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Short communication

Molecular characterization and functional analysis of tumor necrosis factor receptor-associated factor 2 in the Pacific oyster

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ABSTRACT

Tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) are a family of crucial adaptors, playing vital roles in mediating signal transduction in immune signaling pathways, including RIG-I-like receptor (RLR) signaling pathway. In the present study, a new TRAF family member (CgTRAF2) was identified in the Pacific oyster, *Crassostrea gigas*. Comparison and phylogenetic analysis revealed that CgTRAF2 could be a new member of the invertebrate TRAF2 family. Quantitative real-time PCR revealed that CgTRAF2 mRNA was highly expressed in the digestive gland, gills, and hemocytes, and it was significantly up-regulated after *Vibrio alginolyticus* and ostreid herpesvirus 1 (OsHV-1) challenge. The CgTRAF2 mRNA expression profile in different developmental stages of oyster larvae suggested that CgTRAF2 could function in early larval development. CgTRAF2 mRNA expression pattern, after the silence of *CgMAVS (Mitochondrial Antiviral Signaling) -like*, indicated that CgTRAF2 might function downstream of CgMAVS-like. Moreover, the subcellular localization analysis revealed that CgTRAF2 was localized in cytoplasm, and it may play predominately important roles in signal transduction. Collectively, these results demonstrated that CgTRAF2 might play important roles in the innate immunity and larval development of the Pacific oyster.

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1. Introduction

Tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) are a family of intracellular signaling molecules that are involved in the signaling pathways initiated by members of the RIG-I-like receptor (RLR) family [1,2], the Toll-like receptor (TLR) family [3], the TNFR family [4], and the interleukin-1 receptor (IL-1R) family [5]. To date, seven members of the TRAF family (TRAF1 through to TRAF7) have been identified in mammals. Acting alone or in combination, these TRAFs control many biological processes, including cytokine production and cell survival [6].

Among all TRAFs, TRAF1 and TRAF2 were the first identified members of the TRAF family through their recruitment to TNFR2 [7]. Like other TRAF members, TRAF2 possesses different domains at its amino (RING and zinc finger domains) and carboxyl (TRAF domains) ends. The RING domain was proposed to act as an E3 ligase in TNF receptor signaling complexes. TRAF2 possesses a conserved C-terminal TRAF domain that contains a coiled-coil TRAF-N subdomain and a MATH (meprin and TRAF-C homology) subdomain following the RING and zinc finger domains. The coiled-coil can bind several of its signaling partners including c-IAP1/2 [8], which would be important for TRAF2 mediating Nuclear factor κB (NF-κB) signaling events [9]. The TRAF-C domain could interact with various receptors and mediate signaling transduction. Many research reports reveal that TRAF2 participates in the activation of both canonical and noncanonical NF-κB pathways [10], crucial for cell inflammation and cell survival. For instance, it is well known that RIG-I family of receptors can detect cytoplasmic RNA virus infections and then induce type-I interferons through the mitochondrial adaptor protein MAVS [11]. TRAF2 has been reported as one ubiquitin E3 ligase that could interact with MAVS directly to activate antiviral signaling cascades [1,12]. However, recent findings demonstrate that TRAF2 has an unexpected anti-inflammatory function and limits the proinflammatory TLR signaling by a proteasome-dependent mechanism [13]. Therefore, more research is needed to explore the details of TRAF2 function.

Although the TRAF family is well documented in mammals, there are fewer reports regarding its existence and functional

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analysis in invertebrates. In *Drosophila melanogaster*, DmTRAF1 activates the c-Jun N-Terminal Kinase (JNK) signaling pathway, whereas DmTRAF2 activates the NF-κB pathway and stimulates antimicrobial immunity [14]. Furthermore, there are only weak TRAF homologs in *Caenorhabditis elegans* and they may not function in nematode immune responses [15]. In mollusks, some members of the TRAF family, such as TRAF3 in *Crassostrea gigas* [16] and *Pinctada fucata* [17], TRAF6 in *Chlamys farreri* [18] and *Mizuhopecten yessoensis* [19], and TRAF7 in *Crassostrea hongkongensis* [20], have been identified and characterized. These studies on TRAF homologs would help us to understand and trace the evolution of innate immune response systems in invertebrates.

The Pacific oyster, *C. gigas*, has a global distribution and major economic, as well as cultural significance [21]; therefore research on this species has the potential to have great impact. In addition, these sessile animals survive in the intertidal zone where the environment is extremely stressful, so they have evolved an advanced tolerance to such conditions, which makes the oyster a suitable model for the study of intertidal ecology and stress adaptation. Recent reports revealing the association of pathogens (such as OsHV-1) with occasional high mortality levels among oysters suggest an urgency for studies focusing on oyster immune systems [22,23].

Analysis of the oyster genome reveals that there are 15 TRAF family members in the *C. gigas* genome suggesting the expansion of this immune gene family [24]. However, except for *TRAF3* and *TRAF7*, other members of this important gene family have not been characterized in oysters. In the present study, the first molluscan full-length *TRAF2* gene (*CgTRAF2*) was identified and characterized from the Pacific oyster *C. gigas*. We investigated the expression profile of CgTRAF2 transcripts in multiple tissues, as well as following bacterial and viral challenge in hemocytes. To better understand TRAF2 function in oysters, CgTRAF2 transcript expression patterns were examined in different developmental stages, as well as following the silencing of *CgMAVS-like*. Finally, we investigated the distinct subcellular localization of this CgTRAF2 protein.

