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Short Communication

Molecular characterization of a p38 mitogen-activated protein kinase gene from *Scylla paramamosain* and its expression profiles during pathogenic challenge

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ABSTRACT

A novel p38 MAPK gene from *S. paramamosain* was cloned and characterized by rapid amplification of cDNA ends (RACE) technology. *S. paramamosain* p38 (*Sp*-p38) MAPK gene consists of an open reading frame of 1095 bp encoding a 365-amino-acid protein, which showed close phylogenetic relationship to *Litopenaeus vannamei* p38 MAPK. The tissue distribution patterns showed that *Sp*-p38 MAPK was widely expressed in all examined tissues, with the highest expression in hemocytes and intestines. The expression levels of *Sp*-p38 MAPK in hemocytes was up-regulated post-stimulation, which reached the peak at 6 h and 12 h after bacteria (*S. aureus* and *V. harveyi*) and WSSV infection, respectively. In conclusion, our data contributed to define the biological characteristics of *Sp*-p38 MAPK and further demonstrated the critical role of *Sp*-p38 MAPK in vivo during the viral and bacterial infection.

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1. Introduction

Mitogen-activated protein kinases (MAPKs) are a class of evolutionarily conserved serine/threonine protein kinases (Coulthard et al., 2009). MAPK family is composed of three major groups: extracellular signal-regulated kinases (ERKs), C-Jun N-terminal or stress-activated protein kinases (JNKs/SAPKs) and p38 MAPKs (Raman et al., 2007). Among them, p38 MAPKs play a vital role in numerous biological processes including cell differentiation, cell migration, cell cycle regulation, inflammatory response and apoptosis (Hedges et al., 1999; Ono and Han, 2000; Singhal et al., 2002; Yee et al., 2004). Till date, four P38 isoforms, which are P38α, P38β, P38γ and P38δ, have been identified. p38α and p38β express ubiquitously, while p38γ and p38δ are only expressed in certain tissues including muscle, glandular tissues, lung, and kidney (Kumar et al., 2003; Ashwell, 2006; Zhang et al., 2007). Increasing evidences have indicated the activation of p38 MAPK can be activated by various extracellular stimuli, including inflammatory cytokines, environmental stress, UV irradiation and pathogen infections (Cuadrado and Nebreda, 2010; Cai et al., 2011; He et al., 2013; Yan et al., 2013; Zhu et al., 2014). p38 MAPK is activated through simultaneous phosphorylation of the Thr180 and Tyr182 in the conserved Thr-Gly-Tyr (TGY) motif by its upstream MAPK kinase (Gong et al., 2010).

The activation of p38 MAPK induces the expression of various pro-inflammatory cytokines such as IL-1 and TNF-α and initiates innate immune responses (Hansen and Jørgensen, 2007). In *Litopenaeus vannamei*, p38 MAPK can be activated by bacterial pathogen (e.g. *V. parahaemolyticus*, *V. alginolyticus* and *S. aureus*) stimuli (Yan et al., 2013). Previous studies have demonstrated that p38MAPKs are also activated during mammalian viral infections, and that these play important roles during viral replication *in vitro* (Johnson et al., 2000; Banerjee et al., 2002; Hirasawa et al., 2003). During iridovirus infection, p38 MAPK from *Epinephelus coioides* plays a crucial role in the regulation of virus replication (Cai et al., 2011; Huang et al., 2011). These results indicate the p38 MAPK pathway play a critical role in the defense against bacterial or viral infection.

Mud crabs (*Scylla paramamosain*) are mainly distributed throughout the Indo-Pacific and Indian Ocean regions (Keenan et al., 1998) as an important farmed species in the southeast coasts of China. However, bacterial and virus infections are threatening the survival of mud crabs with high culture densities, causing high mortality and significant economic loss (Escobedo Bonilla et al.,

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2008). Compared to mammals and shrimps, information on the p38 MAPK signaling pathway in response to bacterial and viral infection in *S. paramamosain* is limited. In this study, a novel p38 MAPK gene (designated *Sp*-p38) was cloned and characterized from *S. paramamosain*, and its roles in response to bacterial and viral infections were also investigated. The results presented here will provide important information on *Sp*-p38 MAPK defense against pathogen and on the role of the p38 pathway in innate immunity in *S. paramamosain*.

2. Materials and methods

2.1. Animals and pathogens

Healthy adult mud crabs (body weight:220 g ± 15.6 g) were purchased from the commercial crab farm in Chongming county (Shanghai, China) and maintained in 300L tanks supplied with a continuous flow of aerated seawater (salinity 1.0%, temperature 18–20 °C, pH 8.0–8.2) for a week.

S. aureus, *V. harveyi*, and WSSV were conserved in Aquatic Animal Disease Lab, East China Sea Fisheries Research Institute. *S. aureus* and *V. harveyi* were grown overnight in Luria-Bertani (LB) media at 37 °C and 28 °C, respectively. Afterwards, the bacteria were collected by centrifugation at 2000*g* for 20 min, washed twice with Phosphate Buffer Saline (140 mM NaCl, 10 mM sodium phosphate, pH 7.4) medium and resuspended herein at a density of 10^9 CFU/ml (McFar Land). The WSSV inoculum was extracted from gills of *Penaeus chinensis* infected with WSSV. Gill tissue was homogenized with a glass homogenizer, diluted to 1:5 with PBS, followed by freeze-thawing thrice and then centrifuged. Supernatant was diluted to 10^7 viral particles per milliliter and quantified as described previously (Wang et al., 2009).

