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RESEARCH ARTICLE

Molecular profile and expressional modulation of a Toll like receptor-1 homolog from rock bream (*Oplegnathus fasciatus*)

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Abstract Toll-like receptors (TLRs) are well-described pattern recognition receptors that recognize different pathogen-associated molecular patterns (PAMPs) to activate innate immune responses. TLR1 forms a heterodimer with TLR2 that works to identify PAMPs including bacterial components. In the present study, we identified a *TLR1* homolog from rock bream *Oplegnathus fasciatus* (*RbTLR1*) and characterized it at the genomic level. The complete coding sequence of *RbTLR1* was 2406 bp in length and encoded an 802 amino acid protein possessing a calculated molecular mass of 90.7 kDa and typical TLR domain architecture. A single exon spanned the genomic length (2931 bp) of *RbTLR1*. Phylogenetic analysis showed a close evolutionary relationship between RbTLR1 and its fish orthologs. Using quantitative real time polymerase chain reaction, we detected *RbTLR1* mRNA in all the tissues examined, suggesting its merely universal tissue expression. *RbTLR1* was expressed strongly in the spleen, kidney, and liver. Following immune stimulation by bacterial pathogens and mitogens, *RbTLR1* transcript levels were elevated at various time points post stimulation. Moreover, injection of a viral stimulant, polyinosinic:polycytidylic acid, and rock bream iridovirus altered the basal transcription of *RbTLR1*. Collectively, these data showed that *RbTLR1* is transcriptionally regulated in response to bacterial and viral stimulation, suggesting its putative importance in host immune defense.

Keywords Rock bream · TLR1 · Genome organization · Immune stimulants · Transcriptional profiling

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Introduction

Pathogenic microorganisms including bacteria and virus are freely available in the environment and can cause acute infections in host organisms. Therefore, natural defense mechanisms have been established to prevent the deleterious effects of these pathogenic encounters. Innate immunity acts as a basic defense mechanism against the majority of microbial infections. This first-line defense system is one arm of the immune system in fish, whereas it is the exclusive immune system in invertebrates. Teleost fish possess innate immune parameters that are more active and diverse than those of their mammalian counterparts (Magnadottir 2006).

Pattern recognition receptors (PRRs) play a crucial role in innate immunity by contributing to the identification of different pathogen-associated molecular patterns (PAMPs) (Medzhitov 2007) and damage-associated molecular patterns (DAMPs) (Matznger 2002). Toll-like receptors (TLRs) are one of the predominant type of evolutionarily conserved

PRRs that recognize microbial PAMPs (Rebl et al. 2010) such as lipopolysaccharides (LPS), lipoproteins, lipoteichoic acid, flagellin, and nucleic acids (Akira et al. 2006). TLRs are composed of a characteristic extracellular domain, which harbors up to 26 leucine-rich repeats (LRRs) flanked by N- and C-terminal cap motifs (Bell et al. 2003); a transmembrane domain; and an intracellular C-terminus, which includes a Toll/Interleukin (IL)-1 receptor domain (TIR) (Palti 2011).TLRs are diverse among fish species. In this regard, teleost fish show remarkable diversity, rendering them as the most diverse group of vertebrates which bear TLRs (Venkatesh 2003; Volff 2005). The rainbow trout was the first fish in which the IL/TLR super family was identified (Sangrador-Vegas et al. 2000). Typically, fish lineages lack TLR6 and TLR10, whereas TLR20, TLR21, TLR22, and TLR23 are virtually exclusive to fish species (Rebl et al. 2010; Takano et al. 2011; Priyathilaka et al. 2014).

TLR1 and TLR2 belong to the TLR1 family (Magnadottir 2006) and form heterodimers that detect lipoproteins on Gram-positive bacteria (Hajjar et al. 2001) and LPS on Gram-negative bacteria (Takeuchi et al. 2002).

Rock bream is currently a very popular sashimi in East Asia, especially in Korea and Japan, rendering its increasing economic value. Rock bream inhabit the coastal areas of the Pacific and Indian Oceans. In the recent years, the prevalence and virulence of bacterial and viral pathogenic infections have negatively affected this teleost fish species, enhancing its mortality and resulting in its shortage (Zenke and Kim 2008; Park 2009). Therefore, it is necessary to elucidate disease resistance mechanisms in rock bream to develop effective preventive and therapeutic strategies. Moreover, characterization of TLRs in fish like rock bream is a basic approach in comparative studies on fish and mammalian innate immune systems.

Investigation of TLRs in bony fish is a crucial undertaking. Because fish species serve as models of physiology, immune mechanisms identified in fish can be applied to other organisms as well. However, studies characterizing the complete genomic sequence of *TLR1* in bony fish are limited. In this study we identified and characterized the complete rock bream *TLR1* (*RbTLR1*) gene at the genomic level. To understand the relationship between immune stimulants and TLR1 mediated host defense in rock bream, we examined the regulation of *RbTLR1* mRNA in the spleens of fish, stimulated with LPS, *Edwardsiella tarda, Streptococcus iniae*, rock bream iridovirus (RBIV), and polyinosinic:polycytidylic acid (poly I:C).

Materials and methods

Identification of partial cDNA sequences of *RbTLR1*

A rock bream sequence database was established using the Roche 454 genome sequencer FLX system (GS-FLX^{TM}), a next-generation DNA sequencing technology (DNA Link, Republic of Korea). Using the Basic Local Alignment Search Tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/BLAST), we identified partial-length TLR1 cDNAs in the rock bream sequence database.

Identification of the complete *RbTLR1* genomic sequence

A random-shear bacterial artificial chromosome (BAC) library of rock bream genomic DNA was constructed (Lucigen, USA). We screened this library to identify the BAC clone bearing RbTLR1 gene using a two-step polymerase chain reaction (PCR) based genomic library screening approach with gene specific-primers (RbTLR1-F and RbTLR1-R; Table 1). The putative *RbTLR1* containing clone was then sequenced using the GS-FLX^{TM} system (Macrogen, Korea) to obtain full-length genomic sequence. The open reading frame (ORF) of *RbTLR1* was then identified from the obtained genomic DNA sequence by using a National Center for Biotechnology Information (NCBI)-BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). Annotated sequence information for *RbTLR1* was deposited in the NCBI GenBank database (Accession Number JQ754308).

In silico analysis of the rock bream TLR1 sequence

RbTLR1 orthologs were compared using NCBI-BLAST. Pairwise sequence alignments (http://www.ebi.ac.uk/Tools/psa/emboss_needle) and multiple sequence alignments (http://www.ebi.ac.uk/Tools/msa/clustalw2) were performed using the ClustalW2 program. The phylogenetic relationship of RbTLR1 was determined by the neighbor-

Table 1 Primers used in this study

Name	Purpose	Sequence (5′ → 3′)
RbTLR1-F	BAC library screening and qPCR amplification for *RbTLR1*	CCAAACGCACCAGTCACAGAACTA
RbTLR1-R	BAC library screening and qPCR amplification for *RbTLR1*	TGTTTCCTCACGCAAACTCTCCCT
RbTLR1-βF	qPCR amplification for β-actin	TCATCACCATCGGCAATGAGAGGT
RbTLR1-βR	qPCR amplification for β-actin	TGATGCTGTTGTAGGTGGTCTCGT

joining method, supported by 1000 bootstrapping replicates, using Molecular Evolutionary Genetics Analysis (MEGA) software version 3 (Kumar et al. 2004). Protein domain predictions were carried out using the ExPASy-prosite database (http://prosite.expasy.org), SMART online server (http://smart.embl-heidelberg.de), and MotifScan scanning algorithm (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The signal peptide was predicted using the signalP server (http://www.cbs.dtu.dk/service/signalP) and the physicochemical properties of the deduced protein were predicted using the ExPASy prot-param tool (http://web.expasy.org/protparam). The tertiary structure of RbTLR1 was modeled based on the homology modeling strategy using SWISS-MODEL online server (Schwede et al. 2003; Arnold et al. 2006). The three-dimensional (3D) image was generated using the RasMol software version 2.5.7.2 (Goodsell 2005).

