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# Molecular characterization and functional analysis of TRAF6 in orange-spotted grouper (*Epinephelus coioides*)

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ABSTRACT

Tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) is a crucial signal transducer in both the TNFR superfamily and Toll-like receptor/interleukin 1R family. Although significant progress has been made in clarifying the role of TRAF6 in mammals, the function of TRAF6 in fish is still poorly understood. In this study, we cloned the orange-spotted grouper (*Epinephelus coioides*) TRAF6 (EcTRAF6) cDNA, with an open reading frame of 1713 bp encoding 570 amino acids. Sequence analysis indicated that EcTRAF6 contains the four characteristic domains conserved in the TRAF family, including an N-terminal RING finger, two zinc fingers, a coiled-coil domain, and a C-terminal MATH domain. Homology alignment and phylogenetic analysis demonstrated that EcTRAF6 shares high sequence identity with TRAF6 of other fish species. The EcTRAF6 gene contains seven exons and six introns, which is similar to the organization in ayu, but not in the common carp, human, or mouse (six exons and five introns). EcTRAF6 transcripts were broadly expressed in all tissues tested, and increased after infection with *Cryptocaryon irritans*. Intracellular localization showed EcTRAF6 was distributed mainly in the cytoplasm. Over-expression of wild type (WT) EcTRAF6, truncated forms of EcTRAF6, including ΔZinc finger 2 and ΔMATH, and a mutant of C78A activated NF-κB strongly in HEK293T cells; whereas truncations, including ΔRING, ΔZinc finger 1 and Δcoiled-coiled, and a mutant of K132R induced the activity of NF-κB slightly compared to WT EcTRAF6, implying the latter has a more crucial role in downstream signal transduction. Together, these results suggested EcTRAF6 functions like that of mammals to activate NF-κB, and it might have an important role in host defense against parasitic infections.



## 1. Introduction

Tumor necrosis factor receptor-associated factor (TRAF) family members (TRAF1–7) were initially identified as crucial signal transduction docking proteins of the tumor necrosis factor receptor (TNFR) superfamily (Inoue et al., 2000; Xu et al., 2004). They regulate a diverse array of biological functions in mammals, including in adaptive immunity, innate immunity, and osteoimmunity (Wu and Arron, 2003). TRAF6, a unique member of the TRAF family, was first isolated with a yeast two-hybrid system using mouse CD40 as bait (Ishida et al., 1996) and by screening an expressed sequence tag (EST) library (Cao et al., 1996). TRAF6 is the only TRAF family member that participates in both the TNFR superfamily signaling pathways and the Toll-like receptor/interleukin 1 receptor (TLR/IL-1R) family signaling pathways (Chung et al., 2007). Following dissection from MyD88 in the TLR/IL-1R signal, IRAKs interact with TRAF6 to form a signal transduction complex. TRAF6 functions as an E3 ubiquitin protein ligase, together with an E2 ubiquitin-conjugating enzyme (Ubc13/Uev1A), to catalyze the formation of a lysine 63 (K63)-linked polyubiquitin chain on itself, which then activates the TAK1 kinase complex (Lamothe et al., 2008; Xia et al., 2009; Yang et al., 2004). TAK1 then phosphorylates and activates IKK and MAPK, which leads to the activation of transcription factors NF-κB and AP-1, which in turn regulate the expression of cytokine genes (Takeuchi and Akira, 2010).

In recent years, significant progress has been made in understanding the crucial role played by TRAF6 in mammals (Inoue et al., 2007; Kobayashi et al., 2004). Although many piscine TRAF6 sequences can be retrieved from GenBank, including that of the Nile tilapia (*Oreochromis niloticus*; XM_003450798), three-spined stickleback (*Gasterosteus aculeatus*; BT027611), Japanese medaka (*Oryzias latipes*; XM_004066849), fugu (*Takifugu rubripes*; XM_003969622), spotted snakehead (*Channa punctata*;

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JQ004871), ayu (*Plecoglossus altivelis altivelis*; AB516400), zebrafish (*Danio rerio*; AY616583) (Phelan et al., 2005), common carp (*Cyprinus carpio*; GU985443 and HM535645) (Kongchum et al., 2011), and grass carp (*Ctenopharyngodon idella*; KC465198) (Zhao et al., 2013), the function of piscine TRAF6 is still largely unknown. Phelan et al. (2005) demonstrated that TRAF6 expression was induced in the zebrafish after bacterial and viral infections, and that the adaptor protein activated NF-κB in zebrafish liver (ZFL) cells. Two other research groups have reported that piscine TRAF6 expression increases after pathogen infections (Basu et al., 2012; Zhao et al., 2013). To our knowledge, no other study of the role of TRAF6 in fish has been reported.

The orange-spotted grouper (*Epinephelus coioides*) is a commercially important fish species, cultured in South China. It suffers serious diseases, especially infection by *Cryptocaryon irritans*. *C. irritans* is a common obligate protozoan ecto-parasite ciliate that infects salt-water fish (Colorni and Burgess, 1997). In our laboratory, the grouper and *C. irritans* are used as a model to study the immune mechanisms of fish against parasitic infections (Bai et al., 2008; Dan et al., 2013; Luo et al., 2007). Recently, we found that the expression of some TLRs, including TLR21 and TLR9, is up-regulated after *C. irritans* infection, and speculated that the TLR signaling pathway is involved in the host resistance to *C. irritans* infection (Li et al., 2011, 2012). In the present study, to further investigate the role of the TLR signal pathway in fish, we first identified the TRAF6 cDNA and its gene sequence from *E. coioides*. The expression of TRAF6 was then determined in healthy fish tissues and in the immune system organs and local infection sites after infection with *C. irritans*. Last, TRAF6 (wild type (WT) or mutants) was over-expressed in HeLa and HEK293T cells to detect the intracellular localization and signal transduction function, respectively.

