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The Molecular Profiling of a Teleostan Counterpart of Follistatin, Identified from Rock Bream *Oplegnathus fasciatus* which Reveals its Transcriptional Responses against Pathogenic Stress

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Abstract

The follistatin (FST) gene encodes a monomeric glycoprotein that plays a role in binding and inhibiting the functions of members of the transforming growth factor (TGF)-β superfamily. Thus, FST facilitates a wide variety of functions, ranging from muscle growth, to inflammation and immunity. In this study, we sought to characterize an FST counterpart, *RbFST*, which was identified from rock bream *Oplegnathus fasciatus*. The *RbFST* cDNA sequence (2,419 bp) contains a 933-bp open reading frame (ORF) that encodes a putative amino acid sequence for RbFST (35 kDa). The putative amino acid sequence contains a Kazal-type serine protease inhibitor domain (51-98 residues) and an EF-hand, calcium-binding domain (191-226 residues). Additionally, this sequence shares a high identity (98.7%) with the *Siniperca chuatsi* FST sequence, with which it also has the closest evolutionary relationship according to a phylogenetic study. Omnipresent distribution of RbFST transcripts were detected in the gill, liver, spleen, head kidney, kidney, skin, muscle, heart, brain, and intestine of healthy animals, with significantly higher expression levels in the heart, followed by the liver tissue. Under pathogenic stress caused by two bacterial pathogens, *Streptococcus iniae* and *Edwardsiella tarda, RbFST* transcription was found to be significantly up-regulated. Altogether, our findings suggest the putative role of *RbFST* in immune related responses against pathogenic infections, further prefiguring its significance in rock bream physiology.

Key words: *Oplegnathus fasciatus*, Rock bream, Follistatin, Tissue distribution, Immune challenge

Introduction

Aquaculture has been practiced in Korea for several hundred years, with a significant contribution from mariculture farming. As such, it is important to maintain a sustainable mariculture industry in order to gain economic and environmental benefits. The mariculture field has encountered significant production losses in recent years due to the occurrence of numerous infectious diseases. Although there is a high demand for rock bream *Oplegnathus fasciatus*, especially in eastern Asia, culturing them in bulk has become a great challenge due to the infectious diseases primarily caused by *Edwardsiella tarda* (Mohanty and Sahoo, 2007) and rock bream iridovirus (RBIV) (Do et al., 2004). In this regard, exploring

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fish genes that are involved in growth related pathways and immunogenic mechanisms has become a trend in current marine genetic investigations as a primary step of developing a sustainable mariculture industry.

Follistatin (FST) is a monomeric glycoprotein that is considered an inhibitor of follicular stimulating hormone. FST has the ability to suppress other proteins in a manner similar to that of inhibin (Welt et al., 2002). Moreover, it can antagonize several growth factors, including activins, a group of TGF-β superfamily members (Tortoriello et al., 2001). FST is expressed in all types of tissues and exhibits a wide range of tissue distribution. This diversified tissue expression pattern also provides clues to understanding the primary function of FST, which can effectively modify the paracrine/autocrine roles of activin in a variety of tissues. To date, two major FST subfamilies have been identified (Clabaut et al., 1988). Subfamilies are classified based on the two major characteristics of FST-like proteins: (a) a relatively strong activin binding ability and (b) structural homology to other FST-like proteins. Members of the first subfamily have both properties while members of the second subfamily lack the ability to bind activin.

It is known that FST plays a major role in fish development, since significant levels of FST expression have been detected in a zebrafish model (Bauer et al., 1998). FST was found to be expressed at relatively high levels during advanced stages of embryonic development (Nakamura et al., 1990; Michel et al., 1993; Amthor et al., 2002). Moreover, according to previous studies on activin A, FST is involved in fish reproduction and muscle development. By antagonizing and interacting with the functions of members of the TGF-β superfamily, FST can regulate a variety of TGF-β superfamily proteins in growth- and development-related pathways. Early detection of FST transcripts in sea bream embryos also suggested a possible physiological role in fish development (Funkentein et al., 2009). In addition to activin A, extensive studies on FST suggest its involvement in antagonizing the action of several other members of the TGF-β superfamily. FST itself can also interact with bone morphogenetic proteins (BMPs) (Fainsod et al., 1997; Iemura et al., 1998; Amthor et al., 2002), growth differentiation factor-9 (GDF-9) (Lin et al., 2003), and myostatin [MSTN, or growth differentiation factor-8 (GDF-8)] (Zimmers et al., 2002) in order to abolish their functions during particular growth stages.

