Fish and Shellfish Immunology 84 (2019) 927–936

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Molecular cloning and functional characterization of TRAF6 and TAK1 in rainbow trout, *Oncorhynchus mykiss*

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ARTICLE INFO

Keywords:
Tumor necrosis factor receptor-associated factor 6
Transforming growth factor β-activated kinase 1
Innate immunity
Teleost
Rainbow trout
RTH-149

ABSTRACT

TRAF6 and TAK1 are known to play important roles in vertebrate innate immunity as molecular bridge, linking upstream toll-like receptors (TLRs) with the downstream MAPK and NF-κB signalling pathways. However, their roles in TLR signalling pathway have yet to be fully described in fish. Here we identified genes encoding TRAF6 (OmTRAF6) and TAK1 (OmTAK1) from rainbow trout, *Oncorhynchus mykiss*, and examined their roles during pathogenic infections. The deduced amino acid sequences of OmTRAF6 and OmTAK1 contained the characteristic domains conserved in the TRAF and TAK1 families, respectively (OmTRAF6: RING, two TRAF-type zinc fingers, CCR and MATH domains; OmTAK1: STKc and CCR domains). In RTH-149 cells, the expression of OmTRAF6 and OmTAK1 was increased by stimulation with *Edwardsiella tarda* and LPS. Silencing of OmTRAF6 and OmTAK1 in RTH-149 cells negatively regulated the LPS-induced phosphorylation of p38 MAPK and JNK. TAK1 inhibitor (5z)-7-Oxozeaenol significantly decreased the LPS-induced activation of NF-κB in RTH-149 cells. In addition, silencing of OmTRAF6 and OmTAK1 significantly decreased the expression of MAPKs and NF-κB downstream target genes induced by LPS in RTH-149 cells. These findings suggest that OmTRAF6 and OmTAK1 might function like those of mammals to regulate bacteria-triggered signalling pathway in fish.

1. Introduction

The innate immune system constitutes the first line of defense against infection [1]. The main role of this system is to recognize invading pathogens at an early stage and trigger an appropriate inflammatory response. The innate immune response relies on the recognition of evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs), by a variety of germline-encoded pattern recognition receptors (PRRs) that are expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids [2,3]. Among various PRRs, the TLR family is one of the best-characterized PRR families and is responsible for sensing invading pathogens outside of the cell and in intracellular endosomes and lysosomes [4]. Upon recognition of respective PAMPs, TLRs recruit a specific set of adaptor molecules that harbor Toll/interleukin-1 receptor (TIR) domain, such as myeloid differentiation primary response protein (MyD88) and TIR domain-containing adaptor inducing interferon-β (TRIF), and initiate downstream signalling events that leads to the secretion of inflammatory cytokines, type I IFN, chemokines and antimicrobial peptides [2].

The mechanisms underlying the ligand specificity, signalling pathways and cellular trafficking of TLRs have been extensively characterized in mammals. In TLR signalling pathways, tumor necrosis factor receptor-associated factor 6 (TRAF6) and transforming growth factor-β-activated kinase 1 (TAK1) are two important molecules [5,6]. Upon stimulation of TLRs, TRAF6 is recruited to the receptor complex, and activated by IL-1 receptor-associated kinase (IRAK) 1 that binds to the TRAF domain of TRAF6. Then, the IRAK1/TRAF6 complex dissociates from the receptor and associates with TAK1 and TAK1-associated binding proteins, TAB1 and TAB2, at the membrane portion. IRAK1 stays in the membrane and is degraded, whereas the complex of TRAF6, TAK1, TAB1 and TAB2 moves into the cytoplasm, where it forms a large complex with the E2 ligases ubiquitin-conjugating enzyme 13 (Ubc13) and ubiquitin E2 variant 1a (Uev1A) [7]. TRAF6 functions as an E3 ubiquitin protein ligase together with Ubc13/Uev1A to catalyse the formation of a K63-linked polyubiquitin chain on itself, which then activates TAK1 [8–10]. Subsequently, TAK1 phosphorylates and activates mitogen-activated protein kinase (MAPK) and IκB kinases (IKKs), which leads to the activation of transcription factors activator protein 1 (AP-1) and nuclear factor (NF)-κB, which in turn regulate the expression of genes involved in the inflammatory response [3].

To date, 21 TLRs (TLR1-5, 5S, TLR7-9, TLR13, TLR14, TLR18-23

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https://doi.org/10.1016/j.fsi.2018.11.002
Received 13 August 2018; Received in revised form 29 October 2018; Accepted 1 November 2018
Available online 02 November 2018
1050-4648/

and TLR25-28) have been identified in many different teleost fish species, including both, orthologs of mammalian TLRs and teleost-specific TLRs [11–14]. Key features of teleost fish TLRs and the factors involved in their signalling cascade have high structural similarity to the mammalian TLR system. Recently, orthologs of human TRAF6 and TAK1 have been reported in several teleost fish species, including TRAF6 of zebrafish (*Danio rerio*) [15], common carp (*Cyprinus carpio*) [16], grass carp (*Ctenopharyngodon idella*) [17], orange-spotted grouper (*Epinephelus coioides*) [18], grouper (*Epinephelus tauvina*) [19], blunt snout bream (*Megalobrama amblycephala*) [20], rock bream (*Oplegnathus fasciatus*) [21] and black carp (*Mylopharyngodon piceus*) [22] and TAK1 of grass carp [17], orange-spotted grouper [23] and large yellow croaker (*Larimichthys crocea*) [24]. TRAF6 and TAK1 are known to play important roles in vertebrate innate immunity as molecular bridge, linking upstream TLRs with the downstream MAPK and NF-κB signalling pathways. However, their roles in TLR signalling pathway have yet to be fully described in fish. The innate immune response has been considered an essential component in combating disease incidents in fish due to the constraints placed on the adaptive immune response by their poikilothermic nature plus the limited antibody repertoires, affinity maturation, memory and relatively slow lymphocyte proliferation [25]. Therefore, understanding the mechanism that underlie pathogen recognition and subsequent orchestration of the innate immune response in fish is of significant importance for both basic research and management of health in aquaculture. In this study, we identified genes encoding TRAF6 (OmTRAF6) and TAK1 (OmTAK1) from rainbow trout, *Oncorhynchus mykiss*, which is one of the most important cold water fish species due to its importance for food production, sport fisheries and as a research model [26], and investigated their roles in eliciting innate immune response to bacterial infections in a rainbow trout hepatoma cell line RTH-149.

2. Materials and methods

2.1. Reagents

Staphylococcus aureus peptidoglycan (PGN) and lipoteichoic acid (LTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) was purchased from InvivoGen (San Diego, CA, USA). The antibodies against TAK1, phosphorylated TAK1 (Thr184/187), phosphorylated p38 MAPK (Thr180/Tyr182), jun-amino-terminal kinase (JNK), phosphorylated JNK (Thr184/Tyr187) and β-actin were from Cell Signaling Technology (Beverly, MA, USA). The antibodies against p38 MAPK (ab170099) and TRAF6 (ab33915) were from Abcam (Cambridge, UK). The TAK1 inhibitor (5z)-7-Oxozeaenol was from Calbiochem (San Diego, CA, USA).

2.2. Fish cell culture

The rainbow trout hepatoma cell line RTH-149 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Eagle's minimal essential medium, supplemented with non-essential amino acids, 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 25 mM HEPES, 10,000 units/ml penicillin and 10 mg/ml streptomycin. Cells were grown at 19 °C in the absence of CO_2. Trypsin-EDTA (0.05%) was used to detach cells for subculturing. All the cell culture media and reagents were purchased from Lonza (Basel, Switzerland).

2.3. cDNA cloning of the genes encoding rainbow trout TRAF6 and TAK1

Total RNA was extracted from rainbow trout fingerlings (10 g each, provided from a fish farm near Geochang, Korea), using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). A first-strand cDNA was synthesized from 1 μg of total RNA using a CapFishing Kit (Seegene, Seoul, Korea) according to the manufacturer's procedure. To clone rainbow trout TRAF6 and TAK1, two pairs of degenerate primers (TRAF6-FDP/TRAF6-RDP for TRAF6; TAK1-FDP/TAK1-RDP for TAK1) were designed based on the conserved sequences of TRAF6 and TAK1 homologues from zebrafish, pufferfish, salmon and grass carp, and used as gene specific primers for amplifying partial rainbow trout TRAF6 and TAK1 cDNAs. The PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and the selected clones were sequenced in both directions by using T7 and SP6 primers. The 5′- and 3′-ends were obtained by 5′- and 3′-RACE with four intragenic primers (TRAF6-IGP-R and TRAF6-IGP-F for 5′-RACE and 3′-RACE of TRAF6, respectively; TAK1-IGP-R and TAK1-IGP-F for 5′-RACE and 3′-RACE of TAK1, respectively). The 5′- and 3′-RACE products were cloned and sequenced as described above. Then, two pairs of primers (TRAF6FL-F/TRAF6FL-R for TRAF6; TAK1FL-F/TAK1FL-R for TAK1) were designed based on tentative full-length cDNAs, and the sequences of resulting PCR products (named OmTRAF6 and OmTAK1) were verified again. All primers used in the study were listed in Table 1.

Table 1
List of primer sequences used in the study.

Name	Sequence	Length (nt)
TRAF6-FDP	5′-GAT AAC TTT GCC AAR MGR GAG A-3′	22
TRAF6-RDP	5′-CCY TGC ATR GTG TGM ACR AA-3′	20
TRAF6-IGP-F	5′-CTT GGA GGG CTT CTC GGG CTA C-3′	22
TRAF6-IGP-R	5′-GGT GCA TCT CCA GCT GGC TC-3′	20
TRAF6FL-F	5′-ATG TCC TGC TTT GAG AGT GAT AAG-3′	24
TRAF6FL-R	5′-TCA CAG CGA GGC CTC GGG CC-3′	20
TAK1-FDP	5′-TAT CTC CAY GSC ATG AAA CC-3′	20
TAK1-RDP	5′-GGT RGA SAG ACT CTT GTT C-3′	19
TAK1-IGP-F	5′-GAG CAG CAC TGT AAA ATG GCC-3′	21
TAK1-IGP-R	5′-CTT CAA ACA CCT CTG GGG CCA-3′	21
TAK1FL-F	5′-ATG TCT CTG CCA TCC GCT GAT-3′	21
TAK1FL-R	5′-TCA CGA GGT GCC CTG TCT C-3′	19
TRAF6F	5′-TGT CCG AAG GCT CCG ATT GCC TG-3′	23
TRAF6R	5′-GTA CCC GGT AAG GCT GTG CCT G-3′	22
TAK1F	5′-AGC ATG GGG ACT CTC CAG GGA G-3′	22
TAK1R	5′-AGG CCA CCA GGC TGT GGG TTC-3′	21
IL-1βF	5′-ACA TTG CCA ACC TCA TCA TCG-3′	21
IL-1βR	5′-TTG AGC AGG TCC TTG TCC TTG-3′	21
TNF-αF	5′-GGG GAC AAA CTG TGG ACT GA-3′	20
TNF-αR	5′-GAA GTT CTT GCC CTG CTC TG-3′	20
IL-6F	5′-CCT TGC GGA ACC AAC AGT TTG-3′	21
IL-6R	5′-CCT CAG CAA CCT TCA TAT GGT C-3′	22
IL-8F	5′-AGA ATG TCA GCC AGC CTT GT-3′	20
IL-8R	5′-TCT CAG ACT CAT CCC CTC AGT-3′	21
EF1-αF	5′-GCT GGA CAA GCT GAA GGC TGA G-3′	22
EF1-αR	5′-AGC GCA ATC AGC CTG AGA GGT A-3′	22

Note: Y = C/T; S = G/C; R = A/G; M = A/C.

2.4. Sequence analysis

The deduced amino acid sequences of OmTRAF6 and OmTAK1 were searched for similarity using BLAST program at the ExPASy Molecular Biology Server (http://www.expasy.org/tools/blast/). The RING, TRAF, coiled-coil region (CCR) and meprin and TRAF homology (MATH) domains of OmTRAF6 and the serine/threonine protein kinase catalytic domain (STKc) and CCR of OmTAK1 were predicted using SMART (http://smart.embl-heidelbergde) followed by Pfam (http://pfam.sanger.ac.uk/). Alignment of multiple sequences was performed using the MEGALIGN program within Lasergene (DNASTAR, Madison, WI, USA) and a phylogenetic tree was constructed using the MEGA6 Neighbor-joining. All sequences used in the analysis were listed in Table 2.

2.5. RNA extraction, cDNA synthesis and quantitative real-time PCR

To determine tissue expression patterns of OmTRAF6 and OmTAK1, skin, eye, gill, head kidney, intestine, kidney, liver and spleen were

Table 2
TRAF6 and TAK1 protein sequences used for multiple sequence alignment and phylogenetic tree construction.

Species	Protein	Accession no.	Species	Protein	Accession no.
Homo sapiens	TRAF6	AAH31052	*Cyprinus carpio*	TRAF6a	ADF56651
	TAK1	NP_003179		TRAF6b	ADM45856
Macaca mulatta	TRAF6	EHH22980	*Anas platyrhynchos*	TRAF6	XP005011386
	TAK1	AFI35225	*Takifugu rubripes*	TRAF6	XP003969671
Rattus norvegicus	TRAF6	XP230377.4	*Epinephelus tauvina*	TRAF6	AHN13885
	TAK1	NP001101390.2	*Paralichthys olivaceus*	TRAF6	AJE25833
Mus musculus	TRAF6	NP033450	*Lutjanus sanguineus*	TRAF6	AIC37510
	TAK1	Q62073.1	*Oplegnathus fasciatus*	TRAF6	AJW66354
Sus scrofa	TRAF6	NP001098756	*Plecoglossus altivelis altivelis*	TRAF6	BAI68387
	TAK1	NP001107752	*Xenopus tropicalis*	TRAF6	AAH82342
Bos Taurus	TRAF6	AAI02523	*Alligator sinensis*	TRAF6	XP006014704
	TAK1	NP001075064	*Carassius auratus auratus*	TRAF6	AHG97567
Gallus gallus	TRAF6	XP421089.2	*Pelodiscus sinensis*	TRAF6	XP006124531
	TAK1	XP419832.3	*Meleagris gallopavo*	TRAF6	XP003206439
Xenopus laevis	TRAF6	BAE44508	*Euprymna scolopes*	TRAF6	AAY27978
	TAK1	AAC14008	*Chlamys farreri*	TRAF6	DQ350773
Oryzias latipes	TRAF6	XP004066897	*Mytilus galloprovincialis*	TRAF6	AHI17288
	TAK1	XP004083957	*Mylopharyngodon piceus*	TRAF6	AUW56621.1
Oreochromis niloticus	TRAF6	XP003450846	*Salmo salar*	TAK1	ACN10470
	TAK1	XP003450846	*Equus caballus*	TAK1	XP001503830
Epinephelus coioides	TRAF6	AGQ45557	*Capra hircus*	TAK1	XP005684732.2
	TAK1	AGQ48129	*Chrysemys picta bellii*	TAK1	XP005299794
Oncorhynchus mykiss	TRAF6	KY489770	*Maylandia zebra*	TAK1	XP004564764
	TAK1	MF671982	*Ciona intestinalis*	TAK1	NP001071829
Ctenopharyngodon idella	TRAF6	AGI51678	*Litopenaeus vannamei*	TAK1	ANR02615
	TAK1	AGI51677	*Branchiostoma floridae*	TAK1	XP002589769
Danio rerio	TRAF6	AAT37634	*Bombyx mori*	TAK1	XP004931740
	TAK1	NP001018586	*Apis mellifera*	TAK1	XP006572357
Drosophila melanogaster	TRAF6	AAF46338	*Culex quinquefasciatus*	TAK1	EDS27685
	TAK1	NP524080	*Caenorhabditis elegans*	TAK1	CAB01866
			Larimichthys crocea	TAK1	ATN39893.1

collected separately from four healthy fish for RNA extraction. To examine the variation in expression of OmTRAF6 and OmTAK1 in RTH-149 cells following the stimulation with heat-killed *Edwardsiella tarda* (ATCC 15947), 1×10^6 RTH-149 cells were seeded per well into 6-well plates, and stimulated for 0, 6, 12, 24 and 48 h with 2×10^8 cells/ml of heat-killed *E. tarda* (70 °C, 30 min). To examine the variation in expression of OmTRAF6 and OmTAK1 in RTH-149 cells following the stimulation with bacterial ligands (LPS, LTA and PGN), 1×10^6 RTH-149 cells were seeded per well into 6-well plates, and stimulated with different concentrations of bacterial ligands for 6 h. Total RNA was then extracted using an RNeasy kit (Qiagen, Hilden, Germany) as described by the manufacturer. To assure absence of genomic DNA, all RNA samples were treated with RNase-free DNase I. 1 μg RNA from different samples was reverse-transcribed with an iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR) was performed on a CFX96™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). Each 20 μl of reaction mixture contained 5 μl of cDNA (corresponding to 50 ng RNA), 1 μl of each primer (forward and reverse primers), 10 μl of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) and 3 μl of nuclease-free water. The thermocycling parameters were 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. A melt curve was inserted at the end of each run, with readings from 65 °C to 95 °C, every 1 °C for 5 s, to confirm that only the desired product was amplified. The relative expression of target genes (OmTRAF6, OmTAK1, IL-1β, TNF-α, IL-6 and IL-8) was normalized to the expression of elongation factor 1-α (EF1-α), and quantified using the comparative *Ct* method [27]. Data were presented as fold change relative to the corresponding control group. All the primer sets used in the qRT-PCR (TRAF6F/TRAF6R for OmTRAF6; TAK1F/TAK1R for OmTAK1; IL-1βF/IL-1βR for IL-1β; TNF-αF/TNF-αR for TNF-α; IL-6F/IL-6R for IL-6; IL-8F/IL-8R for IL-8; EF1-αF/EF1-αR for EF1-α) were listed in Table 1.

2.6. Western blot analysis

RTH-149 cells were seeded onto 6-well plates at a density of 1×10^6 cells/well in 3 ml of complete medium. After 24 h of incubation, cells were stimulated for 0, 3, 6, 12 and 24 h with 0.1 μg/ml LPS. Cells were harvested and lysed in ice-cold PRO-PREP Protein Extraction Solution (Intron). The protein concentration was quantified by using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA), and equal amounts of protein (40 μg) were subjected to 12% SDS-PAGE. Separated proteins were transferred to polyvinyldifluoride membranes (Millipore, Billerica, MA, USA), the membranes were blocked with 5% (w/v) BSA in TBS containing 0.1% Tween-20, and conventional immunoblot was performed using appropriate antibodies. Chemiluminescence was detected using an ECL kit (Promega).

2.7. NF-κB assay

About 1×10^5 RTH-149 cells cultured in 24-well plates were co-transfected with a total of 1 μg pNF-κB-Luc (Clontech Laboratories, Inc., Mountain View, CA, USA) with 50 ng of the pRL-TK vector (Promega) as internal control using Lipofectamine 2000 reagent (Invitrogen). At 48 h after transfection, cells (except control wells) were stimulated with 0.1 μg/ml LPS for indicated times. In some experiments, cells were pretreated with 1 μM TAK1 inhibitor [(5z)-7-Oxozeaenol] for 60 min before stimulated with LPS. Next, cells were lysed in lysis buffer (Promega) and assayed for firefly and Renilla luciferase activities. Firefly luciferase activity was normalized with that of Renilla and expressed as the relative-fold change compared with the non-treated control. The results were obtained from three independent experiments performed in triplicate.

2.8. Silencing of OmTRAF6 and OmTAK1 expression in RTH-149 cells

siRNA sequences targeting OmTRAF6 [5′-GCC UUA UUC UGU UCA

AUG UUU-3' (sense) and 5′-ACA UUG AAC AGA AUA AGG CUU-3' (antisense)], OmTAK1[5′-CUA CUA CUA GCC AUU GUU AUU-3' (sense) and 5′-UAA CAA UGG CUA GUA GUA GUU-3' (antisense)] and non-targeting negative control sequence [5′-AGA UCC GCU ACU GUC CGA AUU-3' (sense) and 5′-UUC GGA CAG UAG CGG AUC UUU-3' (antisense)] were synthesised at Genolution (Seoul, Korea). RTH-149 cells (1×10^5 cells/well in 6-well plates) at 40% confluence were transfected with each siRNA (60 pmol) using the Lipofectamine 2000 transfection reagent (Invitrogen). After 48 h, cells (except control cells) were stimulated with 0.1 μg/ml LPS for 6 h and collected for the analysis of target gene expression using western blot and qRT-PCR as described above.

2.9. Statistics analysis

All data were analyzed with GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). The data are expressed as the mean ± SEM and the significance of differences was determined using one-way ANOVA followed by Tukey's multiple comparison test. The level of statistically significant difference was set at $P < 0.05$.

3. Results

3.1. Sequences and characteristics of OmTRAF6 and OmTAK1

Using 5′- and 3′-RACE, we identified TRAF6 and TAK1 candidates (named OmTRAF6 and OmTAK1, respectively) from the total RNA of rainbow trout. The cDNA of OmTRAF6 (GenBank Accession number KY489770) was 2391 bp in length, which contained an open reading frame (ORF) of 1656 bp, a 5′-untranslated region (UTR) of 95 bp and a 3′-UTR of 640 bp (Supplementary Fig. S1A). The ORF coded for a deduced protein of 551 amino acids with a predicted molecular mass of approximately 61.7 kDa. Secondary structure prediction by SMART revealed that OmTRAF6 consisted of one N-terminal zinc finger type protein structural domain, named RING domain (76–114 aa), two TRAF-type zinc finger domains (157–209 and 210–267 aa; TRAF) at middle, a coiled-coil region (322–378 aa; CCR) at middle and one C-terminal meprin and TRAF homology (MATH) domain (385–510 aa) (Supplementary Fig. S1A). The cDNA of OmTAK1 (GenBank Accession number MF671982) was 2768 bp in length, which contained an ORF of 1719 bp, a 5′-UTR of 670 bp and a 3′-UTR of 379 bp (Supplementary Fig. S1B). The ORF coded for a deduced protein of 572 amino acids with a predicted molecular mass of approximately 63.8 kDa. Secondary structure prediction by SMART revealed that OmTAK1 comprised a conserved serine-threonine protein kinase catalytic domain (25–273 aa; STKc), a glycine-rich loop of G-x-G-x-x-G (residues Gly32-Gly37) and a hinge region of MEYAEGGS (residues Met93-Ser100) at its N-terminus, and a CCR (501–559 aa) at its C-terminus (Supplementary Fig. S1B).

The alignment of OmTRAF6 with zebrafish, grass carp, african clawed frog (*Xenopus laevis*), human and mouse TRAF6 showed that RING domain is most conserved among five domains of vertebrate TRAF6 (Fig. 1A). To understand the evolutionary relationship of OmTRAF6, a phylogenetic tree was constructed with full-length TRAF6 protein of various species (Fig. 2A). The result revealed that these homolog proteins could be divided in to three groups: teleost, tetrapod (amphibians, birds and mammals) and invertebrate (mollusks and insect). Within the teleost group, rainbow trout, zebrafish, black carp, grass carp, common carp and goldfish (*Carassius auratus auratus*) formed a sub-cluster, and were well separated from other sub-cluster formed by groupers (*E. coioides* and *E. tauvina*), olive flounder (*Paralichthys olivaceus*), snapper (*Lutjanus sanguineus*), rock bream, tilapia (*Oreochromis niloticus*), medaka (*Oryzias latipes*), pufferfish (*Takifugu rubripes*) and ayu (*Plecoglossus altivelis altivelis*). OmTRAF6 branched with zebrafish showing high evolutionary relationship among them. The alignment of OmTAK1 with salmon (*Salmo salar*), zebrafish, african clawed frog, human and mouse TAK1 showed that OmTAK1 had high level of sequence identity with other fish TAK1s (Fig. 1B). Similarly, OmTAK1 and the other fish TAK1s were grouped together to form a teleost cluster distinct from other vertebrate and invertebrate clusters, and OmTAK1 shared the closet evolutionary relationship with salmon TAK1 (Fig. 2B).

3.2. Expression patterns of OmTRAF6 and OmTAK1 in fish tissues and E. tarda- or bacterial ligands-stimulated RTH-149 cells

To investigate the tissue-dependent expression pattern, we performed qRT-PCR using gene-specific primers for OmTRAF6 and OmTAK1 (Fig. 3). OmTRAF6 and OmTAK1 were expressed in all of the tissues tested, although differences in transcript levels occurred. TRAF6 transcripts were most abundant in the liver, and at a low level present, in increasing order, in the skin, eye, head kidney, intestine, spleen, kidney and gill of rainbow trout. TAK1 transcripts were abundant in the liver, intestine and kidney, and at a low level present, in increasing order, in the gill, skin, head kidney, spleen and eye of rainbow trout. Then, to evaluate the effect of bacterial infection on OmTRAF6 and OmTAK1 expression, we stimulated RTH-149 cells with heat-killed *E. tarda*, which is an important pathogen associated with diseases in teleost fish. After stimulation with *E. tarda* for 6 h, the expression of OmTRAF6 and OmTAK1 in RTH-149 cells was increased by 3.52- and 2.06-fold, respectively, compared with the control unstimulated cells (Fig. 4A). We next stimulated RTH-149 cells with various bacterial ligands, including LPS, LTA and PGN. Among the tested bacterial ligands, LPS induced dose-dependent increase in the expression of OmTRAF6 and OmTAK1 in RTH-149 cells, whereas no effect was observed in the presence of LTA and PGN (Fig. 4B).

3.3. Involvement of OmTRAF6 and OmTAK1 on the activation of MAPKs and NF-κB in LPS-stimulated RTH-149 cells

The high conservation of TRAF6 and TAK1 among fish species and other vertebrates might indicate conservation of the TLR signalling pathway, though fish have more TLRs than mammals and fish TLR sequences diversified greatly during evolution [28]. In mammals, TRAF6 and TAK1 are two crucial factors in the TLR signalling pathway, the ubiquitinated TRAF6 activates TAK1, which subsequently phosphorylates MAPKs and IKK, leading to AP-1 and NF-κB activation, respectively [29]. Therefore, we examined whether OmTRAF6 and OmTAK1 are involved in the activation of p38 MAPK and JNK in LPS-stimulated RTH-149 cells. When RTH-149 cells were stimulated with LPS, the phosphorylation of TAK1, p38 MAPK and JNK was enhanced at 6 h after stimulation (Fig. 5A). In addition, NF-κB activity was increased by 9.42-fold compared with the control unstimulated cells at 6 h after stimulation with LPS (Fig. 5B). We then evaluated the effect of decreased expression of OmTRAF6 and OmTAK1 on the activation of p38 MAPK and JNK using RNA interference in RTH-149 cells stimulated with LPS for 6 h. The expression of OmTRAF6 and OmTAK1 was suppressed by transfecting RTH-149 cells with siRNAs targeted to OmTRAF6 and OmTAK1, respectively (Fig. 5C). Transfection with non-specific siRNA did not influence the expression of OmTRAF6 and OmTAK1; thus the gene silencing specificity was confirmed. The silencing of OmTRAF6 and OmTAK1 significantly blocked the phosphorylation of p38 MAPK and JNK in LPS-stimulated RTH-149 cells (Fig. 5C). In addition, TAK1 inhibitor (5z)-7-Oxozeaenol markedly reduced NF-κB activity by ~73.5% in LPS-stimulated RTH-149 cells (Fig. 5D). Next, to investigate the effect of OmTRAF6 and OmTAK1 on the expression of MAPKs and NF-κB downstream target genes IL-1β, TNF-α, IL-6 and IL-8 in LPS-stimulated RTH-149 cells, we evaluated the influence of decreasing OmTRAF6 and OmTAK1 expression on the expression of IL-1β, TNF-α, IL-6 and IL-8 by using RNA interference in the cells stimulated with LPS for 6 h. The expression of OmTRAF6 and OmTAK1 was suppressed by transfecting RTH-149 cells with siRNAs targeted to OmTRAF6 and OmTAK1, respectively (Fig. 6A). Specificity

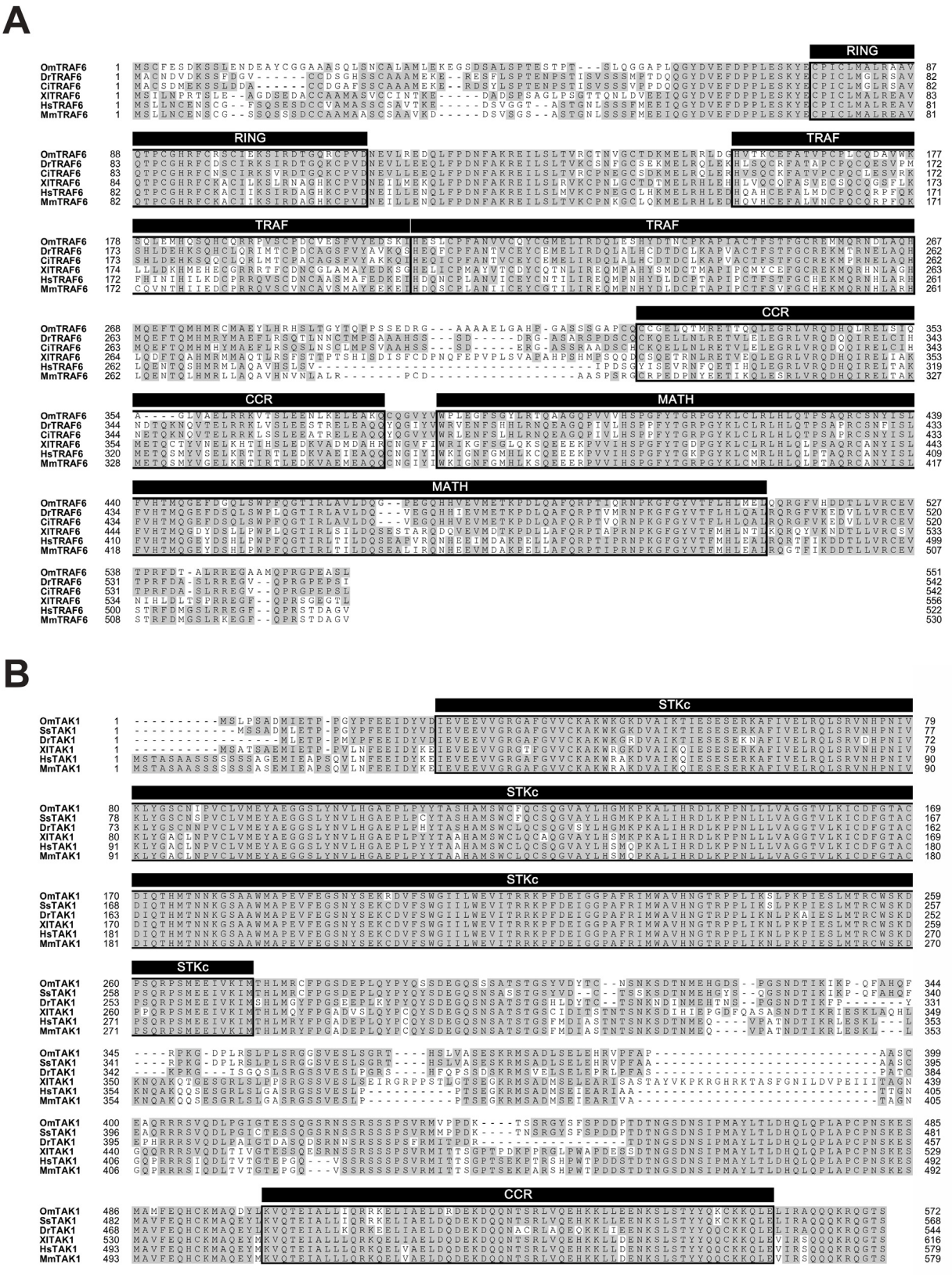

Fig. 1. Alignment of deduced amino acid sequences of rainbow trout TRAF6 (A) and TAK1 (B) (*O. mykiss*, Om) with other species including zebrafish (*D. rerio*, Dr), grass carp (*C. idella*, Ci), african clawed frog (*X. laevis*, Xl), human (*H. sapiens*, Hs), mouse (*M. musculus*, Mm) and salmon (*S. salar*, Ss). Identical amino acids are shaded medium grey. Predictions of RING, TRAF, CCR, MATH and STKc domains were performed by the SMART program, and these domains are indicated as black bars above the alignment.

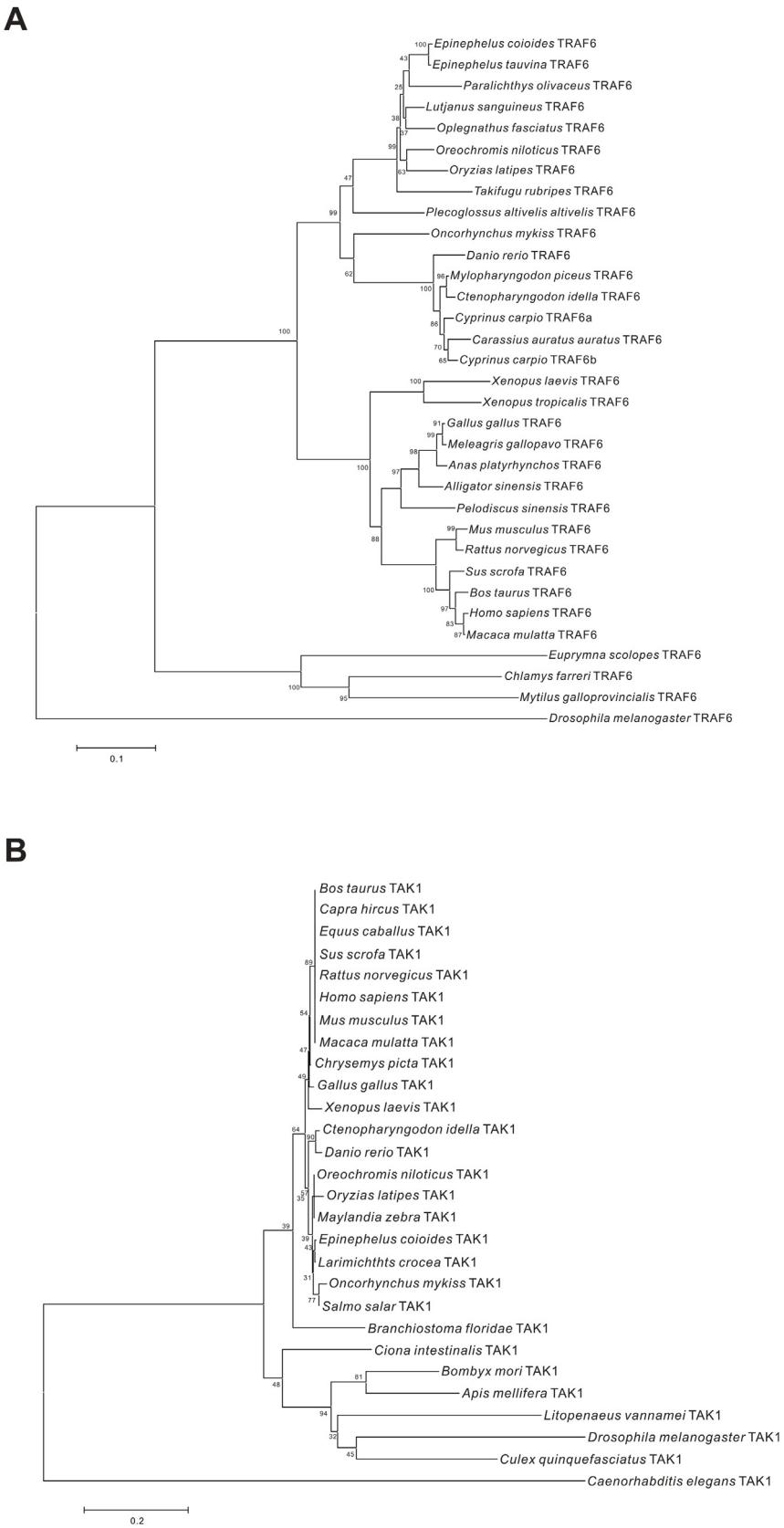

Fig. 2. Phylogenetic tree analysis of TRAF6 (A) and TAK1 (B) proteins from various species. Phylogenetic trees were constructed using MEGA software 6 by the Neighbor-joining method and 10000 replications of bootstrap. The bar indicates the distance. The GenBank accession numbers of the genes used in the analysis are listed in Table 2.

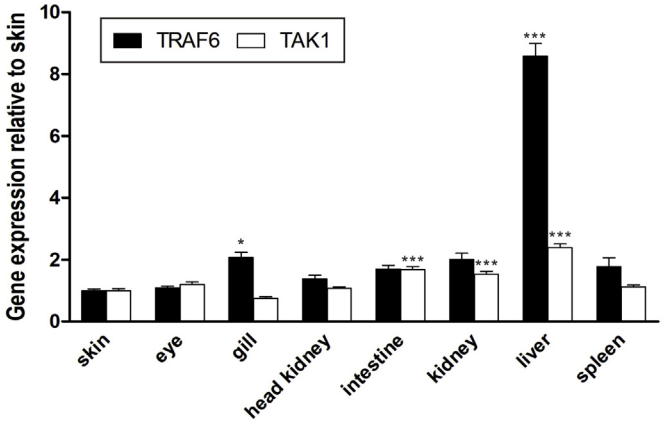

Fig. 3. Expression of OmTRAF6 and OmTAK1 at mRNA level in tissues of rainbow trout as measured by qRT-PCR. EF1-α was used as internal control for producing relative fold value compared to that in skin. Data are shown as the mean ± SEM of four independent experiments performed in triplicate. Asterisks indicate significant differences at $^{*}P < 0.05$, $^{***}P < 0.001$, compared with skin by one-way ANOVA.

of gene silencing was confirmed, since transfection with non-specific siRNA did not influence the expression of OmTRAF6 and OmTAK1. After stimulation with LPS for 6 h, the expression of IL-1β, TNF-α, IL-6 and IL-8 in RTH-149 cells transfected with non-specific siRNA was increased by 5.85-, 6.76-, 4.07- and 5.77-fold, respectively, compared with the control unstimulated cells transfected with non-specific siRNA (Fig. 6B). Silencing of OmTRAF6 and OmTAK1 significantly reduced the expression of IL-1β (2.33- and 1.94-fold, respectively), TNF-α (2.99- and 3.1-fold, respectively), IL-6 (2.41- and 2.71-fold, respectively) and IL-8 (2.09- and 2.64-fold, respectively) in LPS-stimulated cells. Overall, these data suggest that TRAF6 and TAK1 might function like those of mammals as mediators of TLR signalling in rainbow trout.

4. Discussion

The vertebrate innate immune system recognizes microorganisms via germline-encoded PRRs that sense particular structures of microorganisms (PAMPs) and initiate a well-orchestrated immune response [3]. Fish has an innate immune component that shows considerable conservation with higher vertebrates particularly in mammals highlighted by the presence of orthologous PRRs and downstream signalling molecules. However, there is also increasing evidence of fish components and functions that are not observed in mammals suggesting complexity and diversity in fish innate immune function [30,31]. Therefore, knowledge about the network and function of these innate immune-related molecules in model and/or economically important fish is important.

During the past decade, the precise mechanisms underlying TLR signalling have been clarified in mammals by various approaches involving genetic, biochemical, structural, cell biological and bioinformatics studies [2,32]. In TLR signalling pathway, TRAF6 and TAK1 are known to play important roles as molecular bridge, linking upstream TLRs with the downstream MAPK and NF-κB signalling pathways [2,29]. Over the past few years several TRAF6s and TAK1s have been identified and characterized in different fish species [15–24]. However, most of the studies on fish TRAF6s and TAK1s have focused on molecular cloning and expression analysis of TRAF6 and TAK1 genes, thus whether the biological roles of fish and mammalian TRAF6 and TAK1 are similar has yet not been clearly determined. In the present study, we reported the identification and cloning of TRAF6 and TAK1 in rainbow trout, *O. mykiss*. In addition, in order to uncover more in depth functional similarities and divergences of TLR signalling in fish and mammals, we investigated the roles of TRAF6 and TAK1 in eliciting innate immune response to bacterial infections in a rainbow trout hepatoma cell line RTH-149.

Previous structural studies indicated that TRAF6 contains four domains and revealed the functions of individual domains: the N-terminal RING domain and zinc fingers coordinate TRAF6 auto-ubiquitination and its interaction with Ubc13 [33]; the CCR is essential for TRAF6's auto-ubiquitination and downstream signalling events [10]; the MATH domain links TRAF6 to upstream molecules including IRAK4 [34,35]. Similar to the TRAF6s of mammals and other fish, the rainbow trout TRAF6 (OmTRAF6) also contained the classical domains, including an N-terminal RING domain, two TRAF-type zinc finger domains, a CCR domain and a C-terminal MATH domain (Supplementary Fig. S1A and Fig. 1A). Phylogenetic analysis showed that OmTRAF6 belonged to the branch of fishes and was most similar to zebrafish TRAF6 (Fig. 2A). High similarity of amino acid sequence and structural domains indicated that OmTRAF6 encoded a functional homolog of TRAF6, and suggesting similar functions described for mammalian TRAF6s. Likewise, the rainbow trout TAK1 (OmTAK1) had the functional domains present in mammals and other fish, including an N-terminal STKc domain and a C-terminal CCR domain, and shared a high level of sequence identity with other fish TAK1 (Supplementary Fig. S1B and Fig. 1B).