Experimental fish and tissue collection

Healthy rock breams with an average body weight of 50 g were obtained from the Jeju Special Self-Governing Province Ocean and Fisheries Research Institute (Jeju, Republic of Korea). The fish were maintained in a controlled environment (salinity 34 ± 1 ‰; pH 7.6 ± 0.5) at 22–24 °C. Fish were acclimated for 2 weeks prior to the experimentation. Whole blood (1 mL per fish) was collected from the caudal fin by using a sterilized syringe. The samples were immediately centrifuged at 3000×*g* for 10 min at 4 °C to separate the blood cells from the plasma. The collected cells were snap-frozen in liquid nitrogen. The fish were sacrificed and the gill, liver, skin, spleen, head kidney, kidney, skin, muscle, and brain were excised, immediately snap-frozen in liquid nitrogen, and stored at −80 °C.

Immune challenge experiment

To determine the immune responses of *RbTLR1*, pathogenic bacteria *E. tarda* and *S. iniae*, RBIV, poly I:C, and LPS were used as immune-stimulants in time-course experiments. Each rock bream was administered a single intraperitoneal (i.p.) injection of 100 µL LPS (1.25 µg/µL, *Escherichia coli* 055:B5; Sigma) or poly I:C (1.5 µg/µL; Sigma) suspended in phosphate-buffered saline (PBS). *E. tarda* and *S. iniae* used for the bacterial-challenge experiments were obtained from the Department of Aqualife Medicine, Chonnam National University, Korea. The bacteria were incubated at 25 °C for 12 h in brain–heart infusion broth (Eiken Chemical Co., Japan) supplemented with 1 % sodium chloride. The cultures were resuspended in sterile PBS and then diluted to the desired concentration. Each rock bream was i.p.-injected with 100 µL live *E. tarda* (5×10^3 CFU/µL) or *S. iniae* (1×10^5 CFU/µL) in PBS. For the virus-challenge experiment, kidney tissue specimens obtained from RBIV-infected moribund rock bream were homogenized in 20 volumes of PBS. Tissue homogenates were centrifuged at 3000×*g* for 10 min at 4 °C, and the supernatants were filtered through a 0.45-µm membrane. Each rock bream was infected using a single i.p. injection of 100 µL RBIV in PBS. A control group was injected with an equal volume (100 µL) of PBS. Rock bream spleen samples were collected at 3, 6, 12, 24, and 48 h post injection (p.i.) from LPS-, poly I:C-, *E. tarda*-, *S. iniae*- and RBIV-infected rock breams. PBS-injected samples (injection control) were also isolated at each time point. A group of three uninjected animals served as a negative control. Tissue samples from three rock breams were obtained at each time point and pooled. Total RNA was extracted from the pooled tissue samples, and cDNA was synthesized.

Total RNA extraction and cDNA synthesis

Using TRI Reagent™ (Sigma), total RNA was extracted from the blood, gill, liver, spleen, head kidney, kidney, skin, muscle, and brain of healthy rock breams as well as from spleen tissues of immune-challenged fish. cDNA was synthesized from total RNA samples as described previously (Whang et al. 2011).

RbTLR1 mRNA expression analysis by using qPCR

Quantitative real time polymerase chain reaction (qPCR) was used to measure the expression levels of *RbTLR1* in the aforementioned tissues under 'Experimental fish and tissue collection'. The temporal expression of *RbTLR1* in the spleens of fish challenged by different PAMPs and microorganisms (Section 'Immune challenge experiment') was also examined using qPCR. qPCR was performed in a Dice™ thermocycler (Real-Time System TP800; TaKaRa, Japan). Reactions (20 µL total volume) contained 4 µL of diluted cDNA, 10 µL 2× TaKaRa Ex Taq™, SYBR premix, 0.5 µL of each primer (RbTLR1-F and RbTLR1-R; Table 1), and 5 µl of double-distilled H_2O as per the essential MIQE guidelines (Bustin et al. 2009). qPCR was performed under the following conditions: 95 °C for 10 s; 35 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s; and a final cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The baseline was set automatically by using the Dice™ Real-Time System software (version 2.00). *RbTLR1* expression was determined using the Livak ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen 2001). The same qPCR cycle parameters were used for the internal reference gene, rock bream β-actin (GenBank ID: FJ975146). At least three replicates were performed for each sample. *RbTLR1* mRNA levels, relative to the rock bream β-actin expression, are reported. In the immune challenge experiments, *RbTLR1* expression levels were normalized to the corresponding

PBS-injected controls at each time point. The expression level of the uninjected control at 0 h was set as the basal level and was compared with the relative mRNA expression of the injected groups at each time point. All the data are presented as mean ± standard deviation (SD). To determine statistical significance ($P < 0.05$), a two-tailed unpaired Student's *t*- test was performed.

Results

Molecular characterization of *RbTLR1*

The complete ORF derived from the RbTLR21 gene comprised a 2406 bp sequence encoding 802 amino acids (GenBank ID: JQ754308). The predicted molecular mass of RbTLR1 was approximately 90.7 kDa, and the theoretical isoelectric point was 6.39.

Sequence analysis of RbTLR1 revealed that it possessed typical TLR domain organization. RbTLR1 contained eight LRRs (residues 44–66, 91–110, 375–396, 401–426, 447–466, 470–489, and 516–535), a C-terminal LRR (residues 527–587), transmembrane domain (residues 588–610), and TIR domain (residues 646–791) (Fig. 1). Using the SignalP server, we showed that RbTLR1 possess a signal peptide (residues 1–24, Fig. 1).

Pairwise sequence similarity and identity comparisons revealed the homology of RbTLR1 with its vertebrate counterparts (Table 2). RbTLR1 shared 16.2–77.2 % identity with TLR1 proteins of other species. The highest and lowest identity was shared with the orange-spotted grouper and *Caenorhabditis elegans*, respectively. The TIR domain of RbTLR1 exhibited percent identities within the range of 26–95.2 %. The same species, orange-spotted grouper and *C. elegans*, had the highest and the lowest TIR domain identity, with RbTLR1 TIR domain, respectively. As expected, RbTLR1 shared its prominent sequence compatibility with its teleost homologs except with its channel catfish counterpart. Notably, the TIR domain shared greater identity and similarity values than the full-length RbTLR1 sequence. In accordance with the TLR1 multiple sequence alignment, three moderately conserved domains, previously identified in the green spotted pufferfish TIR region (Wu et al. 2008), were also identified in rock bream with minor substitutions, in which third domain harbors highly conserved sequence (FWANL) (Fig. 1).

