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Tumor necrosis factor receptor-associated factor 6 (TRAF6) participates in peroxinectin gene expression in *Fenneropenaeus penicillatus*

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ABSTRACT

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is an important cytoplasm signal adaptor that mediates signals activated by tumor necrosis factor receptor (TNFR) superfamily and the Interleukin-1 receptor/Toll-like receptor (IL-1/TLR) superfamily. In the study, the full-length cDNA of a TRAF6 homolog (FpTRAF6) was identified from *Fenneropenaeus penicillatus*. The full-length cDNA of FpTRAF6 is 2033 bp long, with an open reading frame (ORF) encoding a putative protein of 594 amino acids, including a RING type Zinc finger, two TRAF-type Zinc fingers, and a conserved *C*-terminal meprin and TRAF homology (MATH) domain. The overall amino acid sequence identity between FpTRAF6 and other TRAF6s ranged from 62.7 to 94.1% for crustaceans and from 45.6 to 59.3% for mollusca. Real-time qRT-PCR indicated that FpTRAF6 was constitutively expressed in various tissues of *F. penicillatus*. The temporal expression patterns of FpTRAF6 mRNA were different in the different tissues after microbial challenge. FpTRAF6 was downregulated in the heart, no obvious changes in the gill, intestine and hemocytes, and upregulated in other tested tissues after WSSV challenge. After *V. alginolyticus* injection, FpTRAF6 was downregulated in the heart and intestine, upregulated in the gill, lymphoid organ and hematopoietic organ, and no obvious changes in other tested tissues. RNAi assay was carried out to investigate the function of FpTRAF6. The results showed that silencing FpTRAF6 gene could inhibit peroxinectin expression *in vivo*, and enhance the sensitivity of shrimps to WSSV and *V. alginolyticus* challenge, suggesting FpTRAF6 could play a positive role against bacterial and viral pathogens. In conclusion, the results of the study provide some insights into the function of FpTRAF6 in activating TLRs signaling pathway and the host defense against invading pathogens.

1. Introduction

Fenneropenaeus penicillatus, widely distributed from Pakistan to Indonesia in the Indo-West Pacific, is considered to be one of the most economically important species of shrimps in China, especially along the coast of Fujian and Guangdong provinces [1]. In the past decade, the resource capacity of *F. penicillatus* was seriously threatened by infectious pathogens, predominantly bacterial and viral in the sea. Moreover, the exploitation and utilization of *F. penicillatus* by both fishing and aquaculture may have caused undesirable effects to the species. Therefore, *F. penicillatus* was included in the Red List by the Chinese government as an endangered species in 2005 [2]. To alleviate these problems, a better understanding immune molecules and their crucial functions in *F. penicillatus* immunity is imperative to provide a foundation for enhancement and conservation programs.

Invertebrates including shrimp lack an acquired immune system and their defense mechanisms highly rely on the innate immunity such as the presence of pattern recognition receptors (PRRs) for

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discriminating and eliminating pathogens [3–5]. Among various kinds of PRRs, Toll-like receptors (TLRs) play an important role in innate immunity against infectious agents in both vertebrates and invertebrates, and function as primary sensors for conserved pathogen-associated molecular patterns (PAMPs) of invading pathogens [6,7]. After the PAMPs recognition, TLRs can recruit adaptor molecules myeloid differentiation factor 88 (MyD88) and tumor necrosis factor receptor-associated factor 6 (TRAF6) for signal transduction to activate nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) in the end [8,9]. The activated NF-κB allows some kinases to translocate into the nucleus and leading to the transcription of many pro-inflammatory genes containing chemokines, cytokines, adhesion molecules and proteolytic enzymes [10,11].

Tumor necrosis factor receptor-associated factor (TRAF) is a multifunctional molecule in intracellular signal transduction [12]. TRAFs can be classified into three main groups: two ancestral groups consisting of TRAF4 and TRAF6, and a more recently evolved group consisting of TRAF1, 2 and 5, as well as vertebrate TRAF3 [13]. TRAF6, the most evolutionarily ancient TRAF family member, is a crucial signal transducer of TLRs signal pathway from *Drosophila* to mammalian [14]. Some studies already demonstrated TRAF6 was a crucial cytoplasm signal adaptor that mediates signals activated by tumor necrosis factor receptor (TNFR) superfamily and the Interleukin-1 receptor/Toll-like receptor (IL-1/TLR) superfamily as a central point of convergence, and it could play a pivotal role in the innate immune system [15,16]. In *Drosophila*, activation of the TLRs signal pathway by bacteria and fungi will lead to initiation of the signaling cascade that involves the adaptor proteins MyD88, Tube, Pelle and TRAF6, resulting in the translocation of Dif and Dorsal and the expression of Drosomycin, an antifungal peptide, as well as many others immune response genes [17]. In crustaceans, some studies indicated TRAF6 could participate in TLRs signal pathway in the innate immune system of hosts [18,19], and activate antimicrobial peptide or anti-lipopolysaccharide factors to resist pathogens in some species such as *Litopenaeus vannamei* and *Scylla paramamosain* [11,20].