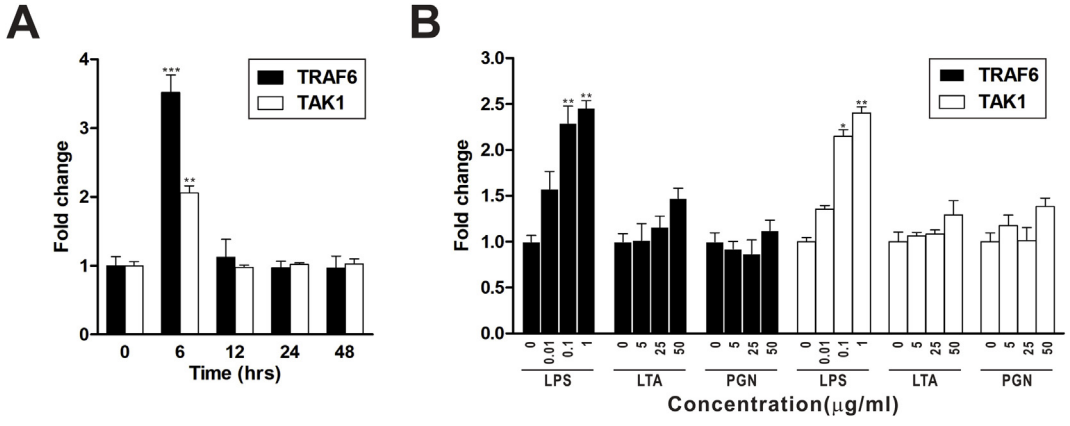

Fig. 4. OmTRAF6 and OmTAK1 expression in RTH-149 cells in response to *E. tarda* (A) and bacterial ligands (B). (A) RTH-149 cells were stimulated with heat-killed *E. tarda*, and the expression of OmTRAF6 and OmTAK1 was determined by qRT-PCR at different time points following stimulation. (B) RTH-149 cells were stimulated with LPS, LTA and PGN at different doses, and the expression of OmTRAF6 and OmTAK1 was determined by qRT-PCR at 6 h following stimulation. The expression levels of OmTRAF6 and OmTAK1 were normalized by the EF1-α level and presented as the relative fold compared with the non-treated control. Data in (A) and (B) are shown as the mean ± SEM of three independent experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, when compared with control unstimulated cells.

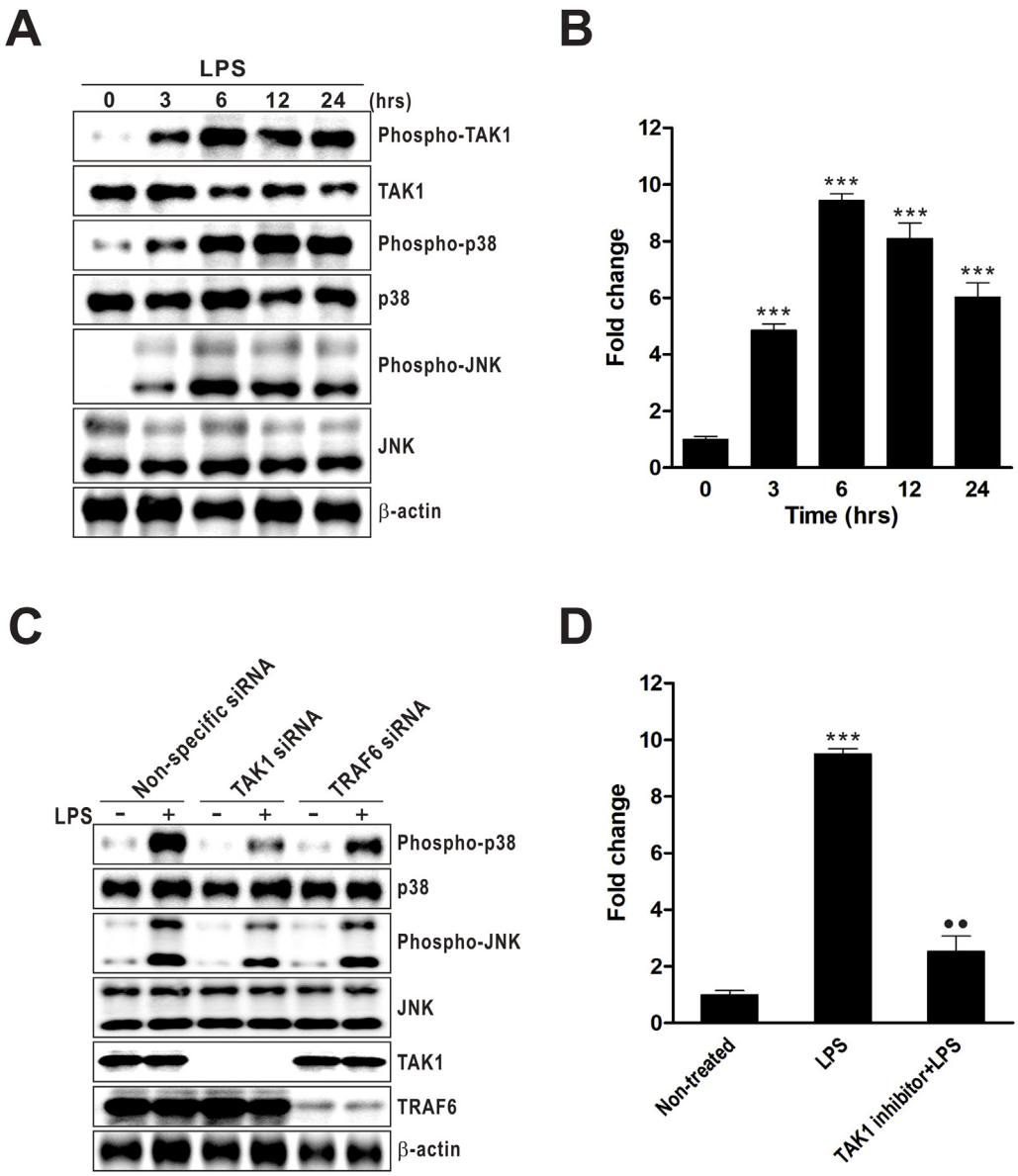

Fig. 5. Involvement of OmTRAF6 and OmTAK1 on the activation of MAPKs and NF-κB in LPS-stimulated RTH-149 cells. (A) RTH-149 cells were stimulated with LPS (0.1 μg/ml) for indicated times. Next, the phospho- and total forms of TAK1, p38 MAPK and JNK were assessed by western blotting. (B) RTH-149 cells were co-transfected with pNF-κB-Luc and pRL-TK vectors. At 48 h after transfection, cells (except control cells) were stimulated with LPS (0.1 μg/ml) for indicated times. Next, NF-κB activity was measured as described in the Materials and methods. (C) RTH-149 cells were transfected with OmTRAF6 siRNA, OmTAK1 siRNA or non-specific siRNA. At 48 h after transfection, cells (except control cells) were stimulated with LPS (0.1 μg/ml) for 6 h. Next, the phospho- and total forms of p38 MAPK and JNK, and total forms of TAK1 and TRAF6 were assessed by western blotting. (D) RTH-149 cells were co-transfected with pNF-κB-Luc and pRL-TK vectors. At 48 h after transfection, cells were pretreated with or without 1 μM TAK1 inhibitor [(5z)-7-Oxozeaenol] for 60 min. After incubation, cells (except control cells) were stimulated with LPS (0.1 μg/ml) for 6 h, and NF-κB activity was measured as described in the Materials and methods. Data in (B) and (D) are shown as the mean ± SEM of three independent experiments performed in triplicate. $^{***}P < 0.001$, control unstimulated versus LPS-stimulated cells; $^{\bullet\bullet}P < 0.01$, non-pretreated versus TAK1 inhibitor-pretreated cells.

