Fish & Shellfish Immunology 61 (2017) 24–33

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

A novel MKK gene (*AjMKK3/6*) in the sea cucumber *Apostichopus japonicus*: Identification, characterization and its response to pathogenic challenge

Yi Wang, Ge Chen, Kaiquan Li, Jingxian Sun, Jian Song, Yaoyao Zhan**, Xiangxiang Zhang, Limeng Yang, Yaqing Chang*

Key Laboratory of Mariculture & Stock Enhancement in North China's Sea, Ministry of Agriculture, Dalian Ocean University, Dalian, Liaoning, 116023, PR China

ARTICLE INFO

Article history:
Received 29 September 2016
Received in revised form
11 December 2016
Accepted 13 December 2016
Available online 14 December 2016

Keywords:
Apostichopus japonicus
Mitogen-activated protein kinase kinase 3/6
Expression analysis
Vibrio splendidus infection

ABSTRACT

The mitogen-activated protein kinase kinases (MKKs) are key components of MAP kinase (MAPK) cascades and function as redox-regulated signaling factors in pathological and physiological processes. In this study, we identified a novel MKK3/6 gene in the sea cucumber *Apostichopus japonicus* (designated as *AjMKK3/6*) by transcriptome database mining and rapid amplification of cDNA ends (RACE) approaches. Sequence analysis and protein structure prediction showed that AjMKK3/6 is highly conserved as compared to those from other invertebrate and vertebrate species. Molecular phylogeny result revealed that AjMKK3/6 exhibited a closest relationship with that from *Strongylocentrotus purpuratus*. Quantitative real-time PCR was employed to determine the expression profiles of *AjMKK3/6* in healthy adult *A. japonicus* tissues and in coelomocytes after *Vibrio splendidus* infection *in vivo*, respectively. As results shown, *AjMKK3/6* was ubiquitously expressed in all examined tissues of healthy adult *A. japonicus* with a relative expression level from high to low as body wall > tube feet > coelomocytes > respiratory tree > intestine > longitudinal muscle. Significant expression changes of *AjMKK3/6* in coelomocytes were observed at 12 h- and 72 h-after *V. splendidus* infection, respectively. In general, the current study will enrich our knowledge of characterizations and immno-functions of MKK3/6 in sea cucumbers.

1. Introduction

Mitogen-activated protein kinase (MAPK)signal cascades are conserved and evolutionary old pathways which could transduce extracellular stimuli into multiple intracellular responses [1,2]. Classic MAPK cascades are composed of three modules named as MAPK, MAPK kinase (MKK/MEK), and MAPKK kinase (MKKK/MEKK). It has been well documented that MAPK signal transduction pathways play a very important role in immune response from yeast to human being [3–5]. As for vertebrates, MAPK modules are involved in both innate and adaptive immunity processes [6,7]. For invertebrates, MAPK modules have been demonstrated to be one of vital signal pathways regulating innate immune system, notably in the immune process against pathogenic infection [8,9]. As the hub of the cascade, MKK consisted of seven members (MKK1, MKK2, MKK3, MKK4, MKK5, MKK6 and MKK7) in mammals [10]. Three subfamilies was selectively phosphorylated by MKK, including extracellular signal-regulated kinases (ERKs), Jun N-terminal kinases (JNKs) and the p38 kinase isozymes [2]. To date, members of MAPK, MKK and MKKK genes were cloned and characterized in various species to deepen our understanding of functions of MAPK signal pathways in multi-cellular organisms [11–13].

As one of representatives of marine invertebrate, the sea cucumber *Apostichopus japonicus*, which belongs to Echinodermata, Holothuroidea, Aspidochirota, Stichopodidae, inhabits along the coastal area of Bohai Sea, Yellow Sea, Japan, Korea and the Russian Far East [14,15]. With the increasing awareness of the nutritional and medicinal value of sea cucumbers, the scale of *A. japonicus* aquaculture industry has enlarged year by year since 1980 [16]. While, for the past decades, the frequent outbreak of pathogen-induced diseases have become one of seriously problems that

* Corresponding author.
** Corresponding author.
E-mail addresses: zyydlou@hotmail.com (Y. Zhan), yqkeylab@hotmail.com (Y. Chang).

http://dx.doi.org/10.1016/j.fsi.2016.12.017
1050-4648/

restricted the development of *A. japonicus* industry [17]. Therefore, much more attention has been paid to understand molecular responses and mechanism of host defense against pathogenic challenge in *A. japonicus* [18]. Many immune-related genes were identified, such as nuclear factor kB (NF-kB) [19], Toll-interacting protein (Tollip) [20], transcription factor activator protein-1 (AP-1) [21], TNF receptor associated factor 3 (TRAF3) [22], lipopolysaccharide-binding protein/bactericidal permeability–increasing protein (LBP/BPI) [23]. Several signaling pathways, such as the TLR [24], JAK/STAT [25], TNF-α signaling pathways [26], have been proven to participate in innate immune response to pathogenic infection in *A. japonicus*. However, little is known about MAPK modules or pathways in the aspect of host defense against pathogens in *A. japonicus* so far.

With the attempt to get more information about MAPK modules in *A. japonicus,* transcriptome database mining and rapid amplification of cDNA ends (RACE) approaches were used to clone the full length cDNA of MKK3/6 gene in *A. japonicus* (designated as *AjMKK3/6*). Bioinformatics software was employed to clarify features of sequence and structure of *AjMKK3/6*. The expression profiles of *AjMKK3/6* in healthy adult *A. japonicus* tissues and in *A. japonicus* coelomocytes after *Vibrio splendidus* infection *in vivo* were determined by quantitative real-time PCR (qRT-PCR).

