

Statistical analyses

The statistical analyses were performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). All the data were statistically analyzed by one-way analysis of variance (ANOVA) for multiple comparisons among groups, and assessed by Duncan's multiple range tests. The differences were considered to be statistically significant at $P < 0.05$. Data were showed as the mean and pooled standard error of mean (SEM).

RESULTS

Cloning and characterization of p38 MAPK

As depicted in Figure 1, the *p38 MAPK* cDNA of black seabream was 2642 bp long, and comprised a 277 bp 5′-untranslated region, a 1083 bp ORF, and a 1282 bp 3′-untranslated region. The 3′-terminal contained a poly A tail, but lacked a polyadenylate AATAA plus tail signal. The ORF of the *p38 MAPK* gene of black seabream encodes a protein of 360 amino acids with an estimated molecular mass of 41.6 KDa and a theoretical isoelectric point of 5.63. Conserved domain analysis revealed that the p38 MAPK protein of black seabream contains a conserved serine/threonine protein kinase catalytic region (S-TKc) structural domain, which possibly belongs to the protein kinase c (PKc) superfamily.

Fig. 1. The cDNA and deduced protein sequences of black seabream. The start (ATG) and stop (TAA) codons are indicated in bold, the MAPK superfamily homology domain of p38 MAPK is shaded in gray, and the predicted TGY phosphorylation motif and ATRW substrate-binding site are indicated by black boxes.

Homology and phylogenetic analyses of p38 MAPK

A multiple sequence alignment of the p38 MAPK proteins of black seabream, spiny-spined gill seabream, spotted grouper, sea bass, zebrafish, human, and protochickens revealed a high degree of sequence conservation in the proteins, as depicted in Table II and Figure 2. Sequence comparison further revealed highly conserved in the Thr-Gly-Tyr (TGY) bisphosphorylation site, substrate Ala-Thr-Arg-Trp (ATRW) binding site, and the ERK docking (ED) site of the aforementioned p38 MAPK proteins.

The phylogenetic tree was constructed and the sequence numbers used are shown in Tables III. The results of phylogenetic tree analyses (Fig. 3) revealed that the p38 MAPK protein of black seabream shared highest similarity with the p38 MAPK protein of *Acanthochromis polyacanthus*, with 94% homology.

Table II. Sequence identity between the p38 MAPK protein of black seabream and those of other organisms selected for constructing the phylogenetic tree.

Scientific name of organism	Amino acid sequence identity (%)
Acanthochromis polyacanthus	94
Epinephelus coioides	92
Dicentrarchus labrax	88
Homo sapiens	88
Danio rerio	86
Gallus sp.	86
Crotalus adamanteus	85
Anolis carolinensis	85
Rattus norvegicus	84
Mus musculus	84
Canis lupus familiaris	84
Felis catus	84
Ailuropoda melanoleuca	84
Oryctolagus cuniculus	84
Sus scrofa	84
Pongo abelii	84
Xenopus tropicalis	83
Litopenaeus vannamei	73
Bemisia tabaci	73
Penaeus japonicus	72
Nasonia vitripennis	72
Apis cerana cerana	72
Acromyrmex echinatior	72
Camponotus floridanus	72
Harpegnathos saltator	72
Danaus plexippus	71
Bombyx mori	71
Aedes aegypti	70
Crassostrea gigas	69
Sarcophaga crassipalpis	68
Drosophila melanogaster	66
Larimichthys crocea	58
Saccharomyces cerevisiae	50

The p38 MAPK protein of black seabream shared 92%, 88%, and 86% sequence homology with the p38 MAPK of spotted grouper (*Epinephelus analogus*), sea bass (*Dicentrarchus labrax*), and zebrafish (*Danio rerio*), respectively, and 58% sequence homology with the p38 MAPK of rhubarb (*Larimichthys crocea*). However, the p38 MAPK protein of black seabream was most distant to that the p38 MAPK of yeast (*Saccharomyces cerevisiae*), with a similarity of 50%. Phylogenetic analysis revealed that the p38 MAPK of black seabream clustered with the p38 MAPK of other vertebrates and was closely related to the p38 MAPK of the spiny chromis (*Acanthochromis polyacanthus*).