Recent studies on FST have provided new insight on its functions, including its involvement in immune-related pathways and developmental physiology. The induction of activin A causes the death of B cells, which are major components of the host defense mechanism (Yu et al., 1987). Additionally, differentiation and proliferation of cells from erythroid lineages was observed following the induction of activin A expression (Broxmeyer et al., 1988). These observations, and its ability to interact with other cytokines such as IL-1 and IL-6 at different regulatory levels, confirm the involvement of activin A in inflammatory pathways (Yu et al., 1987). The interaction of FST with members of the TGF-β superfamily, including activin A, suggests that FST can potentially participate in host immune/inflammatory responses. Moreover, studies under septic conditions (Broxmeyer et al., 1988) showed that because of this FST-activin A interaction, FST must also have a critical role in inflammatory disorders, as it is a stiff inhibitor of activin A. In this study, we have characterized the *FST* gene from rock bream (*RbFST*) at the molecular level and determined the basal mRNA expression levels of *RbFST* in selected tissues. The transcriptional modulation of *RbFST* was also examined in rock bream liver tissues, stimulated with *E. tarda* and *Streptococcus iniae*, in order to elucidate its putative involvement in the host immune response.

Materials and Methods

Identification of the complete *RbFST* cDNA sequence

The full-length *RbFST* cDNA sequence was identified from the previously established rock bream cDNA sequence database (Whang et al., 2011) using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Bioinformatic characterization of RbFST sequences

The open reading frame (ORF) and amino acid sequences of RbFST were derived using DNAssist 2.2. The FST protein sequences from other species were obtained using a BLAST search. These sequences were then used for pair-wise and multiple sequence alignments by utilizing EMBOSS needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) and ClustalW2, respectively (Thompson et al., 1994). Phylogenetic relationships were determined with Molecular Evolutionary Genetics Analysis (MEGA) software version 5 (Tamura et al., 2011) using the Neighbor-Joining method and bootstrapping values taken from 1000 replicates. Characteristic protein signatures in the RbFST sequence were predicted using the ExPASy-prosite server (http://prosite.expasy.org) and the NCBI conserved domain database (CDD) (Marchler-Bauer et al., 2011). Some physicochemical properties of RbFST were determined using the ExPASy ProtParam tool (http://web.expasy.org/protparam).

Experimental animals and tissue collection

O. fasciatus, with an average size of 50 g, were obtained from the Ocean and Fisheries Research Institute (Jeju special Self-Governing Province, Republic of South Korea). The fish were acclimated for one week in 400-L tanks at 22-24°C in a

controlled environment (34 ± 1 practical salinity units, pH 7.6 ± 0.5). Subsequently, gills, liver, kidney, head kidney, spleen, skin, intestine, heart, brain, and muscle tissues were dissected from three healthy fish. Blood samples (~1 mL/fish) were taken from the caudal fin using a 22-G syringe. Samples were immediately centrifuged at 4°C (3,000 *g*) for 10 minutes to separate the blood cells, and then directly frozen in liquid nitrogen before storing at -80°C. Total RNA was isolated from all of the selected tissue samples, separately, using Tri Reagent™ (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol.

Immune challenge experiment

To examine the immune response of *RbFST* upon immune challenges, two immune challenge experiments were performed using *E. tarda* and *S. iniae* as pathogenic stimuli (Umasuthan et al., 2014). The fish were intraperitoneally injected with 100 μL of *E. tarda* (5×10^3 CFU/mL) or *S. iniae* (1×10^5 CFU/mL) that had been re-suspended in 1x phosphate-buffered saline (PBS). The control group was injected with the same volume of PBS. Next, liver tissues of at least three injected animals were collected at 3, 6, 12, 24, and 48 h post-challenge. This process was consistent between the challenged and control groups.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from rock bream tissues that had been pooled from three fish, from both the control and challenged groups, using Tri Reagent™ (Sigma). After quantification by UV spectrophotometry (optical density at 260 nm), the total RNA samples were diluted to 1 μg/μL and used to perform cDNA synthesis using the PrimeScript™ cDNA Synthesis Kit (TaKaRa Bio, Japan), according to the manufacturer's instructions. Finally, newly synthesized cDNA was diluted 40-fold (total volume: 800 μL) and stored at -20°C until use.