Like *Drosophila*, the TLRs signaling pathway composing of the homologs of TLR/MyD88/Tube/Pelle/TRAF6/NF-κB may also exist in crustaceans. Currently, some components of TLRs signaling pathways have been identified in shrimp, including Spätzle [21], Toll [22], MyD88 [9], Tube [23], Pelle and TRAF6 [18,24]. In our previous study, we identified the Pelle molecule (a mammalian like interleukin-1 receptor-associated kinase 1; IRAK-1) and found the molecule could be activated by *Vibrio alginolyticus* and white spot syndrome virus (WSSV) infection in *Fenneropenaeus penicillatus* [24]. In this study, another homologue of TLRs signal pathway components in *F. penicillatus*, FpTRAF6, was cloned and characterized, and RNA interference (RNAi) technique was used to investigate the function. In order to understand the innate immunity of *F. penicillatus* against *V. alginolyticus* and WSSV, we investigated the expression of peroxinectin specifically after FpTRAF6 knockdown. The results will contribute to further understanding of the TLRs signal pathway in *F. penicillatus* immunity and the development of the effective strategies for preventing and controlling the diseases.

2. Materials and methods

2.1. Experimental animals

Healthy shrimps *F. penicillatus* with an average body weight 9 ± 1.7 g were obtained from the East Sea Island Marine Biological Research Center in Guangdong Province, China. All of shrimps were cultured in the fiberglass tanks with air-pumped seawater (2.5% salinity). The shrimp were fed with artificial diet for 7 days and acclimated to laboratory conditions.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from brain, gill, heart, hepatopancreas, lymphoid organ, intestine, hemocytes, stomach, hematopoietic organ, gonad and cephalothoraxes of the experimental shrimps using Trizol reagents (Invitrogen, USA) following the manufacturer's instructions, and the residual genomic DNA was removed using Rnase-free Dnase (RQ1; Promega, Madison, WI). For cloning of the 5′ and 3′ cDNA ends, a cDNA template was prepared with the SMARTer RACE cDNA amplification kit (Clontech, USA). For gene cloning, the first-strand cDNA was prepared with the Prime-Script First-strand cDNA synthesis kit (TaKaRa, China). For real-time quantitative RT-PCR (qRT-PCR) analysis, the first-strand cDNA was prepared using PrimeScript RT reagent kit (TaKaRa, Dalian, China).

2.3. Cloning of F. penicillatus FpTRAF6 cDNA

FpTRAF6 primers were designed (Table 1) based on a partial sequence of TRAF6 from the *F. penicillatus* transcriptome data analyzed by our laboratory. Rapid amplification cDNA ends (RACE) were performed using the SMARTer™ RACE cDNA Amplification kit (Clontech, Japan) according to the manufacturer's protocol. 5′-RACE PCR amplification was performed with Universal Primer A Mix (UPM) and the specific primer TRAF6-5R, and nested PCR was subsequently performed with Nested Universal Primer A (NUP) and TRAF6-5NR using the first-round PCR product as template. 3′-RACE PCR was performed using UPM together with the specific forward primer TRAF6-3F, and then the nested PCR was performed with Nested Universal Primer A (NUP) and TRAF6-3NF. The nest PCR products were cloned into pMD-19T Cloning Vector (Takara) and then sequenced by Shanghai Sangon Biologic Engineering & Technology and Service Co. Ltd.

2.4. Sequence and phylogenetic analysis of FpTRAF6

Sequence analysis was carried out with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The amino acid sequence for FpTRAF6 was deduced using ExPASy software (http://www.expasy.org/). The molecular weight (MW) of the protein was calculated based upon its constituent amino acids, using the Compute pI/MW software tool (http://www.expasy.org/tools/pi_tool.html). The functional domains were presumed using CDD software (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple sequence alignments were performed using the ClustalX 2.0 program (http://www.ebi.ac.uk/tools/clustalw2). The phylogenetic tree was constructed based on the deduced amino acid sequences of FpTRAF6 with the neighbor-joining (NJ) method, applying the Poisson distribution substitution model and bootstrapping procedure with 1000 bootstraps [25].

2.5. Quantification of FpTRAF6 transcript in different tissues

A relative real-time qRT-PCR assay was performed to analyze the expression patterns of FpTRAF6 transcript in different tissues (brain, gill, heart, hepatopancreas, lymphoid organ, intestine, hemocytes, stomach, hematopoietic organ and gonad) by an iCycler iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Totally pairs of specific primers used was shown in Table 1. The elongation factor-1 (FpEF1α) gene was used as internal control in all qPCR experiments. PCR conditions were as follows: 94 °C 2 min, followed by 30 cycles of 94 °C 20 s, 60 °C 20 s, and 72 °C 20 s for the FpTRAF6 and FpEF1α gene. The PCR products were analyzed on 1.2% agarose gel stained with ethidium bromide. Expression level of