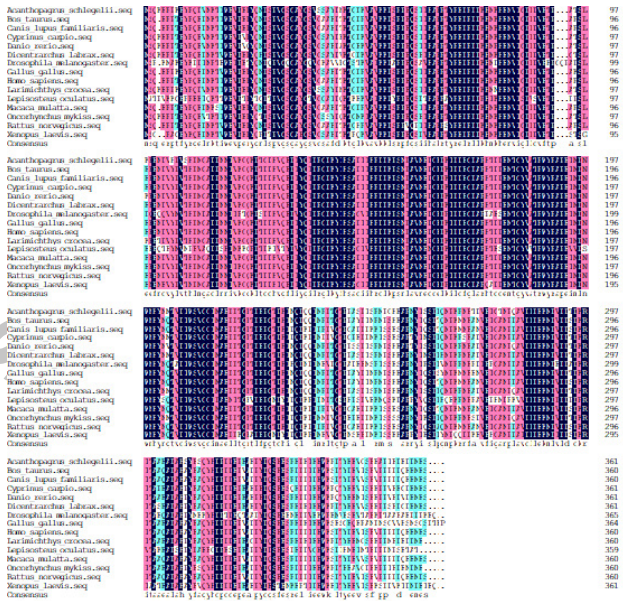

Fig. 2. Multiple sequence alignment of the p38 MAPK protein of black seabream and other species.

Tissue distribution of p38 MAPK

The expression of p38 MAPK mRNA in the different tissues of black seabream is depicted in Figure 4. P38 MAPK mRNA was expressed in the heart, brain, spleen, gill, head kidney, muscles, kidneys, intestines, and liver tissues of black seabream, but a higher expression of p38 MAPK mRNA was observed in the spleen and head kidney, while the expression was relatively low in the heart and brain.

Expression of p38 MAPK mRNA following ammonia stress

The effect of ammonia stress on the expression of p38 MAPK mRNA in the spleen, head kidney, gills, and liver tissues of black seabream is depicted in Figure 5. The relative expression levels of p38 MAPK mRNA were upregulated in the spleen, head kidney, gills, and liver tissues of black seabream following ammonia stress ($P <$

0.05). The findings also revealed that compared to that in the pre-stress (0 h) condition, the splenic p38 MAPK expressed significantly higher after 6 h and 12 h of ammonia stress, while p38 MAPK in the head kidney expressed significantly higher after 12 h of ammonia stress.

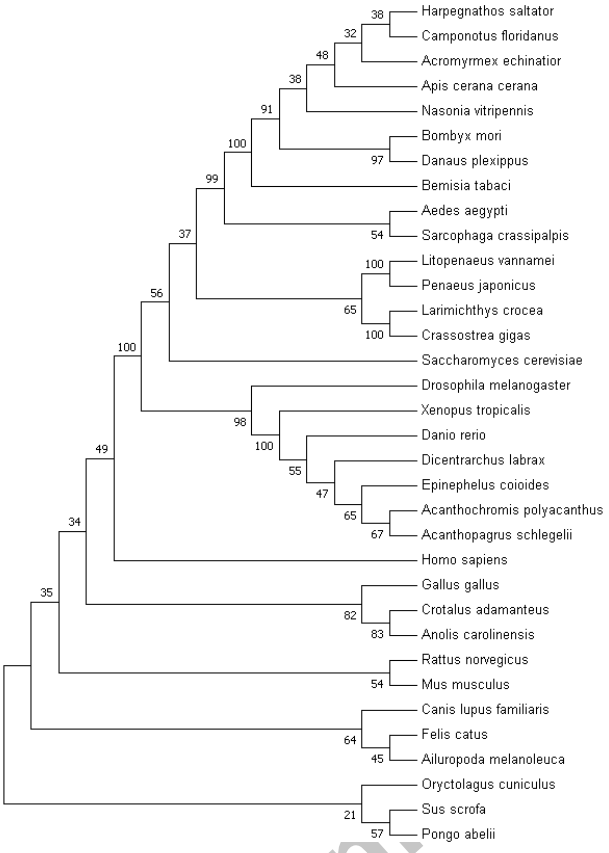

Fig. 3. Phylogenetic tree of the p38 MAPK proteins of black seabream and other organisms.