2. Materials and methods

2.1. Animals, challenge, and sample preparation

Healthy adult oysters, with an average shell height of 60 mm, were collected from a farm in Qingdao, Shandong Province, China. Experimental specimens were acclimatized in aerated and filtered seawater at 22 ± 0.5 °C for more than one week prior to executing the experiment.

One hundred and sixty oysters were randomly divided into four groups of 40 individuals. By filing and making a small hole in the shell, each oyster in one group received an muscle injection of 100 μL phosphate buffered saline (PBS, 0.14 M sodium chloride, 3 mM potassium chloride, 8 mM disodium hydrogenphosphate dodecahydrate, 1.5 mM potassium phosphate monobasic, pH 7.4). Each oyster in the second group received an injection of 100 μL live *Vibrio alginolyticus* at a concentration of 5×10^7 cells/mL, suspended in PBS. Each oyster of the third group received an injection of 100 μL polyinosinic-polycytidylic acid (poly(I:C), InvivoGen, USA) at a concentration of 1.0 mg/mL, also suspended in PBS. Each oyster in the fourth group received an injection of 100 μL filtered oyster tissue supernatant (2×10^5 OsHV-1 viral DNA copies/μL) which was prepared according the description in previous literature [16]. All of the oysters in all four of the groups were injected into the adductor muscle. At 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h after the injection, six individuals from each group were randomly sampled and the hemolymph was collected and immediately centrifuged at 800 g for 10 min at 4 °C, to harvest the hemocytes. Six tissues including gonad, mantle, muscle, gill, hemocytes (blood), and digestive gland of unchallenged oysters were collected from six adult animals to investigate the CgTRAF2 mRNA distribution.

Embryos or larvae from eight stages were identified microscopically. Different developmental stages of samples, including fertilized eggs, blastula (5 h), gastrula (8 h), trochophore (12 h), D-shaped (24 h), umbo larval (8 d and 25 d), and pediveliger stages (35 d) were collected and preserved in TRIzol (Invitrogen, USA) for RNA extraction.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from oyster tissues using TRIzol Reagent followed by treatment with DNase I (Promega, USA). The first-strand cDNA synthesis was then carried out based on Promega M-MLV (Promega) RT Usage information using treated RNA as a template and dTAP adaptor as a primer (Supplementary File 1). The reaction mixture was incubated at 42 °C for 1 h, and the reaction was terminated by heating at 95 °C for 5 min.

2.3. Cloning full-length CgTRAF2 cDNA and sequence analysis

2.3.1. Taking advantage of the C

gigas genome [25], CgTRAF2-F and CgTRAF2-R (Supplementary File 1) were designed for the amplification of the *CgTRAF2* fragment. Based on the *CgTRAF2* fragment sequence, gene specific primers for 3′ and 5′ RACE (rapid amplification of cDNA ends) were designed. The 3′ end of *CgTRAF2* was cloned using the primer 3CgTRAF2-1 (or 3CgTRAF2-2), and the anchor primer AP (Supplementary File 1). The 5′ end of *CgTRAF2* was obtained using primers 5CgTRAF2-1, 5CgTRAF2-2, 5CgTRAF2-3, and the adaptor primer dGAP (Supplementary File 1). Two rounds of nested PCR were performed with the touchdown program in the 5′ RACE cloning. For the first round, the cDNA tailed with dCTP at the 5′ end by terminal deoxynucleotidyl transferase TdT (Invitrogen) was used as the PCR template, with 5CgTRAF2-1 and dGTP as primers. Then 5CgTRAF2-2/AP, or 5CgTRAF2-3/AP, was employed as the second-round PCR primer. PCR products were purified using the E.Z.N.A Gel Extraction Kit (OMEGA, USA) and was cloned into pMD19-T vector (TaKaRa, Japan). The recombinant vector was transformed into Trans1-T1 competent cell (Transgen, China) and sequenced.

Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) was used to analyze *CgTRAF2* cDNA and deduce amino acids. SMART (Simple Modular Architecture Research Tool) (http://smart.emblheidelberg.de) was utilized to predict CgTRAF2 protein domains. TRAF2 and other six TRAF family members protein sequences from different species were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/guide/proteins/) and compared using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A TRAF phylogenetic tree was constructed using the MEGA program (Version 5.05), with the Neighbor-joining algorithm. Reliability of the branching was tested using bootstrap resampling (1000 pseudo-replicates) (http://www.megasoftware.net).

2.4. Transcriptional analysis of CgTRAF2 mRNA expression

As mentioned above, total RNA was extracted using TRIzol Reagent. Reverse transcription was performed using PrimerScript RT reagent kit with gDNA eraser (TaKaRa). Quantitative real-time PCR (qRT-PCR) was performed with the SYBR Green 2 × Master mix (Takara), using an ABI 7500 fast Real-Time Thermal Cycler, according to the manual's instructions (Applied Biosystems, USA), to investigate CgTRAF2 mRNA expression. Primer CgTRAF2-qRT-F and

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