Phylogenetic analysis

To determine the evolutionary position of RbTLR1, a phylogenetic analysis was carried out using deduced amino acid sequences of several vertebrate and one invertebrate TLR1 proteins. *C. elegans* was used as an out-group. The tree we obtained showed three main clades of vertebrate TLR1 proteins: fish, mammals, and birds (Fig. 2). RbTLR1 was positioned in the fish clade, where it sub-grouped with orange-spotted grouper with maximum bootstrap support (100). The tree suggests that *RbTLR1* evolved from a common ancestral vertebrate gene and showed substantial homology with its fish counterparts.

RbTLR1 genomic organization

The putative genomic *RbTLR1* sequence lacked introns. RbTLR1 is encoded by a single exon which was 2931 bp in length. According to the comparison of *RbTLR1* genomic DNA (gDNA) sequence with known *TLR1* gDNA sequences of several other vertebrate species obtained from the NCBI-GenBank sequence database (http://www.ncbi.nlm.nih.gov/gene) (Fig. 3), both non-teleostan and teleostan *TLR1*counterparts have collectively portrayed a distinct genomic arrangements albeit *RbTLR1* had a similar genomic organization to *TLR1* from green spotted pufferfish (Wu et al. 2008) and bovine (GenBank ID: NC007304), including the lengths of their sequences (Fig. 3). Bearing a single exon along their whole genomic gene sequence, these two teleostan similitudes exhibited a clear demarcation from rest of the fish counterparts considered in the comparison. Besides these two TLR1 genes, others showed multi-exonic gDNA structure. However, from those, coding region of some of the teleostan similitudes including zebrafish, Nile tilpaia and zebra mubna TLR1s were found to be split into multiple exons. With the exception of bovine *TLR1*, orthologs in non-fish vertebrates also exhibited multi-exonic gDNA architecture. In general, the multi-exonic genes showed higher variability in total gene length due to the sizes of their introns.

Modeled tertiary structure of the RbTLR1 TIR domain

The 3D molecular organization of the RbTLR1 TIR domain was examined in a model generated using the human TLR1 TIR domain (Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSC-PDB – ID:1FYV) as the template. The model holds valuable information pertaining to RbTLR1, because TIR domains are conserved cytoplasmic components in TLRs that are important for initiating Toll signaling (Anderson 2000). The model resembled the typical TIR domain structure of TLR1, including a five-strand parallel β-sheet surrounded by five α-helices on both sides (Fig. 4). As in human TLR1 TIR (RCSB-PDB, 1FYV; Xu et al. 2000), each α-helix (αA–αE) and β-strand (βA–βE) was labeled in alphabetical order from the N- to C-terminus. Three main loop structures, the BB, CD, and DD loops, connected αB–βB, αC–βD, and αD–βD, respectively. Moreover, we identified four amino acid

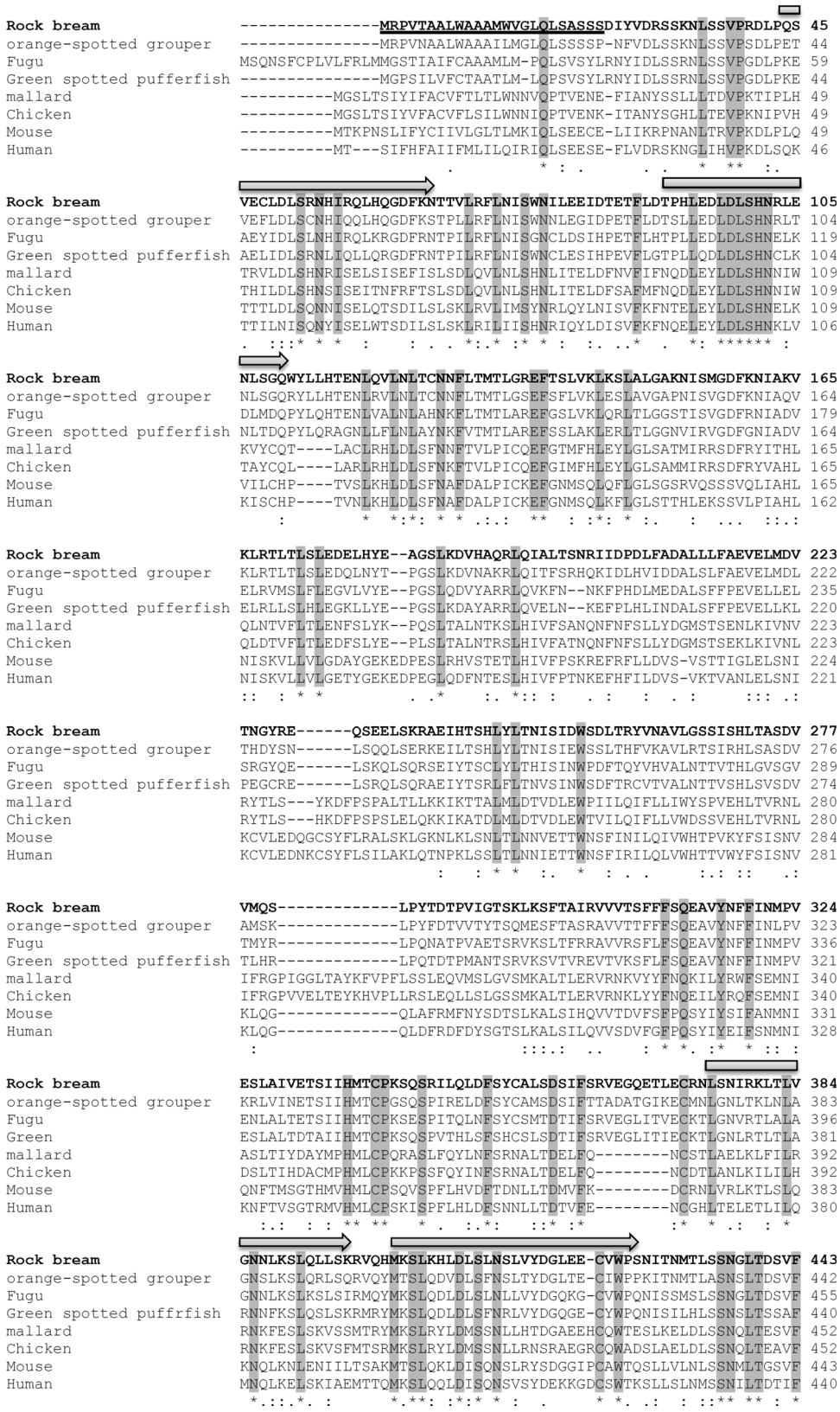
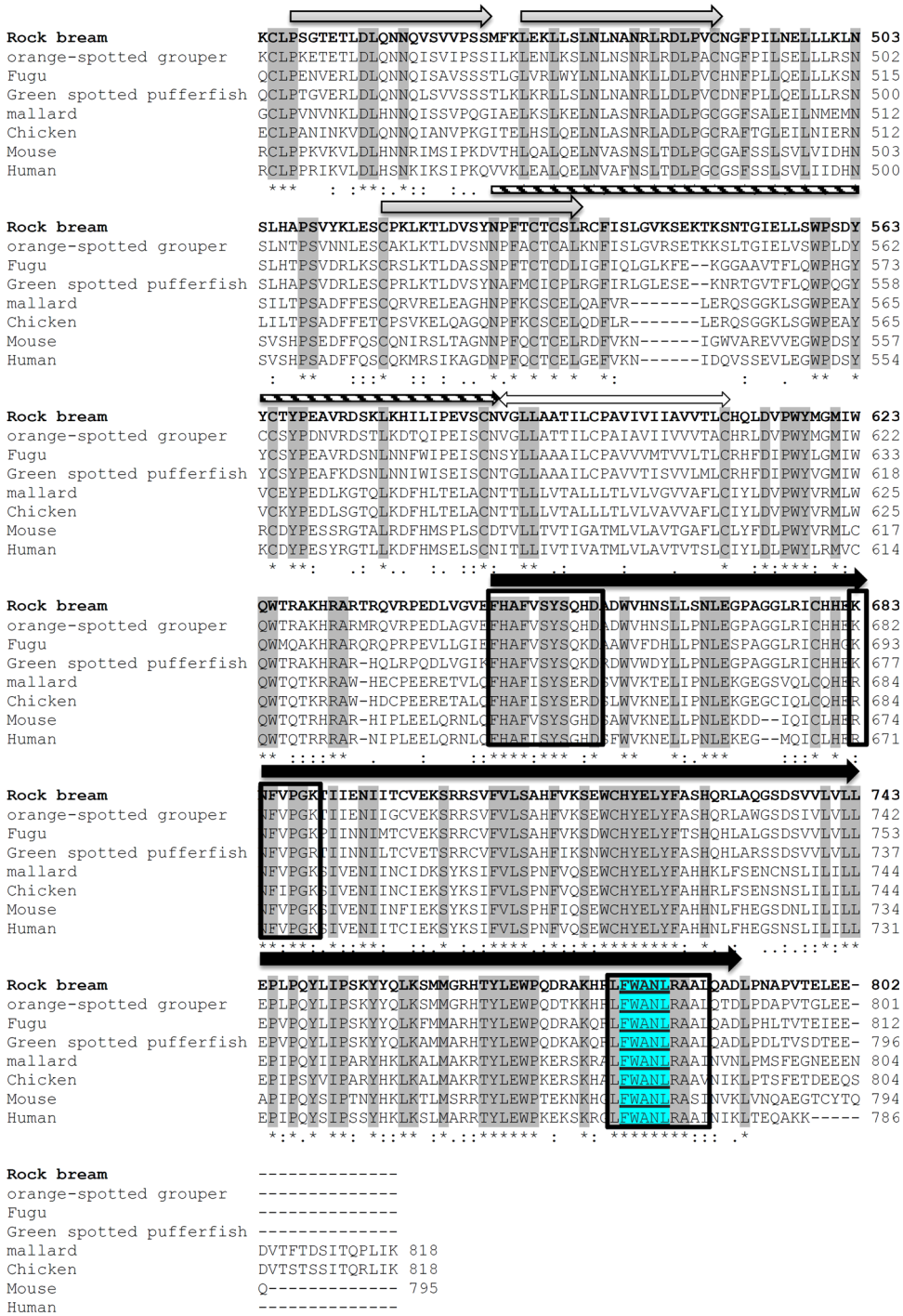