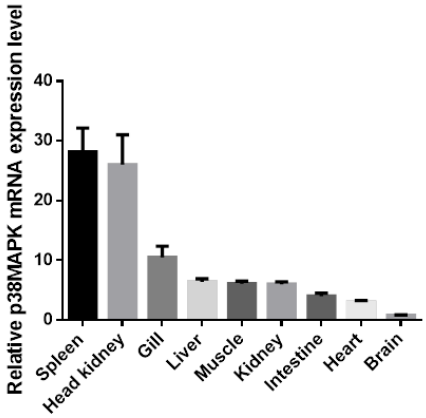

Fig. 4. Relative expression of p38 MAPK mRNA in the different tissues of black seabream.

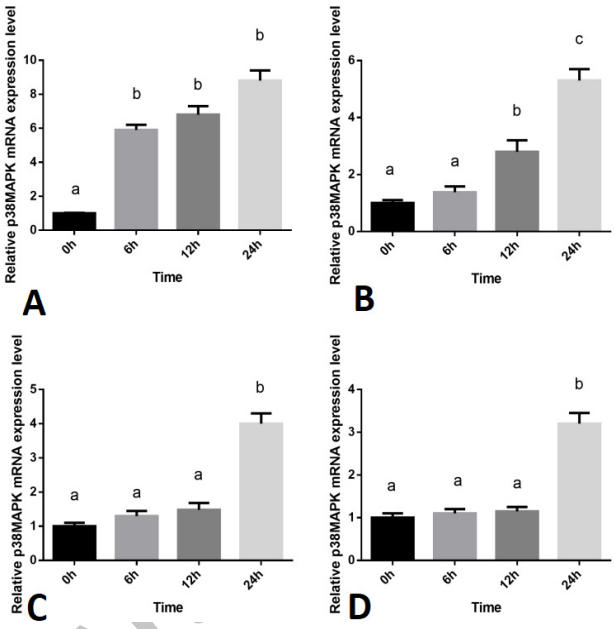

Fig. 5. Relative mRNA expression levels of p38 MAPK in the (A) spleen, (B) head kidney, (C) gills, and (D) liver tissues of black seabream at different time points under ammonia stress.

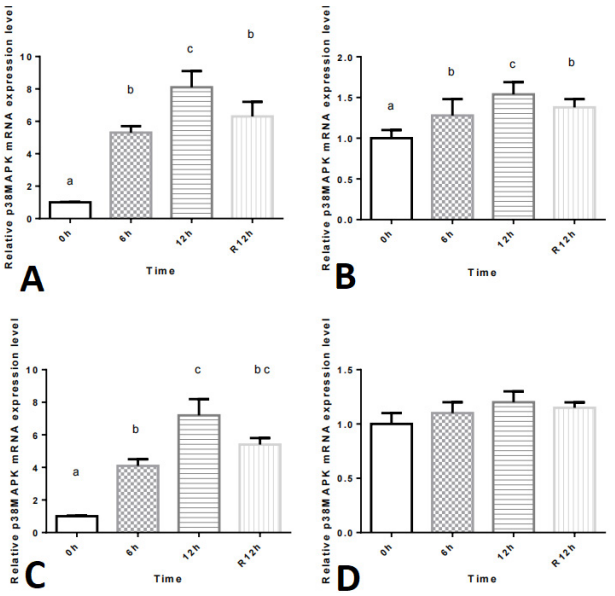

Fig. 6. Relative mRNA expression levels of p38 MAPK in the (A) spleen, (B) head kidney, (C) gills, and (D) liver tissues of black seabream at different time points under hypoxia stress.

Expression of p38 MAPK mRNA after hypoxia stress

The effect of hypoxia stress on the mRNA expression of p38 MAPK in the spleen, head kidney, gills, and liver

tissues of black seabream is depicted in Figure 6. The relative expression levels of p38 MAPK mRNA in the spleen, head kidney, and gills of black seabream were upregulated following hypoxia stress, and the expression was highest after 12 h of hypoxia stress ($P < 0.05$). The expression of p38 MAPK decreased in the spleen and head kidney after 12 h of recovery, and was significantly lower than that after 12 h of hypoxia stress ($P < 0.05$). The findings also revealed that hypoxia stress and post-stress recovery had no effect on the hepatic expression of p38 MAPK ($P > 0.05$).

Table III. Information of the p38 MAPK from other species used for phylogenetic tree construction.