2.2. Rapid amplification of cDNA ends (RACE)

Total RNA was extracted from the *S. paramamosain* tissues using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The integrity and concentration of the RNA was assessed by gel electrophoresis and spectrophotometer. cDNA was synthesized in a 25 μl reaction volume containing 4 μg RNA according to the M-MLVRTase cDNA Synthesis Kit (Takara, Japan) protocol with Oligo-anchor primer.

A pair of primers: *Sp*-P38KF and *Sp*-P38KR was designed to amplify the partial fragment of *Sp*-38 MAPK. The cDNA ends were obtained according to the SMART RACE cDNA Amplification Kit (Clontech, America) protocol. A pair of primers: *Sp*-P38KF1 and anchor R was used to amplify the 3′ end of *Sp*-38 MAPK cDNA. PCR was carried out at the following program: 95 °C for 3 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min, followed by a 10 min extension at 72 °C. The products were extracted and inserted into pMD18-T prior to sequencing by Sunny Biotechnology Co., Ltd, Shanghai. The full-length cDNA sequence of *Sp*-p38 MAPK was obtained by overlapping these two fragments (Table S1).

2.3. Sequence and bioinformatic analysis

General characteristics of the complete cDNA sequence of *Sp*-p38 MAPK and the deduced amino acid sequences were analyzed by DNAstar. The similarities of *Sp*-p38 MAPK with other p38 MAPKs were analyzed by NCBI BLAST program (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). The theoretical isoelectric point (pI) and molecular mass of the deduced protein were predicted by ExPASy Compute pI/Mw program (http://web.expasy.org/compute pi/). The putative domains were predicted by Smart program (http://smart.embl-heidelberg.de/) and SignalP (http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptide. Multiple sequence alignments were generated using the DNAMAN version 5 (Lynnon Corporation, Vandreuil, QC, Canada). A neighbor-joining (NJ) phylogenic tree was constructed using MEGA 6 software based on the deduced amino acid sequences of the related genes in typical species. Bootstrap sampling was reiterated 1000 times.

2.4. Tissue distribution analysis of Sp-p38 MAPK

To investigate expression profiles of *Sp*-p38 MAPK in nine different tissues including hemocytes, heart, hepatopancreas, gills, stomach, intestines, muscle, connective tissue and gonad from *S. paramamosain*, a pair of primer: *Sp*-p38RF and *Sp*-p38RR was used to amplify a 145 bp *Sp*-p38 MAPK fragment, and a pair of primers: *Sp*-18SF and *Sp*-18SR, based on published GenBank *S. paramamosain* 18S gene sequence (FJ646616.1) was designed to use as internal control. RT-qPCR was performed to study the tissue distribution of *Sp*-p38 MAPK, using the cDNA synthetized from the total RNA extracted from the mentioned nine tissues as templates. The RT-qPCR was performed in a volume of 20 μl containing 10 μl 2 × SYBR Premix Ex Taq, 2 μl cDNA, and 4 μl each primer (1 μM). The RT-qPCR was programmed as follows: 95 °C for 10 min, 40 cycles at 95 °C for 10 s and 60 °C for 60 s, and a melt from 60 °C to 95 °C. All reactions were performed in triplicates using individual templates. The relative expression levels of *Sp*-p38 MAPK in different tissues were calculated according to $2^{-\triangle\triangle CT}$. Significant differences were determined using one-way analyses of variance and the Duncan's test for multiple range comparison, with significant levels accepted at $p < 0.05$.

2.5. Expression profiles of Sp-p38 MAPK after challenge with pathogens

The expression profiles of *Sp*-p38 MAPK after pathogen challenge were investigated by injecting 50 μl of *V. harveyi* (1.0×10^6 CFU/μl), or *S. aureus* (1.0×10^6 CFU/μl) suspension, or WSSV inocula (1.0×10^4 copies/μl) into the base of the right fifth leg of each crab. The corresponding control was challenged with 50 μl of PBS (140 mM NaCl and 10 mM sodium phosphate; pH 7.4) or normal tissue extracts. The hemolymph was collected from each crab at 0, 2, 6, 12, 24, 48 h post-injection, and mixed with an equal volume of pre-cooled anticoagulant solution (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, and 10 mM EDTA, pH 4.6). Subsequently hemolymph was centrifuged at 800*g* at 4 °C for 15 min, and hemocyte pellets were immediately suspended in Trizol for RNA extraction. For each sample, three crabs were selected to eliminate individual differences.

Total RNA was extracted to synthetize the cDNA and the expression levels were detected by RT-qPCR with the same primers (Table S1) and methods described previously. All RT-qPCR experiments were performed in accordance with the MIQE guidelines (Table S1) (Bustin et al., 2009).

3. Results and discussion

3.1. Cloning and sequence analysis of Sp-p38 MAPK gene

The 720 bp 5′ end fragment of *Sp*-p38 MAPK obtained from sequencing was confirmed by PCR using the cDNA from hemocytes. Based on the partial cDNA sequence of *Sp*-p38 MAPK, the 3′ ends of the complete sequence were obtained through the RACE technology with one pair of specific primers (*Sp*-p38KF1 and anchor R). Through the overlapping of the two fragments, full-

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