Fig. 1 Multiple sequence alignment of vertebrate TLR1 orthologs. The sequence alignment was generated using the ClustalW method. Signal peptide sequence and LRR motifs are indicated by the *underline* and *gray arrows*, respectively. The C-terminal LRR is denoted by the *pattern-filled arrow*. The transmembrane and Toll/Interleukin (IL)1 receptor (TIR) domains are denoted by *unfilled double-headed* and *solid black arrows*, respectively. Three moderately conserved domains within the TIR are indicated by *boxes*, in which well conserved sequence identified in third domain was *shaded in blue color* and *underlined*. Identical residues are *shaded in gray color*

residues in our model that were present in structural positions corresponding to the conserved surface patch of human TLR1-TIR. Two residues (Asn and Gly) were located in the BB loop, whereas the other two (Phe and Glu) were localized near the beginning of the βA strand and the end of the αA helix, respectively. Conserved (F/Y)DA and FW motifs

Fig. 1 continued



which are prominent features in human TLR1 TIR (Xu et al. 2000), were identified in our model close to the amino- and carboxy-terminal ends, respectively.

Tissue-specific expression profile of *RbTLR1*

To evaluate the variable expression levels of *RbTLR1* mRNA with respect to different tissues in rock bream, qPCR was performed for various tissues using gene-specific primers designed according to the *RbTLR1* coding sequence (Table 1). The relative expression of each tissue was obtained using rock bream β-actin as the non-variant internal control. Relative tissue-specific expression was determined by normalizing values to the expression level in muscle (Fig. 5). *RbTLR1* was expressed in all of the tissues analyzed. Further investigation showed pronounced *RbTLR1* transcript levels in the spleen, kidney, and liver. Moderate levels were detected in the blood, heart and head

Table 2 Percent similarity and identity (with gaps) of RbTLR1 and its TIR domain to TLR1 orthologs of other species

Name of the species	Accession number	Entire sequence				TIR			
		Amino acids (%)	Identity (%)	Similarity (%)	Gaps (%)	Amino acids (%)	Identity (%)	Similarity (%)	Gaps (%)
1. *Epinephelus coioides* (orange-spotted grouper)	AEB32452	801	77.2	85.8	0.1	146	95.2	95.9	0.0
2. *Tetraodon nigroviridis* (green spotted pufferfish)	ABO15772	796	65.6	77.4	0.7	155	80.0	85.8	5.8
3. *Takifugu rubripes* (Torafugu)	AAW69368	812	65.2	76.1	2.2	151	83.4	86.1	3.3
4. *Oncorhynchus mykiss* (rainbow trout)	ACV92063	808	64.2	76.3	1.2	206	84.2	90.4	0.0
6. *Homo sapiens* (human)	BAD67422	786	38.3	57.4	7.7	149	61.6	79.5	4.6
7. *Ictalurus punctatus* (channel catfish)	AEI59662	808	37.8	64.2	3.4	151	67.3	82.7	2.7
17. *Sus scrofa* (pig)	NP001026945	796	37.8	55.6	8.9	163	52.7	72.1	12.7
15. *Canis lupus familiaris* (dog)	NP001139615	789	36.8	55.1	7.9	152	57.1	77.3	6.5
14. *Rattus norvegicus* (Norway rat)	NP001165591	795	36.2	55.7	7.4	152	57.8	76.0	6.5
5. *Anas platyrhynchos* (mallard)	ACS92621	818	36.1	57.0	7.4	152	54.6	79.6	3.9
8. *Gallus gallus* (chicken)	BAD67422	818	36.1	56.6	7.4	151	80.8	56.3	3.3
12. *Mus musculus* (house mouse)	NP109607	795	35.9	54.8	7.6	152	57.1	74.7	6.5
9. *Meleagris gallopavo* (turkey)	ACS92619	818	35.8	55.6	7.4	146	35.6	52.7	15.1
16. *Oryctolagus cuniculus* (rabbit)	XP002709316	796	35.8	52.6	12.4	143	58.2	79.5	2.1
10. *Bos taurus* (bovine)	NP001039969	727	35.6	52.7	15.1	148	57.3	78.7	4.0
13. *Capra hircus* (goat)	ADZ13666	730	34.5	51.6	14.3	147	57.7	77.9	3.4
14. *Caenorhabditis elegans* (*C.elegans*)	NP001020983	1221	16.2	28.9	47.3	132	26.0	44.8	19.5

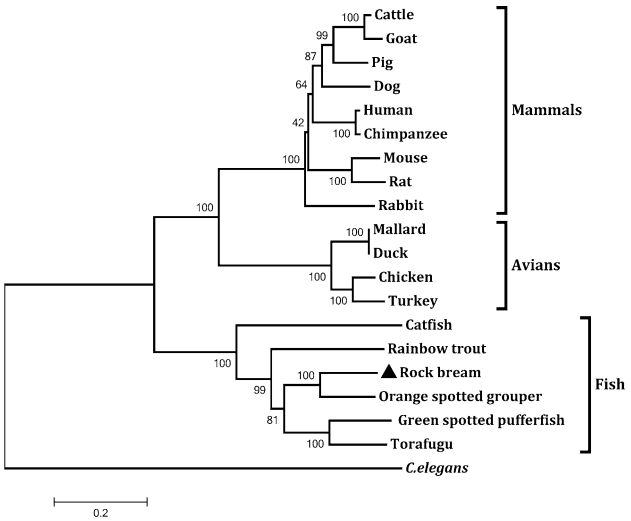


Fig. 2 Phylogenetic tree constructed based on the ClustalW alignment of deduced amino acid sequences of various TLR1 proteins. Trees were estimated using the neighbor-joining method in MEGA version 4.0. Bootstrap values are shown at the *nodes* of the tree and major taxonomic clades are indicated. The corresponding NCBI-GenBank accession numbers of each homolog was indicated in Table 2

kidney ($P < 0.05$). *RbTLR1* transcript was less abundant in the brain, skin, and muscle tissues. Muscle tissue showed the lowest expression level ($P < 0.05$).

Transcriptional regulation of *RbTLR1* in response to immune challenges

The temporal regulation of *RbTLR1* mRNA expression was analyzed in the spleen tissue of rock bream exposed to LPS, poly I:C, *E. tarda*, *S. iniae*, or RBIV. LPS exposure significantly upregulated *RbTLR1* mRNA levels ($P < 0.05$) in spleen tissue 12 and 24 h p.i. by approximately 1.5 and 2.5 fold, respectively (Fig. 6a). At 48 h p.i., transcript levels were downregulated. *E. tarda* challenge also induced the *RbTLR1* transcript levels ($P < 0.05$) that peaked in the early phase (3 h p.i.) and persisted into the late phase (12 and 24 h) (Fig. 6a). In the spleen tissue of fish injected with *S. iniae,* mRNA levels were increased significantly ($P < 0.05$) at each time point tested (3–24 h p.i.), reaching a maximum 3.4-fold difference at 12 h p.i. relative to the unchallenged control (0 h) (Fig. 6a). In contrast to other immune challenges, spleen tissues from poly I:C-challenged fish showed a significant ~2.0-fold elevation in *TLR1* mRNA at 6 h p.i only. Upon RBIV induction, *TLR1* transcription was significantly ($P < 0.05$) upregulated at 3 and 12 h p.i, while significantly downregulated at 24 h p.i (Fig. 6b). After 48 h, transcript levels reached to its basal level.

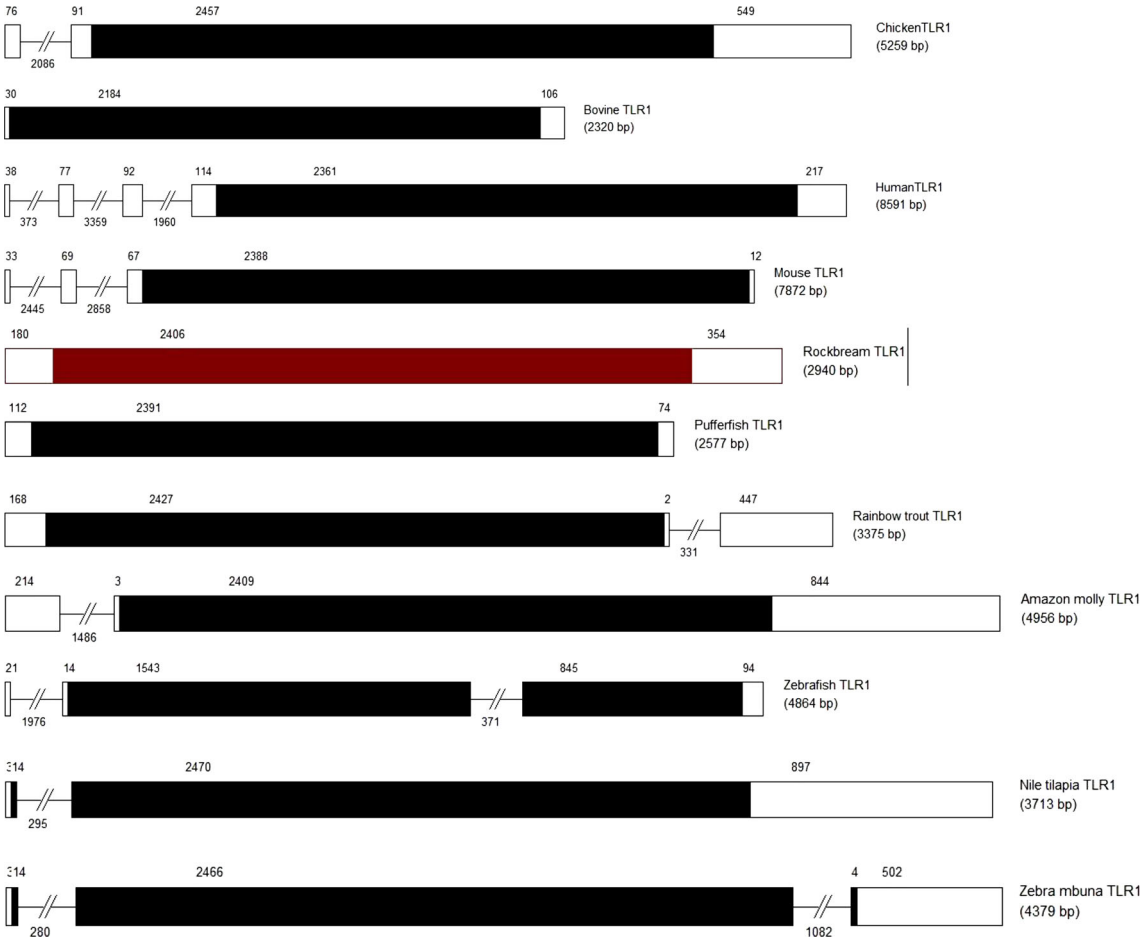


Fig. 3 Genomic organization of *TLR1* genes in different vertebrate species. The exons and introns are indicated by *boxes* and *solid lines*, respectively. The exon sizes are indicated *above the boxes*, and intron sizes are indicated *below the lines*. Regions larger than 200 bp are abbreviated (*diagonal lines*). The sequences are written 5′ → 3′). *TLR1* GenBank Gene-IDs or accession numbers are as follows: chicken, 426274; bovine, 574090; human, 7096; mouse, 21897; rainbow trout, GQ502184; zebrafish, 403127; pufferfish, EF095150; Zebra mbuna, 101475856; Amazon molly, 103135488; Nile tilapia, 102076024

Discussion

Innate immunity plays a crucial role in the first line host defense through identification of PAMPs of invading pathogenic organisms. TLRs are considered as fundamental PRRs involved in innate immunity. Here we characterized TLR1 homolog from rock bream, which is an economically important fish species that is vulnerable to diseases caused by pathogenic microorganisms.