2. Materials and methods

2.1. Samples preparation and bacterial challenge experiment

Healthy adult *A. japonicus* (100–150 g) were collected from Dalian Haibao Fishery Company (Liaoning province, China). Three days prior to experiments, they were maintained in laboratory circulating seawater tanks (~1000 L) at ambient temperature (16–17 °C) without feeding. Tissues of tube feet, coelomocytes, body wall, intestine, respiratory tree, and longitudinal muscle were carefully dissected from 9 healthy animals with 3 mixed individuals as one sample. For coelomocytes collection, the coelomic fluid samples were centrifuged immediately at 800 rpm for 5 min at 4 °C.

To investigate the response of *AjMKK3/6* to pathogenic bacteria infection, coelomic injection with *V. splendidus* was conducted *in vivo* as described by Yang et al. [21,22]. Fifty sea cucumbers were divided randomly and equally to two experimental groups. The control group and the *V. splendidus* challenge group was injected into the cavity of each individual with 100 μL PBS and 100 μL *V. Splendidus* (1×10^7 CFU·mL-1), respectively. Coelomocytes of each individual were harvested at 0, 8, 12, 24, 48, and 72 h post-infection, respectively. Three biological replications were performed for each point.

All tissue samples and coelomocytes were immediately frozen by liquid nitrogen, and then stored at −80 °C before RNA extraction.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was isolated according to the instruction of RNAprep pure Tissue Kit (Tiangen, China). The integrality and concentration of RNA extracted was evaluated by 1.2% agarose gel electrophoresis and spectrophotometer (NanoPhotometer, Munich, Germany), respectively. The cDNA was synthesized using PrimeScript™ RT reagent Kit (TaKaRa, Japan). The PCR reaction system was carried out in a total volume of 20 μL in component with 1000 ng total RNA, 4 μL 5 × PrimeScriptTM buffer, 5 pmol Oligo dT Primer, 100 pmol Random 6 mers and 1 μL PrimeScript RT Enzyme Mix I. The thermal condition of reaction was incubated at 37 °C for 15 min, and followed by a final 5-s denaturalization at 85 °C. All cDNAs were diluted 10 times and stored at −20 °C for subsequent experiments.

2.3. Cloning and sequencing of AjMKK3/6

The expressed sequence tag (EST) was identified from *A. japonicus* transcriptome database. Gene specific primers for *AjMKK3/6* (Table 1) were designed based on the putative unigenes to obtain the full-length cDNA by Primer Premier 5.0. The first strand cDNA for RACE was prepared using the SMARTer® RACE 5′/3′ Kit (TaKaRa, Japan). The PCR reaction system for 5′-RACE and 3′-RACE was conducted following the manufacturer's instructions. Products from RACE were extracted and purified by Quick Gel Extraction kit (Tran, China). Purified RACE products were then ligated into pEASY®-T1 Cloning Vector (Tran, China) and transformed into Trans1-T1 Phage Resistant Chemically Competent Cells (Tran, China). Positive clones were identified by colony PCR using M13 primers (Tran, China). Three independent positive clones were finally sequenced at Sangon (Shanghai, China) for sequence confirmation.

2.4. Bioinformatic analyses of AjMKK3/6

The full-length cDNA sequence of *AjMKK3/6* was blasted against NCBI non-redundant (nr) protein database by translated BLAST (BLASTX, http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify sequence similarities. The open reading frame (ORF) was identified by ORF Finder (http://www.ncbi.nlm.nih.gov/orffinder/). Deduced amino acid sequence and physicochemical properties of AjMKK3/6 protein were analyzed via the Expert Protein Analysis System (http://www.expasy.org/). Domains and the secondary structure of AjMKK3/6 protein were predicted using the simple modular architecture research tool (SMART) online server (http://smart.embl-heidelberg.de/) and PSIPRED v3.3 software (http://bioinf.cs.ucl.ac.uk/psipred/), respectively. Three dimensional (3D) structure modeling of AjMKK3/6 protein was performed using the Swiss-Model Workspace (https://swissmodel.expasy.org/) and then evaluated by Swiss-PdbViewer (version 4.1).

Orthologous sequences of MKK3, MKK6, and MKK3/6 protein from other species, such as *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Danio rerio*, *Xenopus laevis*, etc. were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The amino acid sequences of MKK3/6 protein from these species were aligned by DNAman software (version 6.0). A neighbor-joining (NJ) phylogenetic tree was built using the Molecular Evolutionary Genetics Analysis (MEGA) program (version 5.0). 1000 replications of bootstrapping were used to evaluate the robustness of the phylogenetic tree.

2.5. Expression analysis of AjMKK3/6 by qPCR

qRT-PCR was conducted via the Applied Biosystem 7500 Real-time System (Applied Biosystems, USA) to analyze the expression profiles of *AjMKK3/6*. Cytochrome *b* (Cytb) gene was used as internal control [27]. Primers used in qRT-PCR analyses are listed in Table 1 qRT-PCR was performed in a total volume of 20 μL with 1 μL cDNA, 10 μL of 2 × SYBR Green Master mix (TaKaRa, Japan), 0.4 μL of ROX Reference Dye II, 7 μL PCR grade water and 0.8 μL (10 mM) of each primer (Table 1). The running qPCR program included 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 32 s. At the end of reaction, PCR melting curve analysis was conducted to confirm single PCR products. The relative expression level of *AjMKK3/6* was determined by the comparative $2^{-\Delta\Delta Ct}$ method [28]. The concrete formula was: