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# Genomic structure and immunological response of an STAT4 family member from rock bream (*Oplegnathus fasciatus*)

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ABSTRACT

The Janus tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway plays a critical role in host defense against viral and bacterial infections. STAT proteins are a group of transcription factors that translocate into the nucleus and are critical for the induction of many genes crucial for the allergic cascade and immune defense. In the present study, a member of the STAT4 family was identified from rock bream (*RbSTAT4*) at the genomic level, and its transcriptional regulation in response to different pathological stimuli under *in vivo* conditions was investigated. The genomic sequence of *RbSTAT4* is approximately 15.6 kb in length, including a putative core promoter region and 24 exons interrupted by 23 introns. Bioinformatics analysis of RbSTAT4 identified the presence of typical and conserved features of the STAT4 family, including the STAT_int domain, STAT alpha domain, STAT bind domain, linker domain, SH2 domain, and transcriptional activation domain. According to the phylogenetic analysis, RbSTAT4 exhibited the closest evolutionary proximity with the STAT4 member from mandarin fish (*Siniperca chuatsi*). The *RbSTAT4* transcript in healthy rock breams was detected to have ubiquitous expression in 11 different tissues examined, where liver and spleen tissues showed moderate expressions compared with the highest expression level detected in gill tissue. The time-course *in vivo* immune stimulation of rock bream with lipopolysaccharide, poly I:C, live *Edwardsiella tarda*, and rock bream iridovirus caused significant transcriptional regulation of the *RbSTAT4* expression in gill, head kidney, and spleen tissues, suggesting that *RbSTAT4* is involved in immune regulation mechanisms and/or signaling cascades, orchestrating against both bacterial and viral pathogens.
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## 1. Introduction

Mariculture is a rapidly developing industry worldwide, providing valuable sources of essential fatty acids and essential amino acids along with enriched sources of other nutrients. Many edible fish species are produced either through culturing or capture fisheries. Rock bream (*Oplegnathus fasciatus*) is one of the most economically important and highly consumed fish species, especially in eastern Asia. However, production losses have also increased in recent years with the growing and intensified mariculture industry owing to the occurrence of many infectious diseases. In particular, Edwardsiellosis caused by *Edwardsiella tarda* [1] and iridoviral disease caused by rock bream iridovirus (RBIV) [2] occur frequently, causing a significant mortality of farmed rock breams. In this regard, disease control plays an important and critical role to minimizing production losses, either by pathogen control with chemotherapeutics or by host control with vaccines and immunostimulants [3]. Studies on fish immunogenetics will provide more precise approaches to develop new strategies for efficient disease control.

The immune system of an organism plays a critical and indispensable role, where the innate immune system of fish relies on both cellular and humoral responses that are mediated via the activation of several signaling pathways that have already been identified in mammals [4]. The Janus tyrosine kinase (JAK)/signal

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transducer and activator of transcription (STAT) signaling pathway plays a critical role in host defense against viral and bacterial infections. The JAK/STAT signaling cascade is activated as a response to the various chemical signals induced by interferons (IFNs), interleukins (ILs), growth factors, or other chemical messengers. STAT proteins are a group of transcription factors that transmit signals to the nucleus and are critical for the induction of many genes crucial for the development of allergic inflammations and immune defense [5]. The members of the STAT family have been found to be involved in cell proliferation, apoptosis, survival, immune functions, and certain aspects of tissue differentiation [6,7]. In mammals, STAT proteins are grouped into 7 families (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6), where each STAT member has been found to be bound to different DNA sequences in the promoter region of the target gene.

STAT4 is an important element in mediating IL-12 responses, and it has been most extensively investigated in both human and murine T lymphocytes [8–10]. Many studies have reported that Th1 cell differentiation requires STAT4 activation through the IL-12 signaling cascade [11,12]. Moreover, STAT4 was reported to bind directly with genes involved in Tfh cell differentiation, including *Bcl6* and *IL-21* [13]. STAT4 was also reported to function in conjunction with STAT1 in order to produce IFN-$\gamma$ and to enhance the expression of T-box transcription factor (T-bet) in Th1 cell differentiation through the IL-12 signaling cascade [14]. Although STAT4 is activated by both IL-12 and type I IFN-$\alpha/\beta$ in humans, it is activated only by IL-12 in mice [15–17], and it was also reported that STAT4 is activated by IFN but not by IL-12 in human vascular endothelial cells [18]. In addition to T lymphocytes, STAT4 was also reported to be expressed by different immune cells, including B lymphocytes [19], natural killer (NK) cells [20], dendritic cells, monocytes, and macrophages [21,22]. However, the role of STAT4 in teleosts is not well understood, and no STAT or IL genes from rock bream have been identified yet. In this study, the genomic organization of the STAT4 gene from rock bream was identified and its temporal mRNA expression was investigated in animals challenged with different immune stimuli derived from both bacterial (*E. tarda* and lipopolysaccharide (LPS)) and viral origins (rock bream iridovirus and poly I:C).

## 2. Materials and methods

### 2.1. cDNA sequence identification and bioinformatics analysis

The complete cDNA sequence of rock bream STAT4 (*RbSTAT4*) was identified from the rock bream multi-tissue normalized cDNA GS-FLX database, as described in our previous report [23]. The complete cDNA sequence was used to analyze the open reading frame (ORF) sequence by using the DNAssist version 2.2 software. Both the nucleotide and amino acid sequences were subjected to a homology search using the Basic Local Alignment Search Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/), and functional domains were identified in the protein sequence by analyzing with the Conserved Domain Database (CDD) at the NCBI (http://www.ncbi.nlm.nih.gov/cdd). Identity and similarity percentages were calculated by comparing with other known STAT4 members from different species identified from a BLAST search by using EMBOSS Needle–Pairwise sequence alignment at the amino acid level. Multiple sequence alignment was carried out with STAT4 orthologous sequences by using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A phylogenetic tree was constructed by applying the neighbor-joining method available in the MEGA version 5.0 program, which represents all STAT family members in different taxonomic classes with bootstrap values from 1000 replicates.

### 2.2. Genomic sequence comparison and promoter sequence analysis

The genomic sequence of *RbSTAT4* was identified from a rock bream bacterial artificial chromosome (BAC) library by screening with the polymerase chain reaction (PCR) and a sequencing-based method, as described in our previous report [24]. Genomic DNA (gDNA) and cDNA sequences were analyzed using the Spidey program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/) in order to identify the intron–exon structure of *RbSTAT4*. The genomic structures of other species obtained from the Ensembl Genome Browser (http://asia.ensembl.org/index.html) were mapped using the GeneMapper version 2.5 software and compared with the *RbSTAT4* genomic structure. A putative core promoter sequence of around 940 bp from the transcription start site towards the upstream of the gene was analyzed with the TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and Alibaba 2.1 Transcription Factor Binding Prediction (http://www.gene-regulation.com/pub/programs/alibaba2/index.html) programs to locate the potential transcription factor binding sites.

### 2.3. Experimental animals and tissue isolation

Rock breams (mean weight 50 g), provided by the National Fisheries Research and Development Institute, Republic of Korea, were maintained in our laboratory in 400-L tanks under controlled conditions at 24 °C with sand-filtered aerated seawater. The fish were acclimatized for a period of 1 week under laboratory conditions and fed daily with standard commercial feed. A total of 11 different tissue samples (muscle, blood, brain, intestine, kidney, head kidney, heart, liver, spleen, gill, and skin) were collected for total RNA extraction. Blood samples (approximately 1 mL/fish) were collected from the caudal vein using a 22 G syringe, and samples were immediately centrifuged at 3000 $\times$ *g* at 4 °C for 10 min to separate the blood cells. All the tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until used for the RNA extraction.

### 2.4. Total RNA extraction and cDNA synthesis

Pooled tissue samples from 3 fish (50 mg/fish) were used for the total RNA isolation using TRIzol reagent (Sigma–Aldrich), according to the manufacturer's protocol. Purified RNA samples were diluted to 1 μg/μL, and 2.5 μg of RNA from each tissue was used for cDNA synthesis using the PrimeScript™ First Strand cDNA Synthesis Kit (TaKaRa Bio Inc., Japan) following the manufacturer's protocol. The resultant cDNA was then diluted 40-fold (total 800 μL) before storage at −20 °C.

### 2.5. Tissue-specific gene expression analysis by qPCR

To analyze the tissue-specific *RbSTAT4* gene expression, 11 types of tissues were collected from 3 healthy rock breams, and the respective cDNAs were synthesized as described in Sections 2.3 and 2.4. Gene expression analysis was carried out by the quantitative real-time PCR (qPCR) technique using the following gene-specific primers: sense primer 5′-TTGTGAGTAAAGAGATGGAGCG-3′ and anti-sense primer 5′-AACTTCACCTCCCCATTGTC-3′. Briefly, the reaction was carried out in a 15 μL reaction volume containing 4 μL of cDNA from each tissue, 7.5 μL of 2 $\times$ TaKaRa Ex Taq™ SYBR premix, 0.6 μL of each gene-specific primer (10 pmol/μL), and 2.3 μL of PCR-grade $H_2O$. The qPCR cycle program consisted of 1 cycle of 95 °C for 10 s, followed by 45 cycles of 95 °C for 5 s, 58 °C for 20 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 to maintain consistency,

and the qPCR was performed using the TaKaRa Thermal Cycler Dice™ Real Time System (TP800) version 3.00B. Beta-actin gene expression was detected as the internal control (Accession No. FJ975145), and all the *RbSTAT4* expression values were normalized to the beta-actin expression values in each tissue. The mRNA expression was calculated according to the $2^{-\Delta\Delta CT}$ (Livak) method [25], and the tissue-specific expression values were compared with that of blood. All the samples were tested in triplicates.

### 2.6. Immune-stimulated gene expression analysis

The immune stimulation experiment was conducted with several stimulants and possible pathogens of rock bream, from both bacteria and viruses. Independent groups of rock breams were administered a single intraperitoneal (i.p.) injection of 100 μL each of suspensions of LPS in phosphate-buffered saline (PBS; 1.25 μg/μL, *Edwardsiella coli* 055:B5; Sigma), or poly I:C in PBS (1.5 μg/μL; Sigma), or RBIV in PBS ($10^3$ $TCID_{50}$), or live *E. tarda* in PBS ($5 \times 10^6$ CFU/mL), as described in our previous report [26]. One other group of rock breams served as a PBS-injected control (100 μL i.p. injection) and another group was maintained as the un-injected (UI) control. Randomly selected triplicates of fish were sampled at 3, 6, 12, 24, and 48 h post-injection/infection (p.i.) for gill, head kidney, and spleen tissues isolation. The tissue isolation, cDNA synthesis, and qPCR analysis were performed as described in Sections 2.3, 2.4, and 2.5, respectively. Beta-actin gene expression was detected as the internal control (Accession No. FJ975145), and the relative expression values were calculated by normalizing the *RbSTAT4* expression values in each challenge experiment with those of the PBS controls at each time point. Relative expressions were statistically analyzed as compared with the UI control.

### 2.7. Statistical analysis

To determine the statistical significance between the experimental and control groups, all the mRNA expression data were subjected to either a Student's *t*-test or one-way analysis of variance (ANOVA) in SPSS version 16.0 for Windows (SPSS, Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$. All the data are presented as the mean ± standard deviation.

## 3. Results and discussion

### 3.1. Genomic and cDNA sequence identification

The complete genomic sequence of *RbSTAT4* (~15.6 kb), including the putative promoter sequence (~1 kb), was identified from rock bream BAC library screening and sequencing. This sequence has been deposited in GenBank under the accession number KC521467. The genomic structure of *RbSTAT4* comprised 24 exons and 23 introns, in which the 5′ untranslated region (UTR) was split into 2 exons (Fig. 1). However, the coding region of *RbSTAT4* was split into 23 exons, which is compatible with the genomic structure organization of several other teleost species (Supplementary Fig. 1). Moreover, almost all the exons exhibited an identical length to either the stickleback or zebrafish genomic architectures, except for the last exon that was found to be a bit smaller in length. Although some exons exhibited identical lengths to other STAT4 orthologs from avian and mammalian species, the number of exons was varied among species. However, all the teleost species compared in this study exhibited a more or less similar genomic structure for STAT4 orthologs. Herein it is also intriguing to note that almost all of the taxonomic lineages including teleost are consisted of multi-exon genomic architecture which splits their genomic gene into significant number of exons. This observation convinces the prominent potential of STAT4 members to undergo post-transcriptional modifications such as alternative splicing to produce different isoforms since stat family members are known to form protein variants, which could be arouse as a result of alternative splicing process leading to alter their functional behavior [27], further depicting a strong evolutionary potential.

Putative promoter region analysis of *RbSTAT4* indicated the presence of several important transcription factor binding sites that are involved in the downstream gene regulation for various stimuli, including cytokines, growth factors, stress, and pathogen infections (Fig. 2). For example, octamer transcription factor-1 (Oct-1), activator protein 1 (AP-1), specificity protein 1 (Sp1), c-jun, interferon consensus sequence binding protein (ICSBP), upstream stimulating factor (USF), nuclear factor of activated T-cells cytoplasmic 3 (NF-ATc3), nuclear factor kappa B (NF-κB), and nuclear factor 1 (NF-1)-like transcription factors are proven to be involved in the activation of immune response genes. Moreover, NF-ATc3 and NF-κB were reported to be involved in the transcriptional regulation of genes acting on T-cell activation and Th1 and Th2 cell differentiation [28–30]. Transcription of most of the protein-coding genes is facilitated by the TATA elements. Although there was no evidence of a TATA box closer to the transcription start site in the putative promoter sequence of *RbSTAT4*, there was a TATA-binding protein binding region at around −285 bp upstream to the transcription start site. However, a considerable number of human core promoters were also recorded to be without TATA-like elements and rich in Sp1-binding sites [31], which were prominently identified in the predicted *RbSTAT4* core-promoter region (Fig. 2).

### 3.2. Sequence characterization

RbSTAT4 amino acid sequence analysis showed the presence of characteristic conserved domains of the STAT4 members, including the N-terminal interaction (STAT_int) domain, STAT alpha (coiled-coil) domain, STAT bind (DNA binding) domain, linker domain, Src homology 2 (SH2) domain, and transcriptional activation domain. Moreover, within the SH2 domain, phosphotyrosine binding pocket, hydrophobic binding pocket, and homodimer interface polypeptide binding sites were identified. Additionally, a crucial element for dimerization, the tyrosine residue ($Tyr^{684}$) that is phosphorylated by JAKs during the activation, was well conserved within the transactivation domain Ref. [32]. However, another conserved phosphorylation residue, serine (Ser), was replaced by threonine ($Thr^{714}$) in the transactivation domain in most of the analyzed fish species except *Salmo salar* (Fig. 3). This phosphorylation site was also reported to be involved in the regulation of STAT4 function by serine/threonine phosphorylation [15,33]. The conserved N-terminal domain of the STATs was reported to play a critical role in DNA binding, which was not essential for dimerization [34]. STAT4 is believed to bind to IFN-γ-activated sites (GASs) as a dimer stabilized through N-terminal domain interactions. However, a mutation of the N-domain in tryptophan

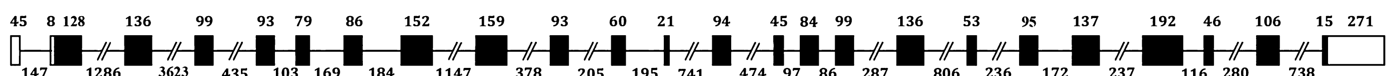


**Fig. 1.** Schematic representation of the RbSTAT4 genomic structure. UTRs, coding regions and introns are denoted by white boxes, black boxes and black lines respectively.

**Fig. 2.** Putative promoter region and transcription factor binding sites of the RbSTAT4. Respective transcription factors are indicated exactly below the TF binding site. Transcription start site and translation start site are marked with a bend arrow. 5′ UTR sequence is boxed and first intron sequence is showed in small letters.

residue W37 was reported to interrupt the dimer formation, which led to the prevention of IFN-α-induced tyrosine phosphorylation and nuclear translocation of the STAT4 [32]. Moreover, methylation of a conserved arginine (Arg-31) at the N-terminus was reported to enhance the DNA binding activity in STAT1 in response to interferon stimulations [35]. Similarly, these 2 conserved $Arg^{31}$ and $Trp^{37}$ residues were found at the same locations in RbSTAT4, suggesting its conserved functional properties. However, the STAT4 N-domain was reported to possess an unusual architecture [36]. The SH2-phosphotyrosine site is important for the interaction between 2 STAT molecules in order to form active dimers that translocate to the nucleus and activate the target gene expression [37].

According to the pairwise sequence alignment, RbSTAT4 showed very high amino acid identity and similarity with other fish species (highest identity to *Siniperca chuatsi*) and moderate identity and similarity with mammalian and avian species (Fig. 3). However, similar to previous reports, the C-terminal region, which plays a critical role for transcriptional activation by phosphorylation, exhibited higher variation among different homologs [38]. The multiple sequence alignment results revealed a clear conservation of most of the characteristic features of the STAT4 members discussed above. In the evolutionary analysis, we used members representing all STAT family proteins distributed among mammal, avian, amphibian, and fish species (total of 42 members). All the STAT families showed closer relation to one another, with higher bootstrap values. RbSTAT4 was clustered within the fish STAT4 clade, suggesting its orthologous nature in the STAT4 family (Fig. 4). Moreover, the STAT4 clade showed a closer evolutionary relation to the STAT1 clade.

### 3.3. Tissue-specific gene expression

*RbSTAT4* was ubiquitously expressed in 11 different tissues examined in healthy rock breams. A lower level of expression was detected in most of the tissues, including kidney, head kidney, intestine, brain, blood, heart, skin, and muscle, where the muscle showed the lowest expression. However, liver and spleen gave moderate expressions, whereas the gill tissue showed the highest (~35-fold compared with blood) mRNA expression (Fig. 5). Gills of teleost species are known to contact with their external environment closely and continuously, further consisting a large surface area which can in turn increase the possibility of occurring frequent pathogenic infections. Therefore, prominent functioning of innate and adaptive immune mechanisms in gill tissues are expected to be occurred, in which STAT4 mediated immunity may also be highly pronounced in gill tissues. Moreover, gills are also comprised of one subdivision of mucosa associated lymphoid tissues, known as “gill-associate lymphoid tissue [39], which are enriched with

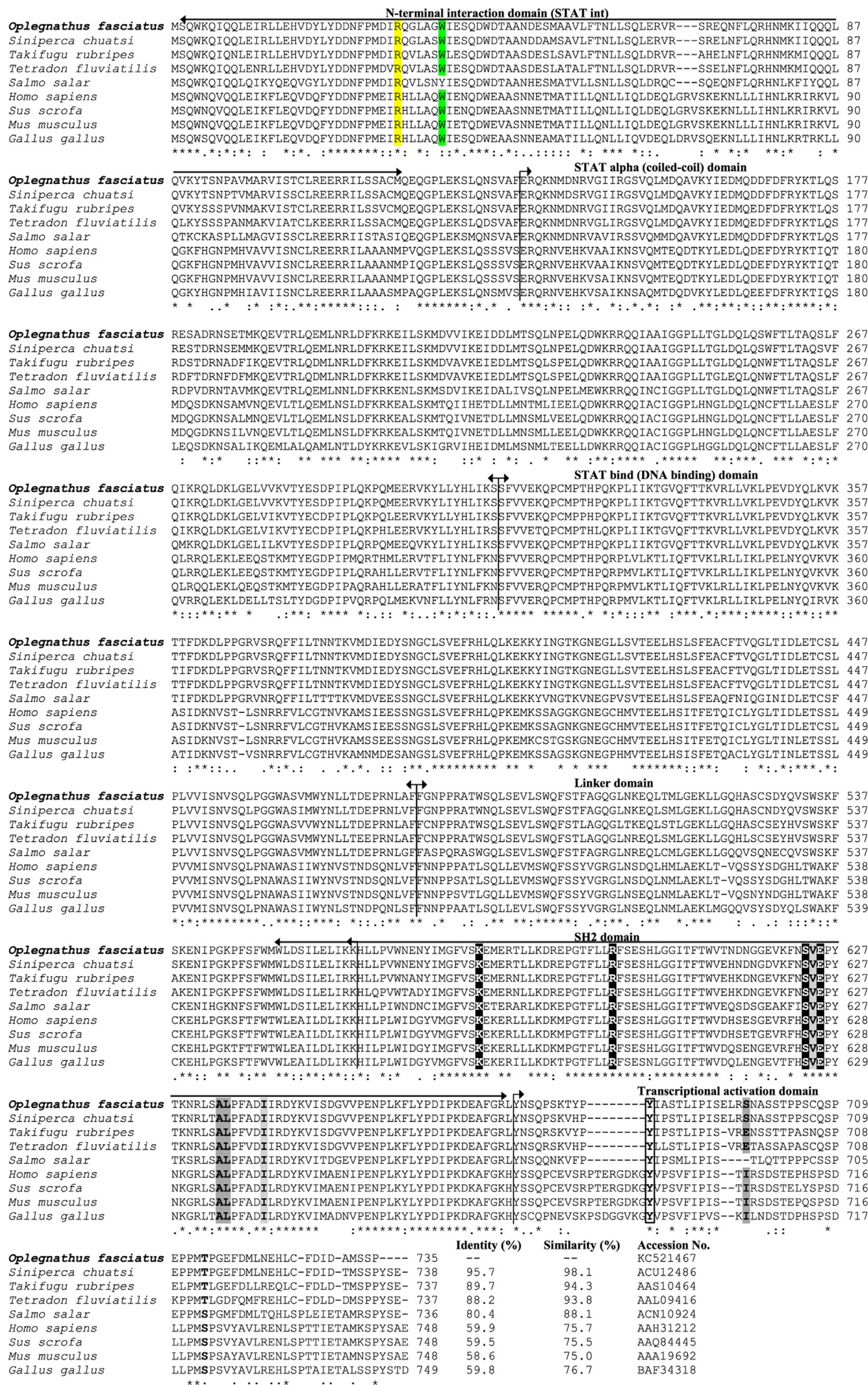


**Fig. 3.** Multiple sequence alignment and pairwise comparison of RbSTAT4 with other STAT4 members. Phosphotyrosin binding pocket, hydrophobic binding pocket and homodimer interface polypeptide binding sites are marked in black, light gray and dark gray backgrounds, respectively. Asterisk (*) indicates identical residues between sequences while colon (:) and period (.) indicate strongly and weakly similar properties of the residues between sequences. Conserved Tyr and Ser phosphorylation sites are showed in boxed and bold face respectively. Two conserved $Arg^{31}$ and $Trp^{37}$ residues are marked in yellow and green background respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