We identified a novel *TLR1* ortholog of rock bream (*RbTLR1*) from our BAC genomic library by using BLAST analysis. The deduced amino acid sequence shared numerous features with its other vertebrate orthologs. Resembling typical TLR architecture, RbTLR1 contained multiple extracellular LRR motifs, a single transmembrane domain, and an intracellular TIR domain (Fig. 1). Eight predicted LRR motifs were encoded by the *RbTLR1* gene sequence. Previous evolutionary studies showed that TLR extracellular domains have evolved more rapidly than TIR domains (Johnson et al. 2003). This might account for the observed numerical variation of LRRs in extracellular domains of teleostan TLR1 similitudes. Pairwise sequence alignment showed that RbTLR1 and its TIR domain shared a high degree of identity and similarity to their respective vertebrate orthologs. The highest percent identity was with the full-length sequence and the TIR domain of the orange-spotted grouper (77.2 and 95.2 %, respectively). This observation validates the primary structure of the novel RbTLR1 protein we identified. In pairwise sequence comparisons, intracellular TIR domains exhibited greater conservation than the full-length sequence (Table 2), suggesting that there was greater structural similarity between TLR1 intracellular domains than the extracellular domains of different vertebrate species. Since intracellular domains of TLRs are responsible for initiating a signaling cascade and extra-cellular domains are involved in identifying

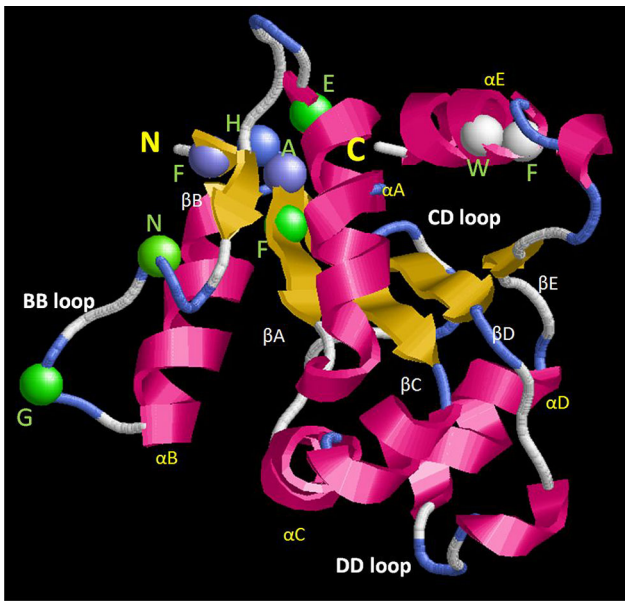


Fig. 4 Predicted three-dimensional structure of the RbTLR1 TIR domain. *Blue* and *green spheres* represent the amino acids of the (F/Y)DA motif and surface patch, respectively. Amino acid residues of FW motif are represented by *two white spheres*. Carboxyl and amino terminals are denoted by the *letters C* and *N*, respectively. β-strands and α-helices are depicted in *yellow* and *pink*, respectively. Each strand and helix is labeled in *alphabetical order*, from the N- to C-terminus

compatible PAMPs, this observation further convinces the activation of similar TLR signaling pathways to different PAMPs (Slack et al. 2000; Bell et al. 2003). As expected, amino acid number (146) of TIR in RbTLR1 lies within the residue range (140–165), conserved among the vertebrate species (Table 2). As detected by our multiple sequence alignment study, among the third domain of three semi-conserved domains identified, 'FWANL' sequence was found to be well conserved in RbTLR1 (Fig. 1) complying with the characteristic FW motif of TLR family proteins, which is known to be important in cellular localization of the receptor molecule (Gangloff et al. 2005).

The phylogenetic tree generated using the TLR1 protein sequences from different vertebrates was in accordance with generally accepted phylogenetic relationships, separating the orthologs into three main clades (Fig. 2). Fish TLR1 members were grouped closely and independently, placing RbTLR1 with TLR1 of the orange-spotted grouper in the fish clade, confirming the common ancestral origin of these species.

Similar to the green spotted pufferfish, the *RbTLR1* gene consists of a single exon (Fig. 3). Deviating from the typical genomic organization of mammalian *TLR1*, bovine *TLR1* also harbors only a single-exon similar to *RbTLR1*. In contrast, all the other teleostan counterparts were found to be multi-exonic, possessing two to three exons. However, in the similitudes of zebrafish, Nile tilpaia and zebra mbuna coding regions were shared among multiple exons, suggesting the potential existence of spliced isoforms of those TLR1s. Moreover, we can further infer that the integration of introns in majority of the teleostan and non-teleostan TLR1s may have a function in substantial regulation of their expression, since some of the intronic sequences are known to harbor regulatory elements which involve in regulation of gene expression (Rose 2008). However, these suggestions merit further investigations.

TIR domains of TLRs are known to be important in the initiation of the intracellular signal transduction process through mediating protein–protein interactions (Kopp and Medzhitov 1999). Through binding different adaptor proteins such as myeloid differentiation primary response factor 88 (MyD88), TIRs play a crucial role in dictating which downstream signaling pathway is activated (Wesche et al. 1997; Medzhitov et al. 1998). Our model of the RbTLR1 TIR domain validated that we correctly identified

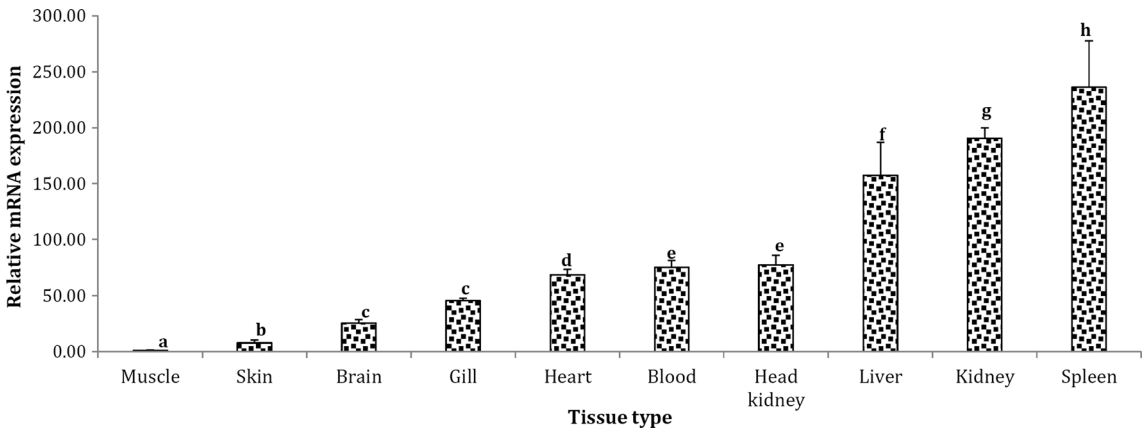


Fig. 5 The tissue-specific expression of *RbTLR1* mRNA by using quantitative polymerase chain reaction (qPCR). *Error bars* represent the SD (n = 3). Data labeled with *different letters* are significantly different ($P < 0.05$)