Species	Gene	Accession number
Litopenaeus vannamei	*p38 MAPK*	AFL70597.1
Penaeus japonicus	*MAPK 14*	BAK78916.1
Harpegnathos saltator	*MAPK 14B*	EFN89763.1
Bombyx mori	*p38 MAPK*	NP_001036996.1
Danaus plexippus	*p38 MAPK*	EHJ76051.1
Camponotus floridanus	*MAPK 14B*	EFN66664.1
Apis cerana cerana	*p38 MAPK*	ADT91683.1
Acromyrmex echinatior	*MAPK 14B*	EGI59042.1
Aedes aegypti	*p38 MAPK*	XP_001653240.1
Nasonia vitripennis	*p38 MAPK*	NP_001136337.1
Bemisia tabaci	*p38 MAPK*	AEA92685.1
Dicentrarchus labrax	*MAPK 14a*	CBN80893.1
Sarcophaga crassipalpis	*p38 MAPK*	BAF75366.1
Xenopus tropicalis	*MAPK 14*	NP_001005824.1
Epinephelus coioides	*p38a MAPK*	AEU04194.1
Danio rerio	*MAPK 14b*	AAH63937.1
Crotalus adamanteus	*MAPK 14-like*	AFJ50620.1
Gallus	*MAPK 14 isoform X1*	XP_001232616.1
Felis catus	*MAPK 14 isoform X1*	XP_003986110.1
Ailuropoda melanoleuca	*MAPK 14 isoform X1*	XP_002914341.1
Canis lupus familiaris	*MAPK 14*	NP_001003206.1
Crassostrea gigas	*MAPK 14*	EKC29510.1
Sus scrofa	*MAPK 14 isoform X1*	XP_001929525.3
Anolis carolinensis	*MAPK 14 isoform X2*	XP_003226523.1
Rattus norvegicus	*p38 MAPK*	AAC71059.1
Pongo abelii	*MAPK 14 isoform X1*	XP_002816848.1
Oryctolagus cuniculus	*MAPK 14 isoform X1*	XP_002714691.1
Mus musculus	*MAPK 14 isoform 1*	NP_036081.1
Drosophila melanogaster	*p38a MAPK*	AAC39030.1
Homo sapiens	*MAPK 14 isoform 1*	NP_001306.1
Saccharomyces cerevisiae	*HOG1 protein*	AAA34680.1
Larimichthys crocea	*MAPK 14A*	XP_010737771.1
Acanthochromis polyacanthus	*MAPK 14A-like transcript variant X2*	XM_022203546.1

DISCUSSION

MAPKs are a class of intracellular serine/threonine protein kinases that transmit extracellular signals into the intracellular compartment via a phosphorylation reaction cascade and had a vital role in cellular responses to extracellular stimuli (Ono and Han, 2000). The MAPK superfamily comprises four subfamilies that are involved in different signaling pathways, of which the p38 MAPK signaling pathway had significant effects in stress and inflammation (Nakamura *et al*., 2006; Kim *et al*., 2011; Sanjeewa *et al*., 2017).

In this study, primers were designed according to the conserved sequence of p38 MAPK of marine fishes, and the *p38 MAPK* gene of black seabream was cloned by qRT-PCR and RACE to obtain the full length 2642 bp cDNA, containing a 1083 bp ORF that encodes a protein of 360 residues. The findings revealed that a high sequence similarity between the *p38 MAPK* gene of black seabream and those of Atlantic salmon (Hansen and Jørgensen, 2007), spotted grouper (Cai *et al*., 2011), groupers (Zhang *et al*., 2019), and silver carp (Li *et al*., 2016), which suggested that the *p38 MAPK* gene is highly conserved in fishes. Further analysis revealed that the cloned p38 MAPK protein of black seabream contained a highly conserved S-TKc domain and three highly conserved sites, including the TGY (Thr-Gly-Tyr) double phosphorylation site, ATRW substrate binding site, and ED site, which mediate the functions of p38 MAPK (Akella *et al*., 2008; Sheridan *et al*., 2008; Robert, 2012). The findings strongly suggested that the functions of the p38 MAPK protein of black seabream cloned in this study are similar to those of the p38 MAPK of other species of fish. The results of sequence homology analysis demonstrated that the amino acid sequence of the p38 MAPK of black seabream cloned herein shared high homology (86–94%) with the p38 MAPK of other marine fishes. Phylogenetic analysis also demonstrated that the cloned p38 MAPK of black seabream was closely related to the p38 MAPK of spiny chromis. Altogether, the findings indicated that the p38 MAPK of black seabream cloned belongs to the p38 MAPK family, which is highly conserved in fishes.