OmTAK1 had well conserved amino acid residues (Thr167, Thr173, Thr176 and Ser181) known to be important as autophosphorylation sites during TAB1-induced TAK1 autoactivation in mammalian TAK1s [36,37]. In addition, the phylogenetic analysis of OmTAK1 showed a highly correlated evolutionary relationship with other mammalian and fish TAK1s, which indicated that these TAK1s have a similar function (Fig. 2B).

Tissue expression pattern detected by qRT-PCR showed that OmTRAF6 and OmTAK1 were expressed in all of the tissues tested, although differences in transcript levels occur (Fig. 3). The constitutive

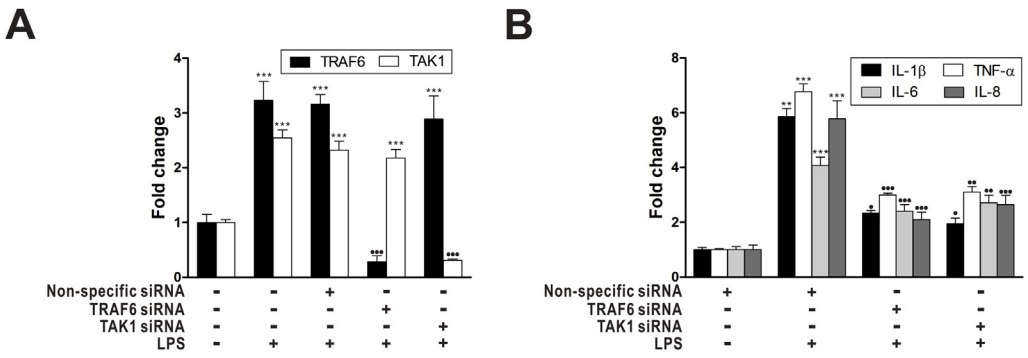

Fig. 6. The effect of OmTRAF6 and OmTAK1 silencing on the expression of MAPKs and NF-κB downstream target genes in LPS-stimulated RTH-149 cells. RTH-149 cells were transfected with OmTRAF6 siRNA, OmTAK1 siRNA or non-specific siRNA. At 48 h after transfection, cells (except control cells) were stimulated with LPS (0.1 μg/ml) for 6 h, and the expression of OmTRAF6, OmTAK1 (A), IL-1β, TNF-α, IL-6 and IL-8 (B) was analysed by qRT-PCR. Data are shown as the mean ± SEM of three independent experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$, control unstimulated versus LPS-stimulated cells; $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$, $^{\bullet\bullet\bullet}P < 0.001$, non-specific siRNA versus OmTRAF6 or OmTAK1 siRNA.

expression of TRAF6 and TAK1 with varied magnitude was previously been reported in various tissues of grass carp [17] and orange-spotted grouper [18,23]. Of note, the expression of OmTRAF6 and OmTAK1 was markedly upregulated in RTH-149 cells stimulated with bacteria *E. tarda* and LPS (Fig. 4). Unlike mammals, fish are known to be resistant to the toxic effect of LPS, the major component of the outer membrane of Gram-negative bacteria and an important endotoxin [38,39]. TLR4, together with accessory molecule, is responsible for the recognition of LPS in mammals [3]. Although several TLRs are present in fish, those molecules specifically involved in TLR4 mediated endotoxin recognition have not been fully established in different fish species. Despite this, LPS has the potency to induce and express several pro-inflammatory cytokines and acute-phase proteins in several fish species [40–43]. Thus, we hypothesized that LPS might be sensed via a receptor other than TLR4 in fish cells, and explored the possible biological activities of TRAF6 and TAK1 in LPS-stimulated RTH-149 cells. In mammals, TAK1 can be activated directly by TRAF6 and then activates both the MKK4/6/7 (p38 MAPK and JNK signalling) and the IKK complex (NF-κB signalling), which are considered as important immune pathways preventing pathogen invasion [2,29]. Ligands for TLR1, TLR5, TLR7 and TLR9 all failed to induce activation of NF-κB and MAPKs to trigger the production of inflammatory cytokines in TRAF6-deficient macrophages [44]. Similarly, TAK1-deficient cells also failed to activate NF-κB and MAPKs in response to TLR ligands [45]. In this study, we confirmed that the phosphorylation of TAK1, p38 MAPK and JNK was enhanced at 6 h after stimulation with LPS in RTH-149 cells (Fig. 5A). The silencing of OmTRAF6 and OmTAK1 significantly blocked the phosphorylation of p38 MAPK and JNK in LPS-stimulated RTH-149 cells (Fig. 5C). Consistently, TAK1 inhibitor (5z)-7-Oxozeaenol markedly decreased the LPS-induced activation of NF-κB in RTH-149 cells (Fig. 5D). In accordance with our results, Bao et al. recently reported that LPS significantly induced the expression of TAK1 in the kidney cell line from large yellow croaker. In that study, they also showed that NF-κB could be activated by LPS stimulation in the TAK1 and TAB1 co-overexpressed human cell line, HEK293T [24].

TRAF6 and TAK1 appear to participate in the activation of MAPKs and NF-κB, which play an important role in the regulation of many genes involved in inflammation and the immune response [3]. To determine whether the TRAF6/TAK1-mediated activation of MAPKs and NF-κB leads to the expression of their downstream target genes, we treated RTH-149 cells with LPS and measured mRNA levels of IL-1β, TNF-α, IL-6 and IL-8. Similar to higher vertebrates [2], LPS induced the expression of IL-1β, TNF-α, IL-6 and IL-8 in RTH-149 cells (Fig. 6B). Silencing of OmTRAF6 and OmTAK1 in RTH-149 cells treated with LPS resulted in downregulation of IL-1β, TNF-α, IL-6 and IL-8 production (Fig. 6B). The data in this fish species and higher vertebrates indicates the conservation of TRAF6/TAK1-mediated TLR signalling from lower to higher vertebrates [2,3].

In conclusion, we have identified a rainbow trout TRAF6 (OmTRAF6) and TAK1 (OmTAK1), which are highly conserved with their respective homologs from other vertebrates. OmTRAF6 and OmTAK1 were widely expressed in the tissues that come in contact with bacteria, and induced by stimulation with *E. tarda* and LPS in RTH-149 cells. Silencing of OmTRAF6 and OmTAK1 in RTH-149 cells negatively regulated the LPS-induced phosphorylation of p38 MAPK and JNK. Inhibitor assay demonstrated that TAK1 is critical for the activation of NF-κB in LPS-stimulated RTH-149 cells. In addition, silencing of OmTRAF6 and OmTAK1 significantly decreased the expression of MAPKs and NF-κB downstream target genes induced by LPS in RTH-149 cells. Although additional *in vivo* studies are required to clarify the current *in vitro* data, our findings indicate that OmTRAF6 and OmTAK1 might function like those of mammals to regulate bacteria-triggered signalling pathway in fish.

Acknowledgments

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (2016R1A6A3A11935245 and 2017R1A2B4007453) and the Intelligent Synthetic Biology Center of Global Frontier Project funded by the Ministry of Science, ICT and Future Planning (2016M3A6A8949166).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.002.

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