RbFST mRNA expression analysis by quantitative real-time PCR (qPCR)

qPCR was performed using the Dice™ TP800 Real-Time Thermal Cycler System (TaKaRa) in a 15-μL reaction volume containing 4 μL of diluted cDNA, 7.5 μL of 2 × TaKaRa Ex*Taq*™ SYBRpremix, 0.6 μL of sequence-specific primers (Table 1), and 2.3 μL of ddH_2O. The following thermal cycling conditions were used: 10 sec at 95°C; followed by 35 cycles of 5 sec at 95°C, 10 sec at 58°C, and 20 sec at 72°C; and a final cycle of 15 sec at 95°C, 30 sec at 60°C, and 15 sec at 95°C. The Dice™ Real-Time System Software (version 2.00) automatically set the baseline. *RbFST* expression was determined using the Livak ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). The same qPCR profile was used for detection of the internal control gene expression, rock bream *β-actin* (Accession No. FJ975145), with gene-specific primers (Table 1). Expression levels were analyzed in triplicate and the data are presented as the mean ± standard deviation (SD) of relative mRNA expression. The relative expression levels of *RbFST* at the 0-h time-point (un-injected control) was used as the baseline for comparison. Expression levels were further normalized to the corresponding PBS-injected controls at each time point. The statistical significance of differences observed between the un-injected control (0 h) and challenged groups was determined with a two-tailed, unpaired t-test at a significance level of $P < 0.05$.

Results and Discussion

Sequence profile and phylogenetic relationship of *RbFST*

The *RbFST* cDNA sequence is a total of 2,419 bp that includes a 933-bp ORF encoding 311 amino acids. The 5'-untranslated region (UTR) is composed of 70 bp and the 3'-UTR consists of 1,416 bp. The molecular mass of RbFST was predicted to be 35 kDa and the theoretical isoelectric point was 4.8. According to the results obtained from NCBI-CDD and ExPASy-prosite servers, RbFST was an FST-like protein because it shares common structural properties with FST-family proteins, including the presence of a Kazal-type serine protease inhibitor domain, which extends from amino acid residues 51 to 98. An EF-hand, calcium-binding domain was also detected, expanding from amino acid residues 191 to 226 (Fig. 1).

Pair-wise and multiple sequence alignments revealed that the putative RbFST protein shares characteristic features with FST protein orthologs from other species. Multiple sequence

Table 1. Oligomers used in this study

Name	Purpose	Sequence (5′ → 3′)
RbFST-F	q-PCR for rock bream RbFST gene	GCTATGCTGCTGACCGCAATGA
RbFST-R	q-PCR for rock bream RbFST gene	GCGTAGGACTGCAACTCCACAAC
Rb-TA	q-PCR for rock bream rock bream β-actin	TCATCACCATCGGCAATGAGAGGT
Rb-TC	q-PCR for rock bream rock bream β-actin	TGATGCTGTTGTAGGTGGTCTCGT

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1    GTCCCGACaAACACA TCCAGACCAGCTCCC CTCCTCCGAGCAGAT CGCCGAGCgGAGCAG
61   CTTTTACGAA
71   ATGATGTCGCGAAGT GTCGCCGTGCTCCTT CTGCTGGCCGTGGCT GTCTGTAACGCGGAG
1     M M S R S  V A V L L  L L A V A  V C N A E
131   GAGCTGCAGAGCAAG AGCAAAGTGTGTGCC AATGTGTTCTGTGGG GCTGGCAGAGAGTGT
21    E L Q S K  S K V C A  N V F C G  A G R E C
191  GCTGTTAACGAGAAg GGGGAGCCCAGCTGT CTGTGCATAGAGAGC TGTAAGCCCCACAAG
41    A V N E K  G E P S C  L C I E S  C K P H K
251  AGGTCAGTGTGTGGC AGCAATGGGAAGACC TACAGGAATCACTGT GAGCTCCACAGGGAT
61    R S V C G  S N G K T  Y R N H C  E L H R D
311  GCTTGTCTGACTGGC TTGAAGATCCAGGTG GCCCACGATGGACAC TGCCAGGAGAAGAAA
81    A C L T G  L K I Q V  A H D G H  C Q E K K
371  ACAGAGCAGGCAGCT GCCAGCCCAGTGGTA TGCTATGCTGCTGAC CGCAATGAGCTGAGG
101   T E Q A A  A S P V V  C Y A A D  R N E L R
431  AGTCGTGTGATCCAG TGGCTGCAGACTGAG GTTATCCCAGATGGC TGGTTTGTCAAGGGA
121   S R V I Q  W L Q T E  V I P D G  W F V K G
491  TCCAACTTCTCTGAC ATCCTGCTCAAATAC TTCAAGTCGTATGAC AACGGTGATTCTCAG
141   S N F S D  I L L K Y  F K S Y D  N G D S Q
551  CTGGACTCCTCAGAG CTGCTCAAATTCATC CAGCAAAATGAGTCG GTTGTGGAGTTGCAG
161   L D S S E  L L K F I  Q Q N E S  V V E L Q
611  TCCTACGCAGACCAG GAGAGCAACAAGCTG CTCAGGAGCCTTTGT GTTGATGCCCTCATT
181   S Y A D Q  E S N K L  L R S L C  V D A L I
671  GAGCTCTCTGATGAG AACGCAGACTGGAAG CTGAGCTTTGATGAG TTCCTCAACTGCCTG
201   E L S D E  N A D W K  L S F D E  F L N C L
731  AAGCCTGGCTTCAAT CCACCAGAGAAGaAA TGTGCCTTGGAGGAT GAGACATATGAGGAC
221   K P G F N  P P E K K  C A L E D  E T Y E D
791  GGAGCAGAGACCCAG GTGGAGTGTAACCGC TGTGTTTGTGCATGC GGCAACTGGGTCTGC
241   G A E T Q  V E C N R  C V C A C  G N W V C
851  ACTGCTATGACCTGC ACTGACAAAACGGCA GCTGTGGATGAGTCA GTAGATGCTGGGGCA
261   T A M T C  T D K T A  A V D E S  V D A G A
911  GAGATGACTGagGAG GAGTGGAACCTCCGT GTGGCTGAGCTCAAC AAGCACCAGGAAACA
281   E M T E E  E W N L R  V A E L N  K H Q E T
971  GTTGAGAAGATGAAG ACCAGCACAAAGGAG GCC
301   V E K M K  T S T K E  A
1004 TAAATAAGGATGCAA TGAAGGAGAGGATGC ACGTCATTACCCTTC CTCCATACTTGATTT
1064 ACCTTGCGGCCATGT GTTGTTATTAGACTG ACTTTTTCATGTTTC TCTGCTATGTTAATT
1124 ATTATACATTTTTTA CAAGTGTTTGTGTTG ATGTTTGTTAGATTC AGGGGCAATCAGAGA
1184 GTATTATAGGATTCC CTCGTGTTCAGTACT GTCATATTTGATTGT ATATGCGCCTCTACA
1244 CTAGCCTTGGGGCTA tTTTTACTACAATCC TTATATTTTTACAGT TATTATGAAAAAAAA
1304 GTCAAGTTGTCACTG TTTTGGAATATGGAA GTCATTGTGTGAGAA GGTAGTGAGGTTAGA
1364 GGTTTGAGGAATCCA CCTGTTGAAATGAGG GAGCTGTCGAAGTAA AGACAAAGCTGACTC
1424 CTTGACACCTGGTGC AGCGTGTTTCTCCAC ACCCTCATGGGTTTC ACGGTCAGCAGTTTG
1484 GGGTAAGAGGGTAAA GCTACTAGTGATTTT GATTTTGTTGCCATT TAGAACTGCCACAGA
1544 AGGCCACTACTTGAC CAATGCACTTCTCTT TCTTTCTCCCTAGCT TTCTTAGGCTTTAGT
1604 TTATCACAAGCCTGA AATTTGTTTGTATCT TACTGCTAAAGTAAT AATTTTGCTGCAAAA
1664 AAGTTTGCTTCTATG TACAGTATGTTATTG GCTTGAGTTGAAACT CATCTTGTTCCCATG
1724 TTATGCTCAAGCAAG TCTCTCTGGTGCTCT TTGGTTCAACATTTC TCTGTCTGTGTCTGA
1784 CATTTCTGAGACAAA GACATAGTGGTCCAG CAGCACAACTGTACC CAGGTGAGACCTGAT
1844 CTGAATATAGCATGG TGAAAAACTGAAACC AAAGTCTTGATGAAG TGTTTGAATGATGTA
1904 CTCCAGGTAAAACAT TACTCTGGACCCAGC AACAACACTATACCT GTGCTTTCAAAATCA
1964 CACTAACAGAAGGAC TAGCCTCAAAGTCAG CTAAAGTAGCTTAGA CGCATCACTAACTGG
2024 GTGCTTATAAATTGT GTGGGTGCGCATGAT TGTGTGTGTGTGCGT GTGTGTGCATTAAAG
2084 CGTGGTGCTGAGAGT AGTTTGGTTTGTTAA ATGTGTTTATTTGTT AAAATCCTGTATCTA
2144 CATTGGGAGGTTCGA CAATGAAAATCAGTG CCTTAGACCTGCAAA CAGTATAGGTCTCTA
2204 CCTTTGCCTGCACAA CGGCTATTAACGCCA CGTGGCACGGCAAGA GTGAGTGTGCTTTGC
2264 TGAAGTTTAGCGGTA AACTAGTGCTTTGTG ACTCTATCCCTGATA AATGTCTGTGTGAAA
2324 GTTGCATTACTAGGT TCAAGCTAAATCTTT ATATTCCACTAAAAG TGCAGAGACTGTAAG
2384 GAATACGTATGTATG CCAATAAAGccCATA CACCAC
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Fig. 1. Nucleotide and amino acid sequences of RbFST. The nucleotide sequence (upper) and the deduced amino acid sequence (lower) are numbered. The start (ATG), stop (TAA) codons and poly (A) signal (AATAAA) are boxed. N-terminal signal peptide is underlined. The FST N-terminal domain-like domain is indicated with double headed arrows (Residues 28-51), Kazal type serine protease inhibitor domain is showed within "(Miyabe et al., 2014)"(Residues 51-96) and EF-hand calcium-binding domain profile is highlighted (Residues 191-226).

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Homo sapiens               -MWKRWLALALALVAVAWVRAEEELRSKSKICANVFCGAGRECAVTEKGEPTCLCIEQCK 59
Pongo abelii               MMWKRWLALA--LVAVAWVRAEEELRSKSKICANVFCGAGRECAVTEKGEPTCLCIEQCK 58
Bos taurus                 MMWRRWLALA--LVAVAWVHAEEQVRSKSKICANVFCGAGRECAVTEKGEPTCLCIEQCK 58
Mus musculus               -MWKRWLALS--LVTIALVHGEEEPRSKSKICANVFCGAGRECAVTEKGEPTCLCIEQCK 57
Gallus gallus              MIWKTLPLLC-ALLAVARLRAEEEPRSKSKICANVFCGRGAECAVTEKGEPTCLCIEQCK 59
Xenopus laevis             MHLMCLPLLV---LMLLCCSAMEEPKSKSKVCANVFCGAGRECAVTEKGDPTCLCIEKCK 57
Silurana tropicalis        MHLSCLPLLA---LLALCS-ALEEPKSKSKVCANVFCGAGRECAVTEKGDPTCLCIEKCK 56
Oplegnathus fasciatus      MMSRSVAVLL--LLAVAVCNAE-ELQSKSKVCANVFCGAGRECAVNEKGEPSCLCIESCK 57
Salmo salar                -MLRSVHFLV--LLAIALCQTE-DVQTKSKVCANVFCGAGRECAVTEKGEPSCLCIESCK 56
Danio rerio                MMFRCLPVFF--LLAVVCCHAEQEAQSKSKVCANVFCGAGRECAVNEKGEPSCLCIEQCK 58
                                   :    :         : ::***:******* * ****.***:*:*****.**

                           Kazal domain profile

Homo sapiens               PHKRPVCGSNGKTYLNHCELHRDACLTGSKIQVDYDGHCKEKKSVSPSASPVVCYQSNRD 119
Pongo abelii               PHKRPVCGSNGKTYLNHCELHRDACLTGSKIQVDYDGHCKEKKSVSPSASPVVCYQSNRD 118
Bos taurus                 PHKRPVCGSNGKTYLNHCELHRDACLTGSKIQVDYDGHCKEKKSVSPSASPVVCYQSNRD 118
Mus musculus               PHKRPVCGSNGKTYLNHCELHRDACLTGSKIQVDYDGHCKEKKSASPSASPVVCYQANRD 117
Gallus gallus              PHGRPVCGSNGKTYLNHCELHRDACLTGSKIQVDYDGHCKEKKSENPAASPVVCYQSDRD 119
Xenopus laevis             SHKRPVCGSNGKNYLNHCELHRDACLTGSKIQVDYDGHCKEKTSDTPAAVPVACYQSDRD 117
Silurana tropicalis        SHKRPVCGSNGKTYLNHCELHRDACLTGSKIQVDYDGHCKEKTSDTPAAVPVACYQSDRD 116
Oplegnathus fasciatus      PHKRSVCGSNGKTYRNHCELHRDACLTGLKIQVAHDGHCQEKKTEQAAASPVVCYAADRN 117
Salmo salar                PHKRSVCGSNSKTYRNHCELHRDACLTGLKIQVAHDGHCQEKKAEHAAASPVVCYVADRN 116
Danio rerio                THKRSVCGSNGKTYRNHCELHRDACLTGLKIQVAHDGHCKEKKKEKAAASPVVCYVADRN 118
                           .* *.*****.*.* ************* **** :****:**.   .:* **.** ::*:

Homo sapiens               ELRRRIIQWLEAEIIPDGWFSKGSNYSEILDKYFKNFDNGDSRLDSSEFLKFVEQNETAI 179
Pongo abelii               ELRRRIIQWLEAEIIPDGWFSRGSNYSEILDKYFKNFDNGDSRLDSSEFLKFVEQNETAI 178
Bos taurus                 ELRRRIIQWLEAEIIPDGWFSKGSNYSEILDKYFKNFDNGDSRLDSSEFLKFVEQNETAI 178
Mus musculus               ELRRRLIQWLEAEIIPDGWFSKGSNYSEILDKYFKSFDNGDSHLDSSEFLKFVEQNETAI 177
Gallus gallus              ELHRRVIRWLEGEIIPDGWFSKGSNYSDVLEKYFKNFDD-DSRLDSTEFLKFVEQNETAV 178
Xenopus laevis             EMRRRVIHWLQTEIIPDGWFSKGSDYSEILDRYFKKYDDGDSQLDSAELQSFLEQS-QST 176
Silurana tropicalis        EMRRRVIQWLQTEIIPDGWFSKGNDYSEILDRYFKKFDDGDSRLDSAELQSFLEQS-QST 175
Oplegnathus fasciatus      ELRSRVIQWLQTEVIPDGWFVKGSNFSDILLKYFKSYDNGDSQLDSSELLKFIQQNESVV 177
Salmo salar                ELRHRVIQWLQTEVVPDGWFVKGSNFTEILVKYFKSYDNGDSQLDSSELLKFIQHNDSNV 176
Danio rerio                ELRRRVIEWLQTEVVPDGWFTKGSNFTDILLKYFKNYDNGDSQLDSAELLKFIQHNETAV 178
                           *:: *:*.**: *::***** :*.:::::* :***.:*: **:***:*: .*:::.

                               EF-hand calcium-binding domain profile

Homo sapiens               N-ITTYPDQENNKLLRGLCVDALIELSDENADWKLSFQEFLKCLNPSFNPPEKKCALEDE 238
Pongo abelii               N-ITTYPDQENNKLLRGLCVDALIELSDENADWKLSFQEFLKCLNPSFNPPEKKCALEDE 237
Bos taurus                 N-ITTYADQENNKLLRGLCVDALIELSDENADWKLSFQEFLKCLNPSFNPPEKKCALEDE 237
Mus musculus               N-ITTYADQENNKLLRSLCVDALIELSDENADWKLSFQEFLKCLNPSFNPPEKKCALEDE 236
Gallus gallus              PTITTYVDQETNKLLRGLCVVALIELSVENADWKLSFNEFLKCLSPSFNPPEKKCALEDE 238
Xenopus laevis             N-ISTYKNEETNRMLKSLCVEALIELSDENADWKLNKNEFLTCLNPDFQPPEKKCALEDE 235
Silurana tropicalis         NITTYKDEETNRMLKSLCVEALIELSDENADWKLSKNEFLKCLNPDFQPPEKKCALEDE 234
Oplegnathus fasciatus      E-LQSYADQESNKLLRSLCVDALIELSDENADWKLSFDEFLNCLKPGFNPPEKKCALEDE 236
Salmo salar                D-MQSYADQESNKLLRSLCVDALIEMSDENSDWKLSFDEFLNCLKPGFNPPEKKCALEDE 235
Danio rerio                Q-MSSYAEEENNRLLRSLCVDALIELSDENADWKLSFDEFLNCLKPGFSPPEKKCALEDE 237
                             : :* ::*.*::*:.*** ****:* :*:****. :***.**.*.*.**********

Homo sapiens               TYADGAETEVDCNRCVCACGNWVCTAMTCDGKN-------QKGAQTQTEEEMTRYVQELQ 291
Pongo abelii               TYADGAETEVDCNRCVCACGNWVCTAMTCDGKN-------QKGAQTQTEEEMTRYVQELQ 290
Bos taurus                 TYADGAETEVDCNRCVCACGNWVCTAMTCDGKN-------QKGAQTQAEEEMTRYVQELQ 290
Mus musculus               TYADGAETEVDCNRCVCSCGHWVCTAMTCDGKN-------QKGVQTHTEEEKTGYVQELQ 289
Gallus gallus              TYEDGAETQVECNRCVYACGNWVCTAMTCEGKNEKMTAHRQQPGQDLTEEELARYVQELQ 298
Xenopus laevis             TYEDGAETQVQCNRCTCACGNWVCTAMACEGKE------------GDEAEDMGRYVEEIK 283
Silurana tropicalis        TYEDGAETQVQCNRCVCACGNWVCTAMACEGKD------------GDHGDDMGRYVEEIR 282
Oplegnathus fasciatus      TYEDGAETQVECNRCVCACGNWVCTAMTCTDKT-AAVDESVDAGAEMTEEEWNLRVAELN 295
Salmo salar                TYKDGAETQVECNRCVCACGNWVCTAMTCNDKT-AVVEETGEAGQEMTEEEWSLRVAELN 294
Danio rerio                TYEDGAETQVECNRCVCACGNWVCTAMTCDEKT-PVFDTEGDQ--EMTEEEWTRRVAELN 294
                           ** *****:*:****. :**:******:*  *                ::    * *:.

Homo sapiens               KHQETAEKTKRVSTKEI 308
Pongo abelii               KHQETAEKTKRVSTKEI 307
Bos taurus                 KHQETAEKSKRVSTKEI 307
Mus musculus               KHQGTAEKTKKVNTKEI 306
Gallus gallus              KHQEQAEKIKKMSTKEM 315
Xenopus laevis             KQQETIENSKSSSSKDA 300
Silurana tropicalis        KQQETVENSKTGSSKDA 299
Oplegnathus fasciatus      KHQETVEKMKT-STKEA 311
Salmo salar                KHQETVEKMKA-ITKEA 310
Danio rerio                KHQETVEKMKT-STKEV 310
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Fig. 2. Clustal W multiple sequence alignment of the deduced amino acid sequence of RbFST in present study with known FSTs. The identical residues are indicated by asterisk (*) and semi conserved residues are indicated by semicolon (:) and dots (.). Kazal domain profile is indicated by thick double headed arrow and highly conserved regions in the domain are shaded. The EF-hand calcium-binding domain profile is indicated with thin double headed arrows and highly conserved residues are boxed.

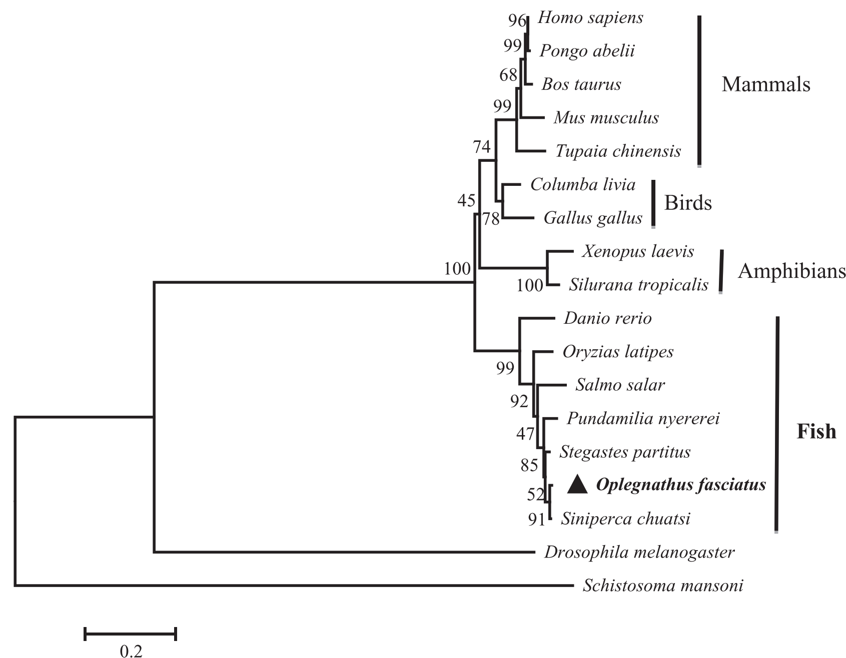

Fig. 3. Phylogenetic analysis of RbFST with selected known full-length folliststin amino acid sequences of other species. Using MEGA v5.2.2 a phylogenetic tree was reconstructed employing the Neighbor Joining method. Branch numbers are the bootstrap values for 1000 replicates. Location of RbFST is indicated with a black triangle and clusters of fish, amphibians, birds and mammals are indicated accordingly.

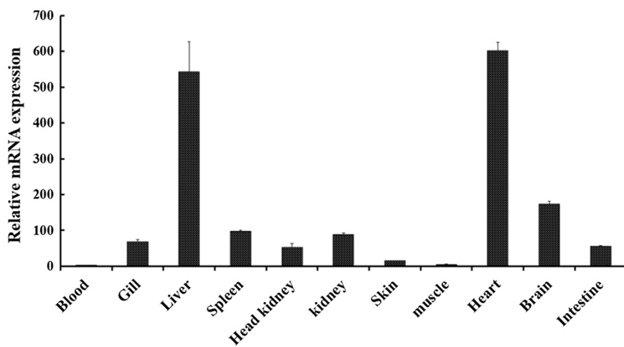

Fig. 4. The tissue specific mRNA expression of *RbFST* determined by qPCR. Expression fold changes were shown relative to the mRNA expression level in blood cells. Error bars represent the SD (n=3).

alignment (Fig. 2) showed the presence of a highly conserved Kazal domain, which is a feature of FSTs. The EF-hand, calcium binding domain is also identified in FSTs as a conserved domain among several species, including rock bream. Pairwise sequence comparisons showed that *Siniperca chuatsi* shares the highest identity (98.7%) and similarity (99.4%) with *RbFST*. The *RbFST* proteins from other fish species are also similar (Table 2). In phylogenetic analyses, *RbFST* clustered with *Siniperca chuatsi* FST within a fish clade. These data indicate that *RbFST* is a counterpart of the fish FST-family of proteins, likely originating from a common ancestor of vertebrates, with a characteristic domain architecture that is conserved among a number of fish species (Fig. 3).