The *p38 MAPK* gene of black seabream was found to be expressed in all the tissues examined, with highest expressed in the spleen, head kidney, and gill. Results here were consistent with the findings of the study by Zhang *et al*. (2019). Spleen and head kidneys are important immune organs in fishes (Bromage *et al*., 2004; Zwollo *et al*., 2008), and the high expression of p38 MAPK in both these immune organs indicated that the p38 MAPK of black seabream has immune-related functions. The findings further revealed that the expression of p38 MAPK was also high in the gills, which could be attributed to the ion regulatory function of p38 MAPK. Marshall *et al*. (2017) reported that p38 MAPK are distributed throughout ionocytes, especially in regions lacking the sodium potassium chloride cotransporter (NKCC). It has also been demonstrated that osmotic stress can upregulate the gene expression of *p38 MAPK* and activate the p38 MAPK signaling pathway (Takei and Hwang, 2016).

The levels of ammonia and DO are important factors that affect the aquatic environment of fishes. Ammonia toxicity causes oxidative stress in fishes and affects their growth (Murthy *et al*., 2001). Hypoxia causes shortness of breath, oxidative stress, subsequent head floating, and can eventually lead to fatality (Raaij *et al*., 1996). The p38 MAPK protein had significant effects in regulating the inflammatory response, environmental stress, and immune response via lymphocytes and macrophages (Raingeaud *et al*., 1995; Regan *et al*., 2009; Huang *et al*., 2011). It has been reported that heat and cold stress as well as microbial infections can induce the expression of the *p38 MAPK* gene (Mizoguchi *et al*., 1996). Therefore, the expression of the *p38 MAPK* gene was investigated in this study following individual exposure to hypoxia and ammonia stress. The study demonstrated that both hypoxia and ammonia stress increased the expression of p38 MAPK, which suggested that the p38 MAPK of black seabream plays a role in the environmental stress response. The findings revealed a tissue-specific variability in the hypoxia and stress response, and the spleen and head kidneys were the most sensitive to these stressors, while the gills and liver were the least sensitive. The tissue distribution of p38 MAPK in black seabream observed in this study was similar to that observed in a previous report on blunt snout bream (Zhang *et al*., 2019). As the spleen and head kidney of fishes are important immune organs, the p38 MAPK-mediated stress response of black seabream could be mediated via inflammatory. Previous studies have in fact demonstrated that p38 MAPK can partake in immunomodulatory responses via Toll-like receptors (Li *et al*., 2013; Yee and Hamerman, 2013).

The present study further revealed that individual exposure to hypoxia and ammonia stress upregulated the splenic expression of *p38 MAPK* at an early stage of stress (6 h), which was possibly mediated by the intracellular inflammatory factor, TNF-α. Stress causes an inflammatory response in the body and triggers cells to secrete TNF-α, which activates p38 MAPK and the p38 MAPK signaling pathway (Waetzig *et al*., 2002). The body also produces large quantities of reactive oxygen species under stress, which can directly oxidize the cysteine residues of MAPK

signaling molecules to activate MAPK and upregulate the expression of *p38 MAPK* (Day and Veal, 2010).

CONCLUSION

The present study identified and characterized a p38 MAPK in black seabream (*Acanthopagrus schlegelii*), which was highly conserved and expressed in various tissues. The ammonia and hypoxia stress tests revealed that the p38 MAPK of black seabream plays a vital role in the environmental stress response of this species. The study provides important insights for further investigation of the mechanism underlying the immune stress resistance of black seabream via p38 MAPK.

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IRB approval

The animal experiments performed in this work were in strict accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and experimental protocol and procedure authorized by the Animal Care and Use Committee of Yibin Vocational and Technical College (AEC-YVTC-20220106).

Ethical statement

This study was conducted in accordance with the requirements of the National Research Council's Guide for the Care and Use of Laboratory Animals and was approved by the Animal Care and Use Committee of Yibin Vocational and Technical College.

Statement of conflict of interest

The authors have declared no conflict of interest.

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