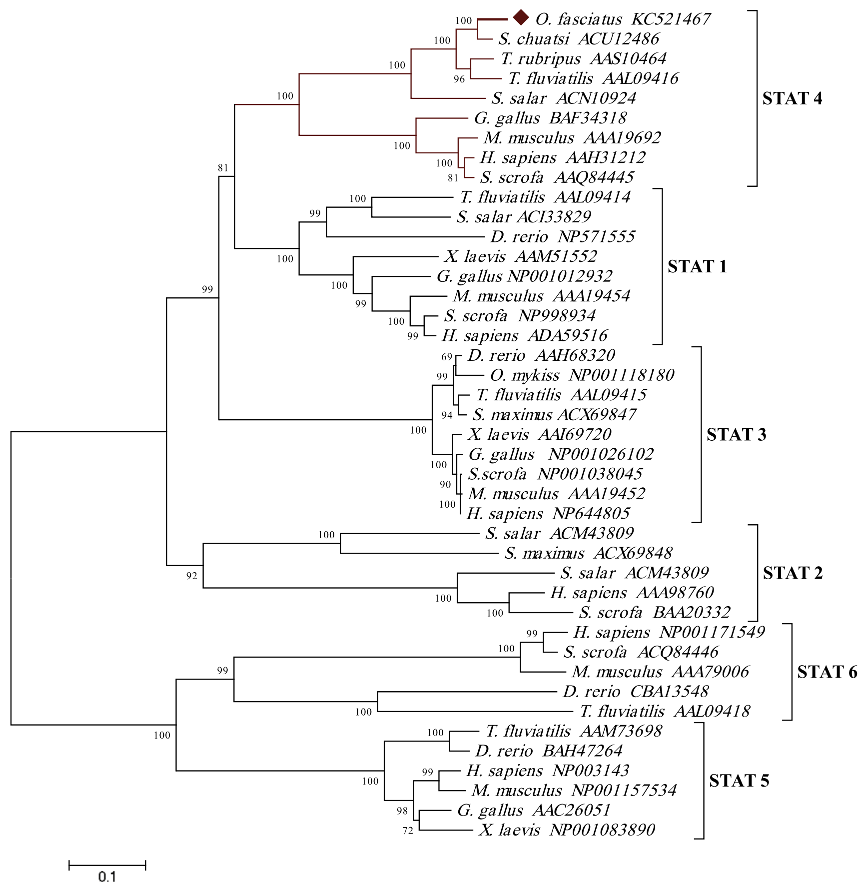


**Fig. 4.** Evolutionary relationship analysis of the RbSTAT4 with other members in the STAT family proteins. The tree is based on the alignment corresponding to the full-length amino acid sequences by ClustalW and MEGA (version 5). The numbers at the branches denote the bootstrap majority consensus values on 1000 replicates.

lymphocytes as well as other immune cells such as macrophages, in which STAT4 molecules were found to be prominently expressed, as mentioned earlier [19,21,22]. Hence, it is not unlike to be observed a pronounced STAT4 expression in gill tissues of teleost, like rock bream. Although, STAT4 was reported to have a restricted distribution, mainly within hematopoietic tissues, including the thymus and spleen, myeloid cells, and developing spermatogonia [9,10], a recent study from mandarin fish showed its ubiquitous expression in different tissues, similar to *RbSTAT4* [40].

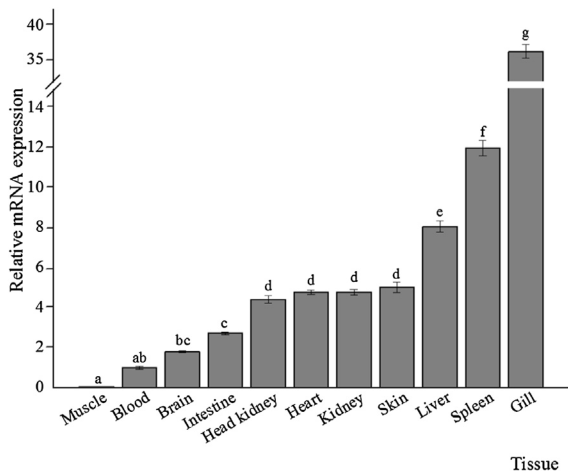


**Fig. 5.** Tissue-specific mRNA expression of the RbSTAT4 in healthy rock breams. Analysis of the mRNA level was carried out by qPCR and relative expressions were calculated compared to the mRNA level detected in blood. Data are represented as means ± standard deviation ($n = 3$). Statistical analysis was performed by one-way ANOVA followed by Duncan's Multiple Range test using the SPSS 16.0 program. Data with different letters are significantly different ($P < 0.05$) among different tissues.

