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Cloning and gene expression of allograft inflammatory factor-1 (*AIF-1*) provide new insights into injury and bacteria response of the sea cucumber *Apostichopus japonicus* (Selenka, 1867)

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ABSTRACT

Allograft inflammatory factor-1 (AIF-1) is an interferon (IFN)-γ-inducible Ca^{2+}-binding cytokine that associates with the immune defense and inflammatory response. In this study, we reported *AIF-1* gene in sea cucumber *Apostichopus japonicus* (*AjAIF-1*). The full-length cDNA of *AjAIF-1* is 1541 bp with an open reading frame (ORF) of 477 bp encoding 158 amino acids. Two EF-hand Ca^{2+}-binding motifs were found in the deduced *AjAIF-1*. *AjAIF-1* was widely expressed in all tested tissues (body wall, intestine, respiratory tree, tube feet, coelomocytes and longitudinal muscle), with the highest expression in respiratory tree. After *Vibrio splendidus* challenge and physical injury, *AjAIF-1* transcripts were significantly upregulated in coelomocytes. The mRNA expression level of *AjAIF-1* in coelomocytes reached to the highest value at 4 h (3.38-folds vs. the PBS control, $P < 0.05$) post injection. After papilla injury, the mRNA level of *AjAIF-1* in coelomocytes was upregulated, and its peak value was found at 4 h (3.88-folds vs. the control, $P < 0.05$). These results indicated that 1) *AjAIF-1* sensitively responds to pathogen infection; 2) *AjAIF*-1 is involved in acute inflammatory response. Our findings gain general information about the role of AjAIF-1 in the innate immunity of *A. japonicus*.

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1. Introduction

The sea cucumber, *Apostichopus japonicus*, is the most economically important species among the 20 edible species of sea cucumbers in China [1]. High economic value leads to great interest in aquaculture and biological study of this species [2–4]. Until 2012, the production of *A. japonicus* has reached 170, 830 tons in China (Chinese fishery statistical yearbook 2013). During the time of cultivation and transplantation, sea cucumbers are prone to operation injury, followed by bacterial infection [5]. Moreover, various diseases caused by pathogens during breeding and cultivation practices occurred frequently, and the major economic losses have happened [6–8]. So, it's very important to identify and characterize the immune-related genes responded to pathogens in *A. japonicus*.

Like all invertebrates, the absence of adaptive immune system in *A. japonicus* makes them exclusively rely on innate immune system to protect them against threats from pathogens, especially in marine environments [9]. The innate immune system could provide the host immediate defense against infection in a non-specific manner, which also lies behind most inflammatory response [10,11].

Inflammation process is defined as a highly complex and coordinated cellular event, which is induced by tissue injury or microbial infection [12]. The inflammation plays an important part in the function of wound reparation or fight against infection which disturbs the state of homeostasis [13]. In human, the allograft inflammatory factor-1 (AIF-1) was identified as one of the key genes associated with inflammatory response that is encoded within the HLA class III genomic region [14]. The AIF-1 is a 17 kDa interferon (IFN)-γ-inducible Ca^{2+}-binding EF-hand protein, which was originally identified and cloned from activated macrophages from rat cardiac allografts with chronic cardiac rejection [15]. It was initially demonstrated that AIF-1 is a modulator of the immune response during macrophage activation. Follow-up studies proposed that AIF-1 plays a significant role not only in the whole immune defense reactions, but also in host responses to inflammatory stimuli [16,17]. The role of AIF-1 has been well documented in vertebrates such as human [18], rat [19] and sea bream [20]. In invertebrate, the first *AIF-1* gene was isolated from sponge and its transcript was upregulated in cytokine-mediated allogenic responses during wound

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repair and allografts [21]. Recently, the *AIF-1* gene of disk abalone (*Haliotis discus discus*) [22], manila clam (*Venerupis philippinarum*) [23], Antarctic sea urchin (*Sterechinus neumayeri*) [9], pearl oyster (*Pinctada martensii*) [24], and pacific oyster (*Crassostrea gigas*) [25] have been identified and they were found to play vital roles in the immune response to pathogenic infection or tissue injury.

Therefore, the main purposes of the present study are to clone the full-length cDNA of *AjAIF-1* from *A. japonicus*, and to investigate the temporal expression pattern of *AjAIF-1* transcript under bacterial challenge and physical injury. The current work contributes to understanding of the role of AjAIF-1 in *A. japonicus* innate immunity, and provides new insights into injury and bacteria response of *A. japonicus*.

2. Materials and methods

2.1. Animals and specimens preparation

A. japonicus in good health conditions with body weight from 4.5 to 5.5 g were collected from a sea cucumber culture farm in Dalian. Animals were maintained at 22–23 °C in the Laboratory under regular management conditions. Tissues of body wall, intestine, respiratory tree, tube feet, coelomocytes, and longitudinal muscle were dissected carefully from nine healthy animals (3 individuals each pool). To harvest coelomocytes, the coelomic fluid was collected and centrifuged immediately at 3000 × rpm for 2 min (4 °C). All of the tissue samples and coelomocytes were immediately snap-frozen in liquid nitrogen, and stored at −80 °C until used for RNA extraction.

