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Identification of tumor necrosis factor receptor-associated factor 6 in the pearl mussel *Hyriopsis cumingii* and its involvement in innate immunity and pearl sac formation

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ABSTRACT

Tumor necrosis factor receptor-associated factor 6 (TRAF6) acts as a central intracellular signal adapter molecule that mediates the tumor necrosis factor receptor superfamily and the interleukin-1 receptor/Toll-like receptor family in vertebrates and invertebrates. In the present study, HcTRAF6, a molluscan homologue of TRAF6 from *Hyriopsis cumingii*, has been cloned and identified. The entire open reading frame of HcTRAF6 was found to comprise a 1965-bp region that encodes a predicted protein of 654 amino acids, which contains conserved characteristic domains including a RING domain, two TRAF-type zinc finger domains, a typical coiled coil and the MATH domain. Phylogenetic analysis revealed that HcTRAF6 was aggregated closely with CsTRAF6 from *Cyclina sinensis* in the invertebrate cluster of mollusks. Further, qRT-PCR analysis showed that HcTRAF6 mRNA was extensively distributed in mussel tissues with a high expression in gills. After immune stimulation with *Aeromonas hydrophila* and lipopolysaccharides, the transcription of HcTRAF6 was obviously induced in the gills and hemocytes. In addition, significant fluctuation in HcTRAF6 expression was observed in the pearl sac, gills and hemocytes after mantle implantation. These findings confirmed its role in the alloimmune response. Dual-luciferase reporter assay showed that over-expression of HcTRAF6 could enhance the activity of the NF-κB reporter in a dose-dependent manner. Further, the RNA interference showed that the up-regulation of antimicrobial peptides in anti-bacterial infection was strongly suppressed in HcTRAF6-silenced mussels and that depletion of HcTRAF inhibited the elimination of *A. hydrophila*. All these findings together prove that HcTRAF6 functions as an efficient regulator in innate immune mechanisms against invading pathogens and the alloimmune mechanism after mantle implantation in *H. cumingii*.

1. Introduction

In China, pearl production is dependent on aquaculture of the bivalve *Hyriopsis cumingii*, which is the main freshwater pearl-producing mussel. However, the high yield of pearls is in sharp contrast to the low value of the output [1]. For pearl culture, allografts of mantle tissue from donor mussels are transplanted into recipient mussels. The recipient mussels undergo a process of healing and regeneration after the graft operation, forming a tenuous layer of epithelial cells and consequently generating fully enclosed pearl sacs [2]. This process is called mantle implantation. Tremendous immunological stress reactions occur in the host mussels as a result of the implantation procedure, which can pose a serious threat as a result of the entry of various invading pathogens during the formation of the pearl sac. In addition, enormous pathogenic microorganisms frequently result in the bacteria infection of mussels during breeding and cultivation. Therefore, understanding the immune mechanisms and pathways underlying mussel formation can help prevent and control diseases that occur during the aquaculture of pearl mussels, and thereby improve the quality of pearls produced.

H. cumingii lacks a specific acquired immune system, so the innate immune system acts as the first line of defense against pathogens [3]. The activation of the initial immune response depends on pathogen-associated molecular patterns (PAMPs), which are recognized by a series of pattern recognition receptors (PRRs) [4]. Toll-like receptors (TLRs), as the most studied and best-characterized PRRs, can recognize and bind a variety of PAMPs and thereby lead to the production of

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Table 1
Primers used in the present study.

Primer name	Primer sequence (5′-3′)
RACE	
HcTRAF6-3′RACE	AAATGCGAAGAAATGTATCAGAGAC
HcTRAF6-5′RACE	CGACAGAAGCGATGACCACA
M13F	GTTGTAAAACGACGGCCAG
M13R	CAGGAAACAGCTATGAC
Real-time PCR	
HcTRAF6-F	CAGACGCAGTGTGGTCATCG
HcTRAF6-R	TGGCACACGAGTTAGGGCAT
EF1-α-F	GGAACTTCCCAGGCAGACTGTGC
EF1-α-R	TCAAAACGGGCCGCAGAGAAT
HcLyso1-F	ATATCGACTGTGGAAGTCC
HcLyso1-R	ATCGTGGCTGGATTTGCAC
HcLyso2-F	TGTATGGAGTGCATATGTC
HcLyso2-R	CAGTCTCCACCTAGACGTC
Hctheromacin −F	GATTCGTTCTCAGTGATTGCT
Hctheromacin −R	GCTCGCTACAGGTGTTCCATA
HcDef-F	GACGATCGCCGAATTACTGG
HcDef-R	TGCATCTGTATGGTGGTGGT
dsRNA template amplification	
dsHcTRAF6 −F-1	TAATACGACTCACTATAGGGGAAATGGAGAGTTTATCTGGAGGAT
dsHcTRAF6 -R-1	TGATGAAATAACCTTTGCTTTTATC
dsHcTRAF6 −F-2	GAAATGGAGAGTTTATCTGGAGGAT
dsHcTRAF6 -R-2	TAATACGACTCACTATAGGGTGATGAAATAACCTTTGCTTTTATC
dsGFP −F-1	TAATACGACTCACTATAGGAAGGGCGAGGAGCTGTTCACCG
dsGFP –R-1	CAGCAGGACCATGTGATCGCGC
dsGFP −F-2	AAGGGCGAGGAGCTGTTCACCG
dsGFP –R-2	TAATACGACTCACTATAGGCAGCAGGACCATGTGATCGCGC
Vector construction	
TRAF6-F	CCGGAATTCTG ATGGCATCGAAAGGTGTGAAC
TRAF6-R	CGCGGATCCCG TGATGAAATAACCTTTGCTTTTATC

multiple diverse effective molecules such as inflammatory cytokines, antibacterial peptides (AMPs) and chemotactic cytokines [4,5]. In mammals, TLRs function as a bridge between the innate immune system and acquired immune system via antigen-presenting cells [6]. The molecular mechanism underlying the intracellular signal transduction function of TLRs (except for TLR3) relies on the myeloid differentiation factor 88 (MyD88)-dependent signaling cascade [7,8]. TLRs interact with the adaptor protein MyD88, which encodes a TIR domain at its N-terminus after PRRs are recognized, triggering an activation cascade. MyD88, which consists of an amino terminal death domain, unites with interleukin-1 receptor-associated kinase (IRAK1) and IRAK4 to form a complex. On phosphorylation of the IRAKs after combination, tumor necrosis factor receptor-associated factor 6 (TRAF6) is activated, and this contributes to the activation of TAK1 [9]. The IKK complex which is necessary for NF-κB is regulated by the combination of TAK1 and TAB1, TAB2, TAB3. Ultimately, the transcription factor NF-κB is translocated to the nucleus, and the activated NF-κB is capable of inducing the expression of multiple immune response genes [10]. Thus, in mammals, TRAF6 plays a central regulatory role as an intracellular signal adaptor molecule both in the tumor necrosis factor (TNF) receptor superfamily and the interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) family based on its distinct receptor-binding specificity [11].

All TRAF proteins are characterized by an N-terminal domain (a RING finger preceded by several putative zinc-finger motifs) and a C-terminal domain (a coiled-coil motif followed by a highly conserved carboxyl terminal TRAF-C domain) [11,12]. Previous studies have demonstrated that the RING finger motif of TRAF6 possesses the ubiquitin ligase catalytic domain, which is crucial for activating the downstream signaling cascades; further, the TRAF domain at the C-terminus can catalyze the oligomerization of TRAF6 and is also required for interaction with signaling proteins and receptors [13]. As a key signaling transducer, TRAF6 mediates a subset of physiological processes associated with acquired immunity, innate immunity and bone metabolism, among other TLR regulation mechanism components [14]. With regard to its immunoregulatory role, it has been reported that in invertebrates, SpTRAF6 from *Scylla paramamosain* is involved in the acute-phase response after immune challenge via regulation of the ALF gene profiles [15]. Further, *Portunus trituberculatus* TRAF6 (PtTRAF6) might contribute to host defense against invasion by gram-negative bacteria [16]. Studies about TRAF6 from the mollusk also suggest that TRAF6 participates in immune defense against stimulation and promotes NF-κB signaling pathways [17–20]. Further, several components of the TLR-mediated signaling pathway, such as Toll [21,22] and MyD88 [23], have been identified in the immune pathways of *H. cumingii*. However, the role of TRAF6 in this mussel species has not been reported to date, and it requires further exploration in freshwater mussels.

In this article, we cloned a TRAF6 homologue from *H. cumingii*, detected the *HcTRAF6* transcripts in different tissues, and determined the dynamic expression of these transcripts after infection with *Aeromonas hydrophila* and lipopolysaccharide (LPS) and after mantle implantation. To further investigate the role of *HcTRAF6* in the triangle sail mussel, HEK293T cells were assessed to determine the function of *HcTRAF6* in the activation of NF-κB. Additionally, we utilized the RNA interference strategy to determine whether *HcTRAF6* could regulate the AMP expression level after challenge with *A. hydrophila* and influence its capacity for bacterial clearance. Overall, our findings shed light on the immune function of *HcTRAF6* in bivalve mollusks and the mechanism of pearl sac formation.

2. Materials and methods

2.1. Experimental mussels

Healthy *H. cumingii* with a shell length of 7–8 cm were obtained from Xuancheng Farm of Zhexing Pearl Trading Co. Ltd. in Anhui Province, China. All the mussels were acclimatized in a laboratory aeration tank at room temperature with recirculating water for one week before further experimentation.