### *3.4. Regulated gene expression after immune stimulation*

Three immune-related tissues were selected for the temporal expression analysis in response to immune stimulations in the present study. The gill is an important organ that is involved in respiration as well as in immune regulation, being frequently exposed to the external environment. Head kidney and spleen are 2 important lymphoid and hematopoietic tissues, which are involved in both innate and adaptive immune responses. LPS is a well-known endotoxin and Gram-negative bacterial cell wall component, and has been reported to induce the JAK/STAT signaling in mosquitoes [41]. After LPS injection, a significant ($P < 0.05$) induction of relative *RbSTAT4* expression was detected in gill tissue from 3 to 48 h post-infection (p.i.), except at 12 h p.i., which was not significantly different from the basal expression (Fig. 6A). Similarly, head kidney also showed a significant induction of relative *RbSTAT4* expression at 3 h p.i. as an acute response, and a significant down-regulation at 24 h p.i., while all other time points showed a basal level of expression (Fig. 6B). However, in spleen, the expression was continuously down-regulated after 3 h p.i. (Fig. 6C). A similar study with LPS stimulation reported only a mild change in STAT transcript level in the shrimp [42]; however, IL-12- or IFN-α-mediated IFN-γ secretion was reported as a result of up-regulated STAT4 expression

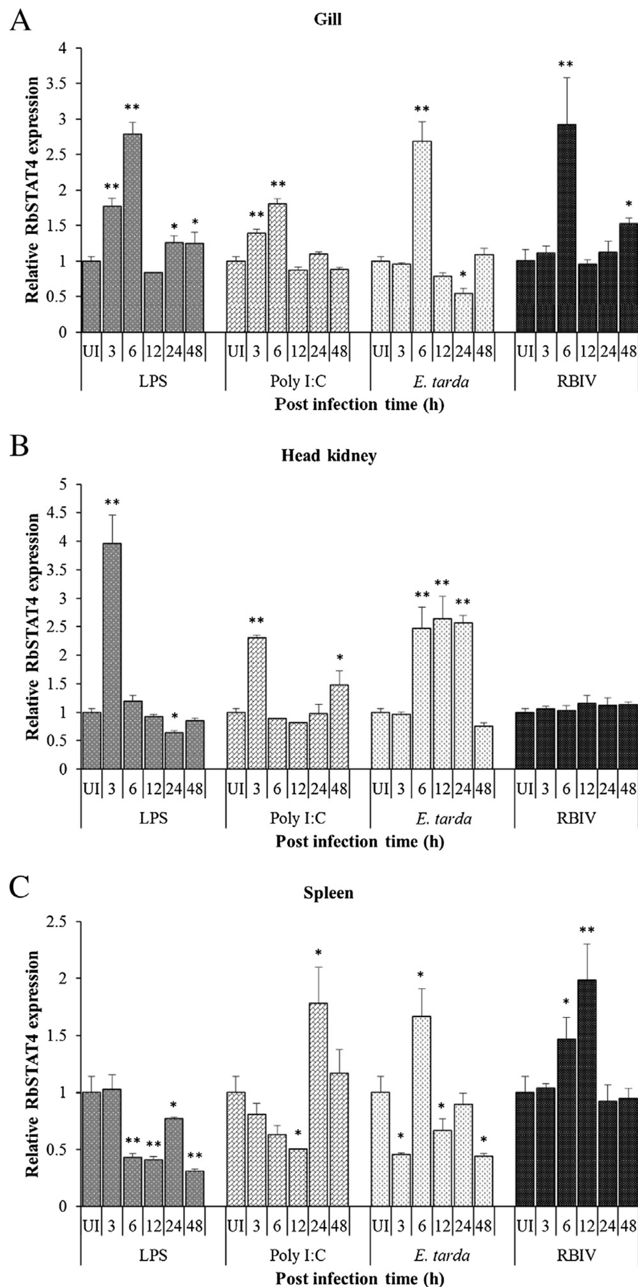


**Fig. 6.** Relative mRNA expression of RbSTAT4 in response to LPS, poly I:C, *E. tarda* and RBIV infections in (A) gill, (B) head kidney and (C) spleen tissues. The mRNA levels were detected by qPCR and data are represented as means ± standard deviation ($n = 3$). Statistical analysis was performed by *t*-test and asterisks indicate significant differences ('*' $P < 0.05$; '**' $P < 0.01$) to the un-injected control (UI).

in response to LPS stimulation during dendritic cell maturation or monocyte activation [21]. The injection with poly I:C, a synthetic analog of double-stranded RNA virus, resulted a generally up-regulated expression pattern of the *RbSTAT4* in all 3 tissues, except at 12 h p.i. in spleen tissue where it was significantly down-regulated (Fig. 6). Poly I:C injection caused both acute and late responses in the head kidney tissue. However, in gill tissue, it was detected as an early phase response compared with the late phase response detected in the spleen. This result is in agreement with a previous time-course study on mSTAT4 expression in poly I:C-induced MFF-1 cells from mandarin fish, which showed a significant induction from 6 h onwards [40].

