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Short communication

Expression and functional analysis of tumor necrosis factor receptor (TNFR)-associated factor 5 from Nile tilapia, *Oreochromis niloticus*

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ABSTRACT

Nile tilapia (*Oreochromis niloticus*) is a pivotal economic fish that has been plagued by Streptococcus infections. Tumor necrosis factor receptor-associated factor 5 (TRAF5) is a crucial adaptor molecule, which can trigger downstream signaling cascades involved in immune pathway. In this study, Nile tilapia TRAF5 coding sequence (named OnTRAF5) was obtained, which contained typical functional domains, such as RING, zinc finger, coiled-coil and MATH domain. Different from other TRAF molecules, OnTRAF5 had shown relatively low identify with its homolog, and it was clustered into other teleost TRAF5 proteins. qRT-PCR was used to analysis the expression level of OnTRAF5 in gill, skin, muscle, head kidney, heart, intestine, thymus, liver, spleen and brain, In healthy Nile tilapia, the expression level of OnTRAF5 in intestine, gill and spleen were significantly higher than other tissues. While under *Streptococcus agalactiae* infection, the expression level of OnTRAF5 was improved significantly in all detected organs. Additionally, over-expression WT OnTRAF5 activated NF-κB, deletion of RING or zinc finger caused the activity impaired. In conclusion, OnTRAF5 participate in anti-bacteria immune response and is crucial for the signaling transduction.

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is a worldwide common commercially farmed fish and is very pivotal in aquaculture due to its great economic value. China's tilapia industry accounts for more than 40% of the world's total output [1]. However, tilapia farming is terribly threatened by streptococci disease, which caused the high cumulative mortality in more than 95% of the farms in china [2,3]. The immediate pecuniary loss included by tilapia streptococci disease in China was 1–1.5 billion [4]. Thus, it is urgent to develop an effective method for preventing tilapia streptococci disease, improve the immunity of tilapia is the most promising approach to prevention and treatment against streptococci infection.

TRAF5 was originally identified as an interacting protein with lymphotoxin-β receptor and CD40, activating of NF-κB pathway [5,6]. Subsequently, TRAF5 was proved to exert signal pleiotropic functions with other receptors, not limiting to CD27, CD30 and interleukin (IL)-1 receptor [7–12]. Additionally, TRAF5 had been implicated in many signal pathways. In RLR pathway, TRAF5 was required for the antiviral immune responses of host. When TRAF5 deletion, the activation of JNK and IRF3/IRF7 was decreased significantly [13–15]. Further evidence for the role of TRAF5 in many cell types was provided by studies. TRAF5 controlled helper T cell differentiation by inhibiting IL-6R signaling in $CD4^+$ T cells [16]. Deletion study in mice has revealed the inhibition of TRAF5 in cytokines and antibodies production [17–20]. Moreover, TRAF5 work as an important negative regulator of IL-6R signaling needed for Th17 development in B lymphocyte [21]. These aforementioned studies demonstrate that TRAF5 play special role in adaptive and innate immunity. Apart from this, TRAF5 forms heterotypic proteasome with other TRAFs. The interaction trigger multiple and complex functional outcomes, which depending on receptor-special, component of multimers or cell types [22,23].

Compared with other TRAF proteins, TRAF5 was large unknown in teleost. In the present study, the whole coding sequence of TRAF5

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Table 1
Sequences of primers used in this study. Restriction enzyme sites were underlined.

Primers	Sequences (5′-3′)
TRAF5-F	ATGGCAACAGCAAATACAAATCAG
TRAF5-R	TTACAGCTGCTCAAAACCTGTCATAT
RT- TRAF5-F	TGCAGCAGGAGGTCCAGACAGA
RT- TRAF5-R	TGAGGATGAAGCGCCCGAGA
β-Actin-S	AACAACCACACACCACACATTTC
β-Actin-A	TGTCTCCTTCATCGTTCCAGTTT
pcDNA-TRAF5F	GG GGTACCATGGCAACAGCAAATACAAATCAG
pcDNA-TRAF5R	CCGGAATTCCAGCTGCTCAAAACCTGTCATAT
TRAF5T1/T2/T3/4T4-F1	GG GGTACCATGGCAACAGCAAATACAAATCAG
TRAF5T1-R1	CGTGATCACAGCTCTAACAAATTCCTCCTTCAGCTTCAAC
TRAF5T1-F2	AGAGCTGTGATCACGCCAACTGAGGTTTTCCAGGATAAC
TRAF5T2-R1	GATAGCTTTGTCTTCCTCCTGCAGATCGTTTAATGTGAA
TRAF5T2-F2	GAAGACAAAGCTATCAATCATCACATGCTGCTGGTCC
TRAF5T3-R1	TAACTTCTCCTCCAA AGGATGAAGCGCCCGAGAC
TRAF5T3-F2	TTGGAGGAGAAGTTAAAGCGTCACTCAGGCCTCCTA
TRAF5T4-R1	ATTTTGGGGAGTTTCGATCAACTTCCCATCATAGGATGTC
TRAF5T4-F2	GAAACTCCCCAAAATGCTGTGTACGTTAAAGACAACACG
TRAF5T1/T2/T3/T4-R2	CCGGAATTCCAGCTGCTCAAAACCTGTCATATC

(OnTRAF5) was obtained from Nile tilapia. Then tissue distribution and expression profiles of OnTRAF5 were detected by qRT-PCR. To analysis the function of OnTRAF5 in signaling pathway, the effect of OnTRAF5 on NF-κB was investigated. Additionally, which structure domains exert function in this processing also explored.

2. Materials and methods

2.1. Bacteria, fish, stimulation, and sampling

In *S. agalactiae* culture, bacteria were inoculated in brain heart infusion (BHI) and incubated in a shaker (120 rpm) at 28 °C overnight. For the challenge, the concentration of the bacteria was 1×10^7 CFU/mL. To make inactivate cells, formalin was added in the culture, which final concentration was 0.2%. The cells were disposed 24 h at 28 °C, the inactive effect was detected by BHI agar plate.

Fifty healthy tilapia (average weight of 100 ± 10 g) were purchased from local commercial market(Shenzhen, China). Fish were acclimated in aerated sand-filtered water at 28 °C and fed daily with commercial feed. Three fish were randomly selected for sample with gill, skin, muscle, head kidney, heart, intestine, thymus, liver, spleen and brain. Then samples were immediately frozen by liquid nitrogen until used for next analysis. Tilapias were injected intraperitoneally with 100 μL of formalin-inactivated *S. agalactiae* ZQ0910 suspension (1×10^7 cells mL^{-1}). Meanwhile, the control group was treated with 100 μL 1 × PBS. At each timepoint of 0 h, 12 h, 24 h, 48 h, 72 h and 96 h following treatment, gill, skin, head kidney, spleen, intestine and thymus from 36 fish were sampled (3 fish per replicate) for real-time PCR. All samples were immediately frozen by liquid nitrogen, and then reserved at −80 °C for quantitative real-time PCR analysis.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from the tissue of Nile tilapia using TransZol Up (Trans, Beijing, China). The quality of total RNA was detected by Qubit3.0. After treatment by EasyScript One-step gDNA Removal (Trans, Beijing, China), those RNA were used for the first-strand cDNA synthesis through cDNA Synthesis SuperMix (Trans, Beijing, China).

2.3. Cloning of cDNA sequence

The special primers were designed according to the whole-genome sequencing data of *O. niloticus* in NCBI. Using the primer TRAF5-F/TRAF5-R, PCR was conducted by Thermal Cycler 2720 (Thermofisher), under the following amplification conditions: 94 °C for 5 min, followed by 35 cycles at 94 °C for 30s, 61 °C for 30 s, and 72 °C for 2 min, with a final extension at 72 °C for 10 min.

2.4. Bioinformatics

The open reading frame (ORF) was analyzed by ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The nucleotide and predicted amino acid sequences of OnTRAF5 were analyzed using Genetyx 7.0 software. The protein structure was analyzed with ExPASy tools (http://expasy.org/tools/). Multiple amino acid sequences alignment of TRAF5 was performed using ClustalX, a phylogenetic tree was constructed through MEGA 5.0 software by the neighbor-joining method.

2.5. Real-time PCR

qRT- PCR was used to analysis the expression profiles of TRAF5 using gene specific primer RT-TRAF5-F/RT-TRAF5-R. And β-actin (GenBank no. XM_003443127.5) was used as a reference gene for normalization of the expression level. The sequences of primer were listed in Table 1. Reaction was performed by IQ5 Real-time PCR System (Bio-Rad laboratories) with SYBR Green Master mix (TOYOBO). The reaction volume contained 0.3 μl cDNA sample, 5 μl SYBR Green Master mix, 0.5 μL (10 μM) each primer and 3.7 μL PCR-grade water. PCR conditions were as follows: 94°Cfor 3 min, followed by 40 cycles of 94°Cfor 30s, 55 °C for 30s and 72 °C for 30s. Each sample was assayed in triplicate. Data of this part was analyzed by $2^{-\triangle\triangle Ct}$ method.

2.6. Construction of expression plasmids

The whole ORF of TRAF5 was amplified with primers pcDNA-5F/5R containing KpnI and EcoRI restriction enzyme sites (Table 1) and inserted into the corresponding sites of the pcDNA3.1 plasmid respectively. Four mutants of recombinant plasmids OnTRAF5 were constructed by special primers (Table 1), including ΔRING, ΔZinc finger, Δcoiled-coil and ΔMATH. The recombinant plasmids were extracted using an E.Z.N.A. Endo-free Plasmid Mini Kit (Promega), which were used for analysis the effect on NF-κB activity.

2.7. Luciferase reporter assays

HEK-293T (1×10^6 cells/well) cells were seeded into 48-well plates and cultured for 24 h at 37 °C, then 254 ng plasmids containing 125 ng recombinant or pcDNA3.1 plasmids, 125 ng NF-κB reporter plasmids and 4 ng pRL-TK Renilla (*Renilla reniformis*) luciferase plasmids were co-transfected using Lipofectamine™3000((Invitrogen, USA). Each sample was run in triplicate. After 48 h, cells were washed with passive lysis buffer (Promega, USA). Relative luciferase activity was calculated as the activity of firefly luciferase relative to Renilla luciferase.

2.8. Statistical analysis

Data were expressed as mean ± SD and statistical analysis was performed using SPSS software. Differences were considered significant if the probability (P) < 0.05.

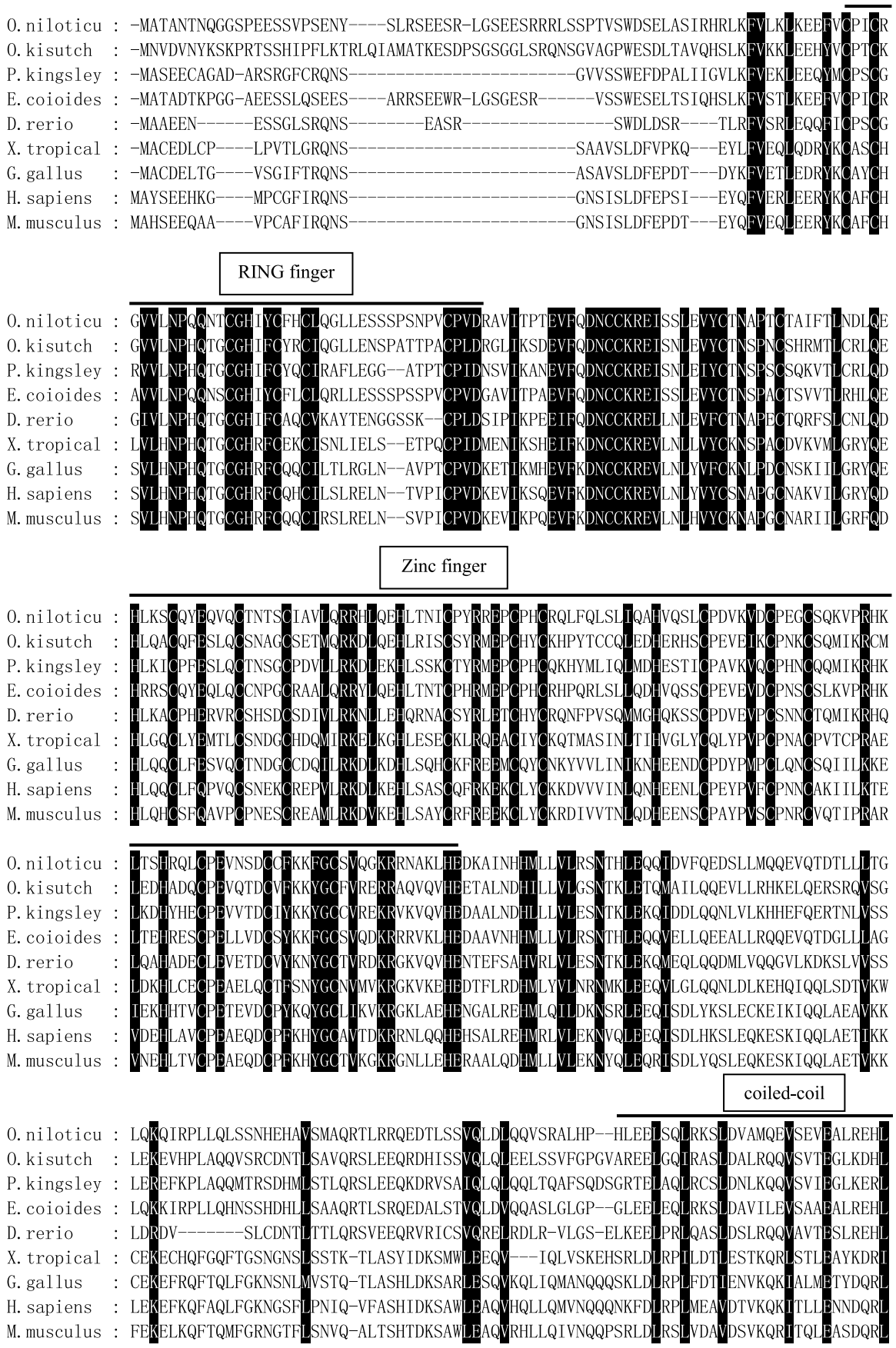

Fig. 1. Multiple alignment of TRAF5 proteins. The relatively conserved functional domains including RING finger, zinc finger, coiled-coil and MATH domain were underlined.

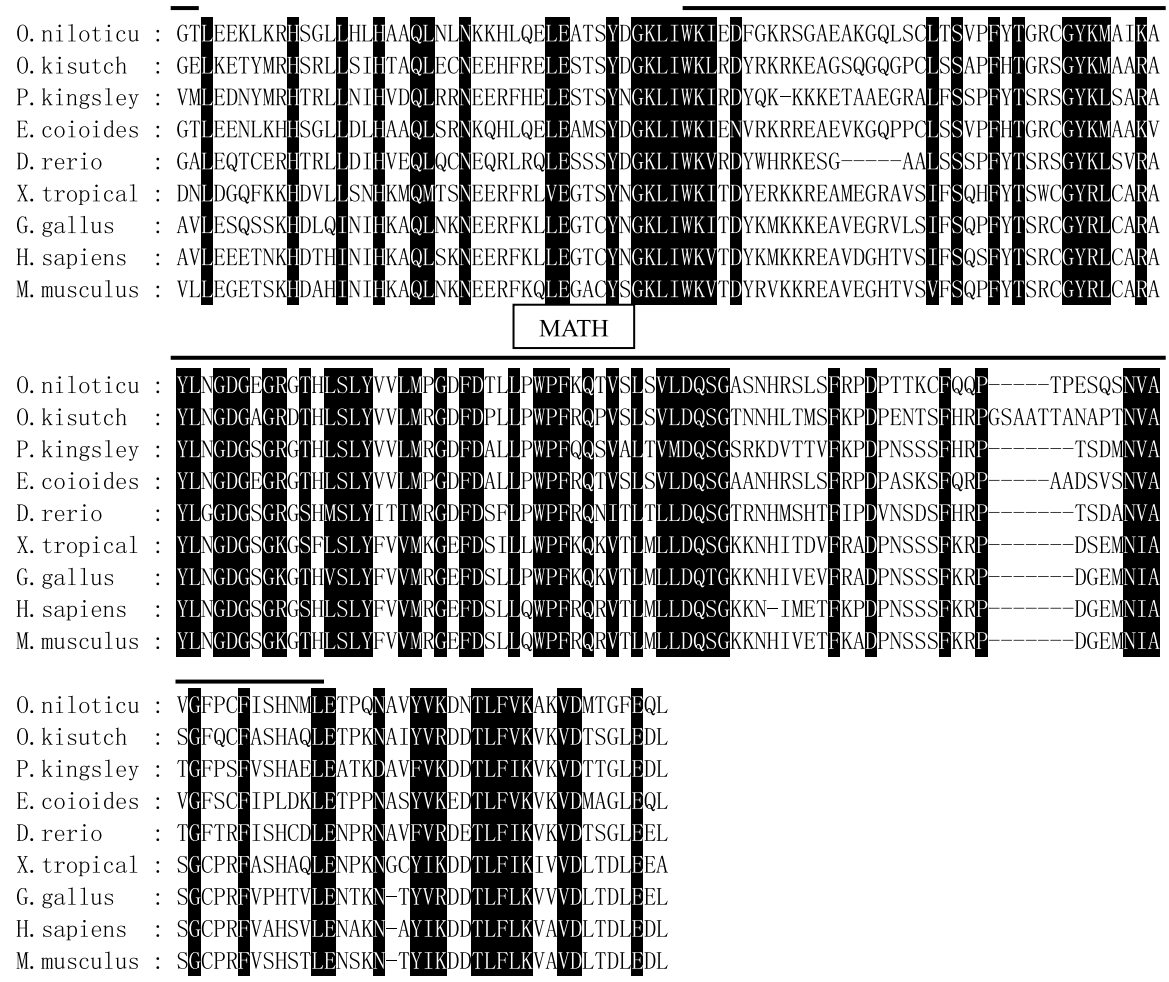

Fig. 1. (*continued*)

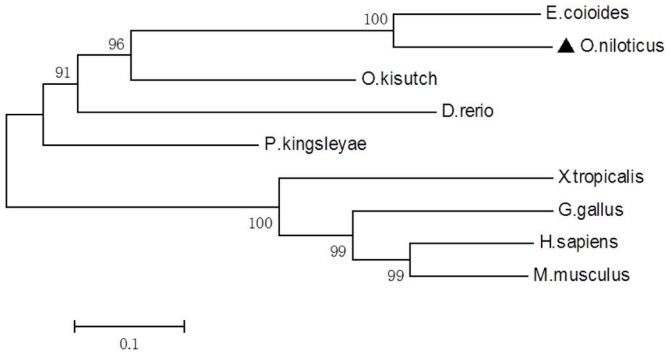

Fig. 2. Phylogenetic analysis of OnTRAF5. Amino acid sequence of OnTRAF5 is marked by solid triangle.

3. Results

3.1. Bioinformatic analysis of OnTRAF5

The complete coding sequence of OnTRAF5 (GenBank no. MN149369) was 1764bp encoding 587 amino acids. By analysis the predicted protein, RING, zinc finger, coiled-coil and MATH, which were highly conserved in other TRAFs, were also found in OnTRAF5 (Fig. 1). The deduced amino acid of OnTRAF5 shared highest identity (95%) to *Maylandia zebra* TRAF5. Additionally, a phylogenetic tree was constructed using MEGA5.0. OnTRAF5 fell into the cluster with other piscine TRAF5, which was separate from mammals (Fig. 2).

3.2. Expression profiles of OnTRAF5

OnTRAF5 was ubiquitously expressed in all the tissues tested. The highest expression level of OnTRAF5 was detected in most of immune organs, including intestine, gill and spleen. While little higher profile was detected in head kidney, skin and brain, which was about > 95 fold the level detected in muscle, the tissue with the lowest level of OnTRAF5 transcript (see Fig. 3).

As shown in Fig. 4, OnTRAF5 expression in the gill was initially down regulated, then increased significantly and had a highest expression level at 12 h following the challenge with *S. agalactiae*, followed by subsequently declined and kept basal level until 96 h. A similar phenomenon was detected in spleen, head kidney and intestine, while the highest expression was found at 24 h, 48 h and 72 h respectively. In skin, the expression of OnTRAF5 also firstly declined and then increased remarkably, dropped to the basic level at last. Meanwhile, the highest level maintained from 48 h to 72 h. Compared to the control, expression level of OnTRAF5 was down-regulated at 0–12 h post-injection, little increased at time points of 24 h followed by once-again up-regulation significantly at 96 h in the thymus (see Fig. 4).

3.3. Activation of NF-κB pathway

To determine whether OnTRAF5 could induce NF-κB activity, like mammals TRAF5, cotransfections were performed with an NF-κB-Luc reporter gene and pcDNA3.1-OnTRAF5. As shown in Fig. 5A, over expression of OnTRAF5 efficiently induced the activation of NF-κB signaling pathway. Moreover, Over-expression WT-OnTRAF5, Δcoiled-coil

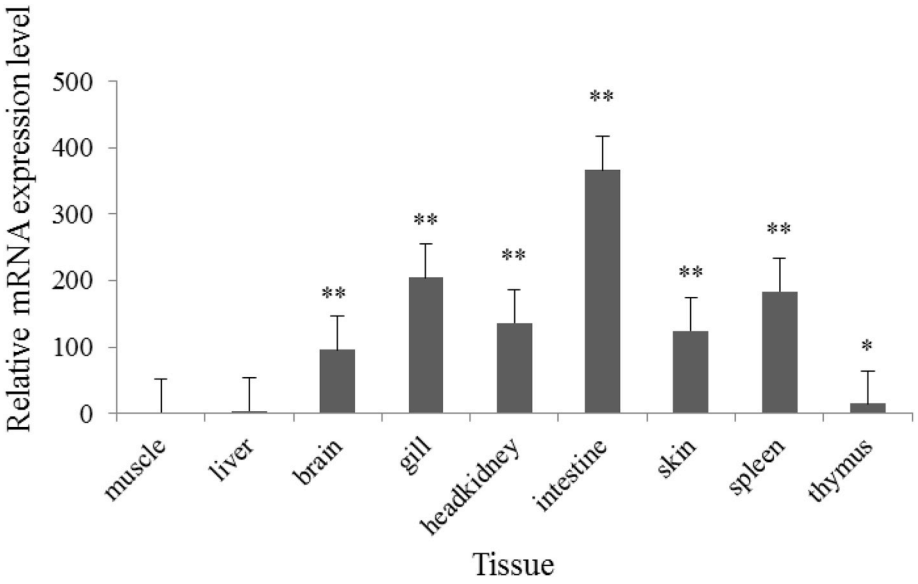

Fig. 3. Constitutive expression of OnTRAF5 in healthy tilapia tissues was determined with qRT-PCR. The mRNA expression levels were normalized to β-actin transcripts. Vertical bars represented the means ± SD. Significant difference was indicated by asterisks, *p < 0.05 or **p < 0.01.

and ΔMATH activated NF-κB activity in a large degree, but not ΔRING or Δzinc finger in Fig. 5B. These results revealed the prominent regulation of OnTRAF5 in NF-κB, which was dependent upon the RING and zinc finger.

4. Discussion

TRAFs have been proved participate in regulation of multiple pathways, which control immune response processing and apoptosis [24]. Although TRAF3/6 was extensively analyzed in teleost, functional study about TRAF5 was only reported in *Epinephelus coioides* [25–42]. In this study, TRAF5 from tilapia was obtained according the genomic data in NCBI. Like other TRAF5 proteins, OnTRAF5 also contained RING, zinc finger, coiled-coil and MATH domain. Protein blast analysis showed that OnTRAF5 had highest identify with *Maylandia zebra* TRAF5. While multiple alignment result showed that OnTRAF5 had lower identifies with TRAF5 from *Epinephelus coioides* (74%), *Oncorhynchus kisutch* (53%) and *Danio rerio* (46%), as shown in Table 2. Phylogenetic analysis showed OnTRAF5 and TRAF5 from other fish species clustered into one group, most tightly clustered with *Epinephelus coioides*.

In mammals, TRAF5 are broadly existed in many immune related tissues. High expression level of TRAF5 was detected in epidermis, spleen, lung, and thymus [6,43]. The expression level of EcTRAF5 was significantly higher in the skin, hindgut and head kidney than other tissues [42]. Similar results were found in this regard. We found that OnTRAF5 had a relatively higher expression level in intestine, gill and spleen. Both intestine and gill are mucosa-associated lymphoid tissue, which function as first line of defense pathogens in teleost fish [44,45]. However, the basic expression level of TRAF5 in healthy intestine tissues needs to be considered, so the amount of induced production of TRAF5 at 12–24 h post injection is also considerable. Additionally, spleen seems to represent a major hematopoietic organ and acts in the initiation of the adaptive immune response [46,47]. Recent studies have demonstrated that mammals TRAF5 play diverse roles for different cell types or variety of receptors. For example, TRAF5-deficient T cells have significant defects in proliferation, IL-2 production, and NF-κB, p38, and ERK1/2 activation [12]. However, TRAF5 negatively regulates TLR-mediated cytokines and antibody production in B lymphocytes [48]. We speculated that OnTRAF5 function varied in distinct immune pathway with different kinetics. That's may be the reason why these expression pattern difference among the tissues at different time post affection. We hypothesized that OnTRAF5 also plays an important role in immunity.

After *S. agalactiae* stimulation, the expression of OnTRAF5 from different tissues was up-regulated in some degree. Especially in thymus, the firstly up-regulation was very tightly, while secondly increase was remarkable. This may explained by OnTRAF5 participate in both innate immune and adaptive immune response.

Although TRAF5 is most structurally similar to TRAF3, it has been suggested to be most functionally similar to TRAF2, as both are positive regulators of NF-κB, stress-activated protein kinase (SAPK) and c-Jun N-terminal kinase (JNK) signaling pathway [8,12,49]. In mammals, TRAF5 has been shown to induce the activation of both NF-κB1 and NF-κB2 [15,50,51]. In grouper, EcTRAF5 activated NF-κB signal, RING and zinc finger play pivotal role in this promote processing [42]. Our findings uncovered that OnTRAF5 induced NF-κB signal, this induction was impaired when RING and zinc finger missing. Those results were in line with previous reports. For example, Hiroyasu found that TRAF5 activate p65-p50 NF-kB complex, which is dependent upon the zinc region [50]. Furthermore, TRAF5 lacking RING domain inhibited the induction of CD23 expression triggered by CD40 [6]. Binding to our study, we can know that RING and zinc finger domain is essential for TRAF5 exert functional role.

5. Conclusions

In this study, the whole coding sequence of TRAF5 was obtained from Nile tilapia. Like TRAF3, OnTRAF5 contained RING, zinc finger, coiled-coil and MATH domain. OnTRAF5 had higher expression level in immune related organs. After *S. agalactiae* challenge, expression level of OnTRAF5 was increased in some degree. Furthermore, RING and zinc finger of OnTRAF5 were pivotal for activating NF-κB pathway. We concluded that OnTRAF5 participate in antimicrobial immune response, which also involved in signaling transduction.

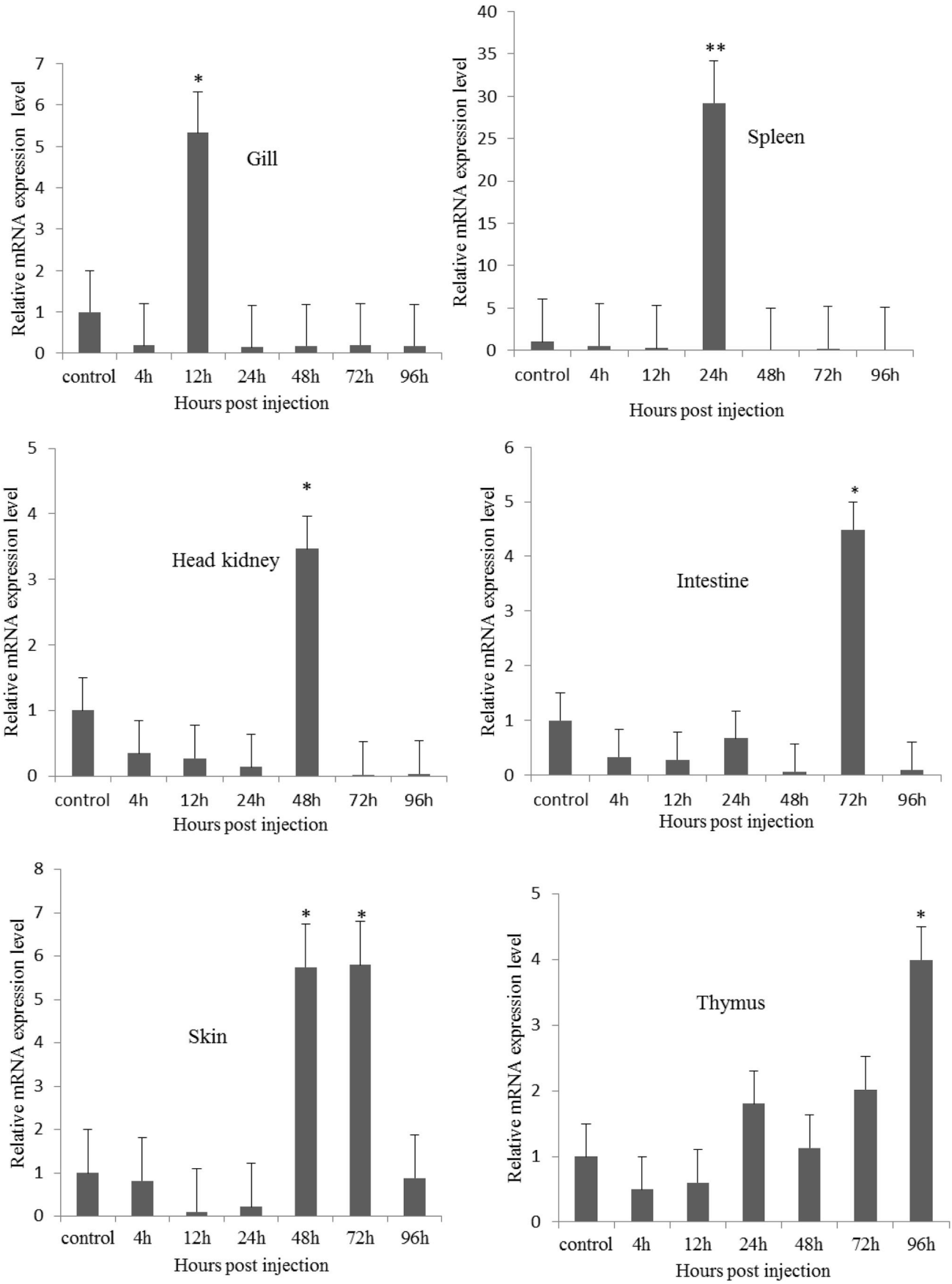

Fig. 4. Expression profiles analyses of OnTRAF5 in different tissues after S. agalatiae stimulation. Relative expression was calculated by the $2^{-\triangle\triangle Ct}$ method. The values are shown as mean ± SD. Significant difference was indicated by asterisks, *$p < 0.05$ or **$p < 0.01$.

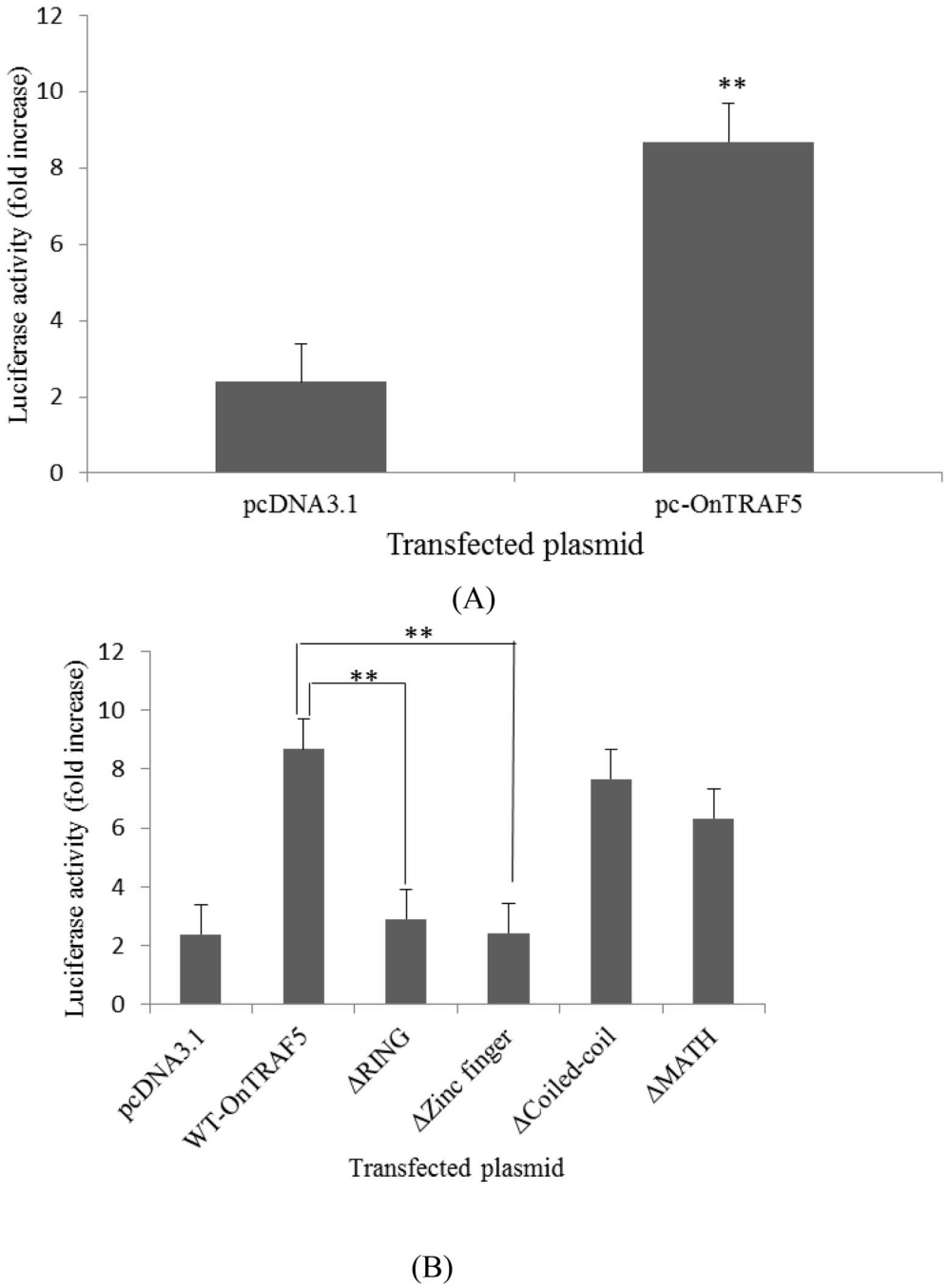

Fig. 5. (A): Over-expression of OnTRAF5 significantly activated the activity of NF-κB in HEK293 cells. (B): While the RING or zinc finger missing, the activation of NF-κB was impaired remarkably. The values are shown as mean ± SD. Significant difference was indicated by asterisks, **p < 0.01.

Table 2
Amino acid identity (%) of TRAF5 between tilapia and other vertebrates.

Species	Genebank No.	Identify (%)
Maylandia zebra	XP_004572683.1	95
Epinephelus coioides	AME21334.1	74
Oncorhynchus kisutch	XP_020323447.1	53
Scleropages formosus	XP_018606186.1	50
Paramormyrops kingsleyae	XP_023686168.1	50
Ctenopharyngodon idella	QDF63035.1	47
Cyprinus carpio	XP_018920623.1	46
Carassius auratus	XP_026053566.1	46
Danio rerio	XP_021325328.1	46

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