2.2. Bacterial challenge and physical injury

To determine the immune responses of *AjAIF-1*, an immune challenge experiment was conducted by coelomic injection with *Vibrio splendidus* (D4501), which was obtained from Key Laboratory of Mariculture & Stock Enhancement in North China's Sea, Ministry of Agriculture. *V. splendidus* was cultured on 2216E plates at 28 °C overnight. A single colony was inoculated in 5 ml of 2216E medium at 28 °C and culture with shaking at 180 rpm for 12 h followed with centrifuged at 2000 × rpm for 2 min (4 °C) to harvest bacteria. Bacteria pellets were re-suspended with 100 μL phosphate-buffered saline (PBS, pH 7.4) for immune challenge. For experimental groups 100 μL (1×10^7 CFU ml^{-1}) *V. splendidus* in PBS was injected into the coelomic cavity [26]. The control group was injected with 100 μL PBS. Nine individuals were sampled at 2, 4, 8, 12, 24, 48 h post-injection.

The physical injury experiment was performed by papilla injury. The papilla injury was done by cutting a dorsal papilla in the middle of sea cucumber (Fig. 1). All injuries were induced at the same position for each individual. Uninjured animals were used as the control group. Nine individuals were randomly selected at 2, 4, 8, 12, 24, 48 h post-injury.

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted using the RNAprep Pure Tissue Kit (TIANGEN, China) according to the manufacturer's protocol. The integrity of isolated RNA was evaluated by visualization on agarose gel, and its purity and concentration were measured by spectrophotometer (NanoPhotometer, Munich, Germany). First strand cDNA was synthesized in a 10 μL reaction system with 300 ng total RNA, 2 μL 5 × PrimeScript™ buffer, 25 pmol Oligo dT Primer, 50 pmol Random 6 mers, 0.5 μL PrimeScript RT Enzyme Mix I (PrimeScript™ RT reagent Kit, TaKaRa, Japan). Reactions were incubated at 37 °C for 15 min, and then at 85 °C for 5 s to deactivate the enzyme. All cDNA were stored at −20 °C for subsequent experiments.

2.4. Cloning and sequencing of AjAIF-1

The partial cDNA sequence of *AjAIF-1* gene was identified from the previous transcriptome analysis of *A. japonicus*. Based on the partial sequence, 3′- and 5′-rapid amplification of cDNA ends (RACE) was performed using SMART RACE cDNA Amplification Kit (Clontech, USA) following the manufacturer's instructions. Primers were listed in Table 1. Products from RACE were extracted and purified. Purified RACE products were ligated into pMD-19T vector (TaKaRa, Japan) and transformed into competent DH5α cells (TIANGEN, China). The positive recombinants were identified by colony PCR and sequenced. Three independent clones were sequenced for sequence confirmation.

2.5. Transcriptional analysis of AjAIF-1 by quantitative real-time PCR

The AjAIF-1 expression levels were analyzed by Applied Biosystem 7500 Real-time System (Applied Biosystems, USA). Real-time PCR was carried out in a total volume of 20 μL containing 1 μL of 1:5 diluted original cDNA, 10 μL of 2 × SYBR Green Master mix (SYBR PrimeScript™ RT-PCR kit II, TaKaRa, Japan), 0.4 μL of ROX Reference Dye II, 0.4 μM of each primer (Table 1). The qRT-PCR program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 32 s. To ensure data validity, the negative PCR control reactions were performed with ddH_2O as templates and 3 repeated wells were set in each sample. The PCR products were examined by melting curve to confirm amplification specificity. In addition, the amplicons were checked by agarose gel in order to confirm the correct amplicon sizes. The relative mRNA level of the *AjAIF-1* was

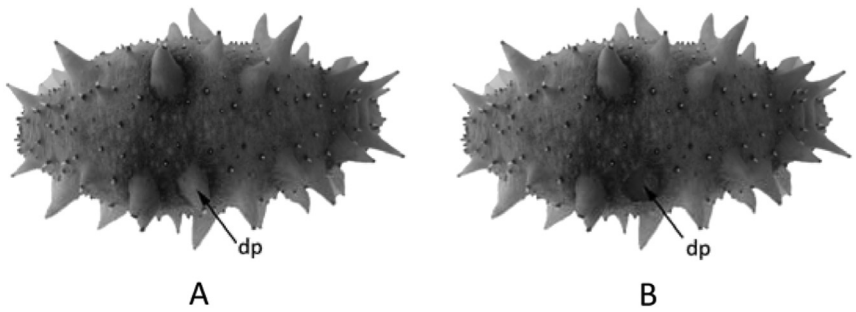

Fig. 1. The physical injury was achieved by cutting a dorsal papilla. (A) Photograph of normal *A. japonicus*. (B) The dorsal papilla was cutted. The arrow indicted the location of dorsal papilla. dp: dorsal papilla.

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