## 2. Materials and methods

### 2.1. Fish, challenge, and sampling

Healthy orange-spotted grouper were obtained from a governmental hatchery (Daya Bay Aquaculture Centre, Guangdong, China), where no disease outbreak has occurred in recent years. The fish were then cultured under laboratory conditions at 27 (±1) °C with re-circulating seawater for two weeks before the trial, and fed twice a day with a commercial grouper diet. Samples of gill, skin, blood, brain, thymus, liver, spleen, head kidney, and trunk kidney were collected from five fish, and snap-frozen in liquid nitrogen, before their use in the analysis of tissue expression patterns.

The grouper were infected with *C. irritans* according to a previously described procedure (Li et al., 2011). In brief, 80 grouper were randomly divided into the *C. irritans*-infected group and the uninfected control group. The fish in the infection group were exposed to *C. irritans* at a dose of 30,000 theronts per fish for 2 h, whereas the fish in the control group were not treated. At 6 h, 1 d, 2 d, 3 d, and 5 d post infection, skin, gill, spleen, and head kidney samples were collected from both groups. Five fish were sampled from each group at each time point.

### 2.2. DNA and RNA isolation, cDNA synthesis

Genomic DNA was isolated from the fins of the groupers using a TIANamp Marine Animals DNA Kit (Tiangen), according to the manufacturer's protocol. The DNA was then dissolved in PCR-grade water and used as the template to amplify the TRAF6 gene. Samples collected as reported in Section 2.1 were first homogenized with a pestle and mortar and the total RNA was then extracted with TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. The samples were then incubated with DNase I (Fermentas) at 37 °C for 30 min to remove any genomic DNA contamination. An aliquot (1 μg) of the total RNA was used to synthesize the first-strand cDNA with ReverTra Ace-α reverse transcriptase (Toyobo), which was used as the template to amplify the TRAF6 sequence and analyze its expression.

### 2.3. Cloning of cDNA sequence

To clone the partial sequence of grouper TRAF6 (EcTRAF6), degenerate primers were designed based on a multiple sequence alignment of the fish TRAF6 genes reported in GenBank, including those of the Nile tilapia, three-spined stickleback, ayu, common carp, and zebrafish. Using primers TRAF6F2/R3 (Table S1), a 1271 bp cDNA sequence was amplified, which showed a high level of sequence identity with TRAF6 of the other fish species.

To amplify the full-length sequence of EcTRAF6, the first-strand cDNA for 5′ or 3′ RACE was synthesized with a SMARTer™ RACE cDNA Amplification Kit (Clontech) using spleen RNA as the template and following the manufacturer's protocol. Primers RATRAF6R1/UPM and RATRAF6R2/NUP (Table S1) were used to clone the unknown 5′-terminal region with primary and nested PCR; primers RATRAF6F1/UPM and RATRAF6F2/NUP (Table S1) were used to clone the unknown 3′-terminal region with primary and nested PCR. The full-length EcTRAF6 open-reading frame (ORF) was amplified using primers TRAF6FLF/R (Table S1).

### 2.4. Cloning the DNA sequence

To analyze the genomic structure of EcTRAF6, the DNA ORF was amplified using the genomic DNA as template. First, a partial ORF containing the stop codon was amplified using gene-specific primers DTRAF6F1/R1 (Table S1), which were designed based on the cDNA sequence. Second, the 5′-terminal ORF was amplified using three gene-specific primers GWTRAF6R1/R2/R3 (Table S1) and a degenerate primer (AP1) supplied with the Genome Walking Kit (TaKaRa), according to the manufacturer's protocol. Last, the full-length ORF was obtained by splicing the partial sequence and 5′-terminal sequence, and the exons and introns were defined by aligning the cDNA and DNA sequences.

### 2.5. Bioinformatics

A multiple sequence alignment was constructed with ClustalX 1.83 and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The theoretical isoelectric point (pI) and molecular mass of EcTRAF6 were computed with an online program (http://web.expasy.org/compute_pi/). A protein structure analysis was performed with the SMART program (http://smart.embl-heidelberg.de/). A phylogenic tree was constructed using the MEGA 4.0 program (http://www.megasoftware.net/mega4/mega.html).

### 2.6. Real-time PCR

To detect the expression of EcTRAF6, primers QTRAF6F1/R1 and >β-actin F1/R1 (Table S1) were designed with the beacon designer 7.80 software (http://www.premierbiosoft.com/molecular_beacons/index.html), and β-actin was used as the internal control. Real-time PCR was performed on a Roche LightCycler® 480 Real-time PCR Detection System (Roche) with the SYBR Green Real-time PCR Master Mix (Toyobo). The PCR conditions were as follows: one cycle of 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 57 °C for 15 s, and 72 °C for 20 s. The specificity of the PCR products was confirmed by melting curve analysis and sequencing. Each sample was amplified in triplicate. The mRNA expression of the