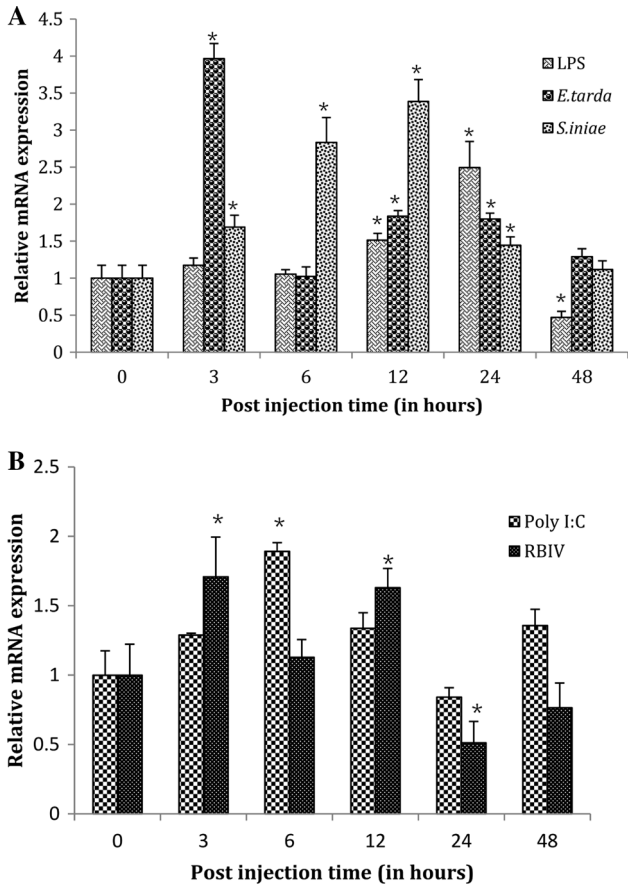


Fig. 6 Expression profile of *RbTLR1* in spleen tissue upon stimulation with **a** LPS, *Edwardsiella tarda*, and *Streptococcus iniae*, and **b** polyinosinic:polycytidylic acid (poly I:C) and rock bream iridovirus (RBIV) determined using qPCR. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using rock bream β-actin as a reference gene. Values were normalized to the corresponding PBS controls at each time point. Expression in uninjected controls at 0 h post injection was set as the baseline. *Error bars* represent SD (n = 3); $^*P < 0.05$

the primary peptide sequence of *RbTLR1*. The model exhibited structural features that resembled the human TLR1 TIR domain (Fig. 4).

In our analysis of tissue-specific expression, the spleen exhibited the highest *RbTLR1* transcript levels compared to the rest of the tissues analyzed (Fig. 5). We attributed this to the key role of the spleen in regulating the immune system (Tarantino et al. 2011). A similar tissue-specific expression profile is observed with *TLR1* orthologs in other vertebrates including the orange-spotted grouper (Wei et al. 2011), rainbow trout (Palti et al. 2010), and chicken (Yilmaz et al. 2005). In contrast, *TLR1* mRNA is scarcely expressed in every tissue examined in healthy green spotted pufferfish, including the spleen (Wu et al. 2008). *TLR1* expression in torafugu is restricted to the kidney, heart and genital gland, with the strongest expression in kidney (Oshiumi et al. 2003). With the exception of torafugu and green spotted pufferfish, constitutive *TLR1* expression was detected in the tissues examined from all the above mentioned species, suggesting its necessity for their survival.

To examine the regulation of *RbTLR1* transcription levels in response to exposure to different pathogenic organisms and molecular patterns, we performed challenge experiments by using *E. tarda*, a Gram-negative bacteria; LPS, a component of Gram-negative bacterial cell walls; *S. iniae*, a Gram-positive bacteria; poly I:C, a PAMP that acts as dsRNA viral mimic; and RBIV, a virulent viral pathogen of rock bream.

As shown in Fig. 6a, RbTLR1 appears as a candidate immune responsive gene under Gram-negative bacterial invasion, because its expression was significantly elevated ($P < 0.05$) after stimulation with LPS and *E. tarda*. Notably, *E. tarda* could trigger an early phase (3 h p.i.) relatively higher (∼2.9-fold) expressional induction compared to LPS, which may attribute to the difference of the strength of two stimulants. *E. tarda* is a live pathogen, whereas LPS is a non-living single chemical component of the bacterial wall. Hence, live pathogens like *E. tarda* can proliferate in host cells and increase their levels of PAMPs to mount more potent response. It is a documented fact that TLRs can lead to induce proinflammatory cytokines including IL-1β (Kawai and Akira 2010). Interestingly, IL-1β identified from rock bream was also found to upregulate at the same time points, 12 and 24 h after LPS injection and 3 h after *E. tarda* injection (unpublished data), reinforcing the potential activation of TLR1 mediated signaling cascade in rock bream spleen cells upon Gram negative bacterial invasion. Inductive response of *RbTLR1* to the aforementioned stimulants is comparable to the modulation of *TLR1* mRNA levels in the spleen of orange-spotted grouper (Oshiumi et al. 2003). Therein, increased expression levels of *TLR1* were detected at 24 and 48 h after stimulation with *Vibrio alginolyticus* and 12 h after LPS induction. A similar positive regulation of *TLR1* expression was observed in green spotted pufferfish after 12 h post challenge with LPS (Wu et al. 2008). As shown in Fig. 6a, the *RbTLR1* transcription profile upon *S. iniae* challenge showed a nearly parabolic-shaped distribution, exhibiting a significant increase at every time point except 48 h p.i. These data suggest that *RbTLR1* is a candidate PRR for sensing invaded Gram-positive bacteria, further convincing its role in activating immune signaling based on our detected upregulated expression of IL-1β in response to *S. iniae* exposure (unpublished data). Consistently, in zebrafish, Gram-positive *Mycobacterium* infection resulted an upregulation of *TLR1* expression, even after 8 weeks of infection (Meijer et al. 2004). In contrast, bacterial TLR agonists such as lipoproteins and flagellins could not significantly upregulate the transcript level of TLR1 relative to the basal level in rainbow trout (Palti et al. 2010).

Since poly I:C is a well-known TLR agonist, it was used as an immune stimulant in our experiments to analyze the effect of immune challenge on *RbTLR1* transcription. This viral PAMP enhanced *RbTLR1* expression significantly ($P < 0.05$) only in the early phase (6 h p.i.). Similarly, poly I:C elevates the mRNA expression of *TLR1* in the head kidney and spleen of the orange-spotted grouper, albeit in the late phase (24 h p.i.) (Wei et al. 2011). Intriguingly, our unpublished data showed a positive transcriptional response of IL-1β upon poly I:C exposure. These data further suggest that RbTLR1 may induce proinflammatory cytokines in rock bream under viral infection.

For the first time in a teleost, we identified a transcriptional inductive response of the *TLR1* gene upon stimulation with a DNA virus, RBIV (Fig. 6b), revealing that TLR1 is potentially involved in recognizing PAMPs on DNA viruses. These data can be fairly supported by the previous evidence which claims that the glycoprotein PAMPs on human cytomegalovirus (a DNA virus), interacts with the TLR1/2 heterodimeric complex (Boehme et al. 2006). The pattern of IL-1β expression induced upon RBIV infection was found to comply with the transcriptional response of *RbTLR1* (unpublished data). These data indicate the existence of a putative cytokine-inducible immune response pathway that can be triggered by the sensitivity of TLR1 to viral pathogens in rock bream. We attribute the downregulation of transcript at 24 h p.i to the host immune evasion mechanisms exerted by the virus during the late phase of the infection, since virus can orchestrate different protective mechanisms against host immunity, ensuring the survival of the virus in the host organisms (Iannello et al. 2006).