In the case of the live bacterial infection (*E. tarda*), head kidney showed significant up-regulation of the relative *RbSTAT4* expression from 6 to 24 h p.i., whereas in the other 2 tissues *RbSTAT4* was expressed only at 6 h p.i. However, in spleen tissue, the *RbSTAT4* expression showed a dynamic behavior in response to the *E. tarda* infection (Fig. 6C). The transcriptional activation and translocation of STATs to the nucleus as a result of bacterial challenges have been reported in insects [43–45]. Moreover, it was reported that JAK/STAT signaling is involved in both antibacterial and antiviral responses in insects [41,44,46]. Although *E. tarda* infection caused a significant induction of *RbSTAT4* expression in head kidney, live RBIV infection did not cause any significant difference to the *RbSTAT4* expression throughout the experimental period (Fig. 6B). However, in gill tissue, significantly induced *RbSTAT4* expression was detected at 6 and 48 h p.i., whereas it was induced at 6–12 h p.i. in spleen tissue (Fig. 6A and C). Similarly, transcriptional induction of STAT4 was reported in infectious pancreatic necrosis virus-infected zebrafish embryonic cells [47]. Moreover, white spot syndrome virus infection also showed an increased level of phosphorylated STAT in the lymphoid organs of shrimp, although it showed a decreased transcriptional level of shrimp STAT [42]. Furthermore, a dynamic regulation of STAT4 phosphorylation through the type 1 IFNs was observed after a viral infection in mice [48].

IL-12 is a key immunoregulatory cytokine that coordinates innate and adaptive immune responses through STAT4 activation, which is produced mainly by macrophages and dendritic cells in addition to the monocytes, B cells, and neutrophils, in response to pathogenic infections, including those of bacteria, virus, fungi, and parasites [49]. In addition, microbial products were also reported to induce T-cell-independent IL-12 production via Toll-like receptor signaling [50,51], which suggests that *RbSTAT4* is involved in immune mechanisms against both bacterial and viral infections. IFN-γ is a well-known effector cytokine that is involved in the control of intracellular viral infections, and is activated by STAT4 through the IL-12 signaling pathway [52]. However, both the IL-12 and IFN-γ genes are yet to be discovered in the rock bream genome.

In addition to IL-12 and IL-23, type 1 IFNs were also reported to activate STAT4 directly to induce IFN-γ production during viral infections in mice, and STAT4 was reported to bind with the 5′-upstream sequence of the murine IFN and human perforin genes [13,48,53]. Hence, we compared the *RbSTAT4* expression results obtained in this study with our previous data on type 1 IFN mRNA expressions against the 4 types of infections in head kidney tissue in the same organism [54]. Similar to the *RbSTAT4* expressions, both IFN-1 and IFN-2 also showed induced transcriptional levels in response to all 4 challenges. More specifically, *E. tarda* infection caused a significant elevation of both IFN-2 and *RbSTAT4* expressions at the same time points, except at 6 h p.i. for IFN-2. Moreover, both IFN-1 and IFN-2 showed only mild regulations at some time points in response to RBIV infection, whereas *RbSTAT4* showed no response to the RBIV infection in head kidney. However a clear relation was observed between STAT4 activation by the IFN-α/β and IFN-γ production in lymphocytic choriomeningitis virus-infected mice [48].

Furthermore, STAT4 was reported to enhance interferon regulatory factor-1 (IRF-1) gene transcription by binding to the IRF-1 promoter in human NK and T-cells [55,56]. IRF-1 is a transcription factor that is involved in the regulation of IFN and IFN-inducible genes in Th1 responses through the regulation of T-cell maturation and Th1 and NK cell development [55]. The transcriptional regulation of IRF-1 has been reported in different fish species in response to viral infections [57–59]. In the present study, we also examined the rock bream IRF-1 mRNA expression (Accession No. GQ903769) in gill tissue after the poly I:C injection. Similar to the

*RbSTAT4* expressions detected in gills, rock bream IRF-1 also showed a significant up-regulation of mRNA expression after the poly I:C injections throughout the experimental period, with a higher magnitude but exactly similar pattern of expression to the *RbSTAT4* (un-published data), suggesting the involvement of *RbSTAT4* in IRF-1 gene regulation.

In conclusion, we have identified a member of the STAT4 family from rock bream (*RbSTAT4*) at genomic level and investigated its time-course expression against *in vivo* immune challenges by 2 different mitogens (LPS and poly I:C) and live pathogens (*E. tarda* and rock bream iridovirus). According to the transcriptional regulation of *RbSTAT4* observed in this study, we suggest that *RbSTAT4* is involved in immune regulation mechanisms and/or signaling cascades, functioning against both bacterial and viral pathogens. Hence, the findings of this study will help to extend the knowledge on the diverse biological functions of the STAT4 family members with respect to their immune regulation mechanisms in teleost species. Nevertheless, further investigations are required to clarify the detailed involvement of STAT4 in immune regulation in teleost's, along with the other members of the respective signaling pathways.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2013.09.011.

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