In summary, the complete coding sequence of *RbTLR1* was identified from our custom constructed BAC genomic DNA library. We structurally characterized RbTLR1 and analyzed its transcriptional profile in healthy and immune-challenged animals. Phylogenetic analysis revealed the evolutionary proximity of RbTLR1 with orthologs of other vertebrates. The predicted genomic structure of RbTLR1 showed distinct arrangement to most of other fish species, considered. The immune response of *RbTLR1* gene expression upon exposure to different PAMPs, bacterial and viral challenges, was similar to that of the proinflammatory cytokine IL-1β, providing evidence for the putative involvement of RbTLR1 in host immune defense. In addition, our data speculate that the PAMPs used in these experiments may be the potential TLR1 agonists in rock bream. RBIV induction increased the levels of *RbTLR1* transcription in this study. However, future research should focus on elucidating the mechanisms of TLR1/2 heterodimeric complex-mediated sensing of DNA virus in teleost species. To fully evaluate the function of TLR1 in rock bream innate immunity, a comparative analysis between rock bream TLR1 and 2 is required to be performed since both TLR1 and 2 receptors together form a heterodimer for subsequent PAMP identification and initiation of the signaling cascade to mount an immune response as evidenced by previous studies.

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References

Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124:783–801

Anderson KV (2000) Toll signaling pathways in the innate immune response. Curr Opin Immunol 12:13–19

Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22:195–201

Bell JK, Mullen GE, Leifer CA, Mazzoni A, Davies DR, Segal DM (2003) Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol 24:528–533

Boehme KW, Guerrero M, Compton T (2006) Human cytomegalovirus envelope glycoproteins B and H are necessary for TLR2 activation in permissive cells. J Immunol 177:7094–7102

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622

Gangloff M, Ludidi PL, Gay NJ (2005) Structure and motifs involved in Toll signaling. In: Rich T (ed) Toll and Toll-like receptors: an immunological perspective. Springer, US, pp 56–93

Goodsell DS (2005) Representing structural information with RasMol. Curr Protoc Bioinform 11:5.4:5.4.1–5.4.23

Hajjar AM, O'Mahony DS, Ozinsky A, Underhill DM, Aderem A, Klebanoff SJ, Wilson CB (2001) Cutting edge: functional interactions between Toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. J Immunol 166:15–19

Iannello A, Debbeche O, Martin E, Attalah LH, Samarani S, Ahmad A (2006) Viral strategies for evading antiviral cellular immune responses of the host. J Leukoc Biol 79:16–35

Johnson GB, Brunn GJ, Tang AH, Platt JL (2003) Evolutionary clues to the functions of the Toll-like family as surveillance receptors. Trends Immunol 24:19–24

Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11:373–384

Kopp EB, Medzhitov R (1999) The Toll-receptor family and control of innate immunity. Curr Opin Immunol 11:13–18

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408

Magnadottir B (2006) Innate immunity of fish (overview). Fish Shellfish Immunol 20:137–151

Matzznger P (2002) The danger model: a renewed sense of self. Science 296:301–305

Medzhitov R (2007) Recognition of microorganisms and activation of the immune response. Nature 449:819–826

Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, Janeway CA Jr (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell 2:253–258

Meijer AH, Gabby Krens SF, Medina Rodriguez IA, He S, Bitter W, Ewa Snaar-Jagalska B, Spaink HP (2004) Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. Mol Immunol 40:773–783

Oshiumi H, Tsujita T, Shida K, Matsumoto M, Ikeo K, Seya T (2003) Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. Immunogenetics 54:791–800

Palti Y (2011) Toll-like receptors in bony fish: from genomics to function. Dev Comp Immunol 35:1263–1272

Palti Y, Rodriguez MF, Gahr SA, Purcell MK, Rexroad CE 3rd, Wiens GD (2010) Identification, characterization and genetic mapping of TLR1 loci in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol 28:918–926

Park SL (2009) Disease control in Korean aquaculture. Fish Pathol 44:19–23

Priyathilaka TT, Elvitigala DA, Whang I, Lim BS, Jeong HB, Yeo SY, Choi CY, Lee J (2014) Molecular characterization and transcriptional analysis of non-mammalian type Toll like receptor (TLR21) from rock bream (*Oplegnathus fasciatus*). Gene 553:105–116

Rebl A, Goldammer T, Seyfert HM (2010) Toll-like receptor signaling in bony fish. Vet Immunol Immunopathol 134:139–150

Rose AB (2008) Intron-mediated regulation of gene expression. Curr Top Microbiol Immunol 326:277–290

Sangrador-Vegas A, Martin SA, O'Dea PG, Smith TJ (2000) Cloning and characterization of the rainbow trout (*Oncorhynchus mykiss*) type II interleukin-1 receptor cDNA. Eur J Biochem 267:7031–7037

Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res 31:3381–3385

Slack JL, Schooley K, Bonnert TP, Mitcham JL, Qwarnstrom EE, Sims JE, Dower SK (2000) Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. J Biol Chem 275:4670–4678

Takano T, Kondo H, Hirono I, Endo M, Saito-Taki T, Aoki T (2011) Toll-like receptors in teleosts. In: Bondad-Reantaso MG, Jones JB, Corsin F, Aoki T (eds) Diseases in Asian Aquaculture VII. Fish Health Section, Asian Fisheries Society, Malaysia, pp 197–208

Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, Akira S (2002) Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 169:10–14

Tarantino G, Savastano S, Capone D, Colao A (2011) Spleen: a new role for an old player? World J Gastroenterol 17:3776–3784

Venkatesh B (2003) Evolution and diversity of fish genomes. Curr Opin Genet Dev 13:588–592

Volff JN (2005) Genome evolution and biodiversity in teleost fish. Heredity (Edinb) 94:280–294

Wei YC, Pan TS, Chang MX, Huang B, Xu Z, Luo TR, Nie P (2011) Cloning and expression of Toll-like receptors 1 and 2 from a teleost fish, the orange-spotted grouper *Epinephelus coioides*. Vet Immunol Immunopathol 141:173–182

Wesche H, Henzel WJ, Shillinglaw W, Li S, Cao Z (1997) MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity 7:837–847

Whang I, Lee Y, Kim H, Jung SJ, Oh MJ, Choi CY, Lee WS, Kim SJ, Lee J (2011) Characterization and expression analysis of the myeloid differentiation factor 88 (MyD88) in rock bream *Oplegnathus fasciatus*. Mol Biol Rep 38:3911–3920

Wu XY, Xiang LX, Huang L, Jin Y, Shao JZ (2008) Characterization, expression and evolution analysis of Toll-like receptor 1 gene in pufferfish (*Tetraodon nigroviridis*). Int J Immunogenet 35:215–225

Xu Y, Tao X, Shen B, Horng T, Medzhitov R, Manley JL, Tong L (2000) Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. Nature 408:111–115

Yilmaz A, Shen S, Adelson DL, Xavier S, Zhu JJ (2005) Identification and sequence analysis of chicken Toll-like receptors. Immunogenetics 56:743–753

Zenke K, Kim KH (2008) Functional characterization of the RNase III gene of rock bream iridovirus. Arch Virol 153:1651–1656