Tissue-specific mRNA expression profile of *RbFST*

In order to determine the transcriptional profile of *RbFST* under physiological conditions, qPCR was performed using gene-specific primers and cDNA was synthesized from different tissues obtained from healthy rock bream. Relative mRNA expression levels were calculated using the expression of rock bream *β-actin* as the reference gene and the expression in each tissue was further normalized to that in blood cells. The results demonstrate that some of the tissues express a considerably higher amount of *RbFST* transcripts, while other tissues show relatively lower levels of *RbFST* expression (Fig. 4). This suggests that *RbFST* functions in a tissue-selective manner. The lowest level of expression was observed in blood cells while the heart and liver showed the highest expression levels. The gill, spleen, kidney and brain showed moderate levels of *RbFST* expression.

The main role of FST in heart tissues remains unclear and studies are being conducted to identify the involvement of FST in cardiac conditions (Ogura et al., 2012; Miyabe et al., 2014). Even though the expression of *FST* under disease conditions, such as myocyte hypertrophic growth, the loss of ventricular performance in response to pressure overload (*FST-like 1*), and ischemic injury (*FST-like 3*) (Shimano et al., 2011), has been detected, FST's expression and involvement in heart tissues under physiological conditions has not yet been extensively studied. As detected in our expressional analysis, transcript levels of *RbFST* were prominent in liver tissues. The liver is

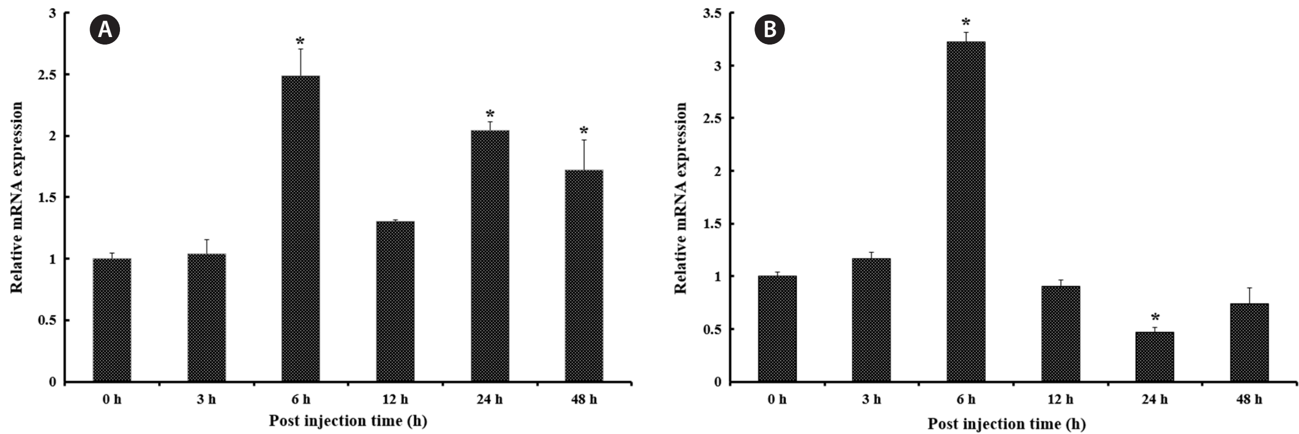

Fig. 5. Transcriptional regulation of *RbFST* in liver tissue upon pathogen challenges. Expression of *RbFST* after *Streptococcus iniae* (A) and *Edwardsiella tarda* (B) challenges. Error bars represent the SD (n=3). Significantly up-regulated or down-regulated time points are marked with "*".

known to be involved in host-immune responses (Racanelli and Rehermann, 2006). FST consistently has the ability to interact with most of the cytokines and components of cell signaling pathways, which in turn can trigger immune responses (Murakami et al., 2012). The presence of *FST* transcripts at high levels under physiological conditions in the liver may indicate the importance of FST as an agent of immuno-modulation. Similarly, moderate levels of *FST* mRNA have been ascertained from the tissue distribution analysis of sea bream *Sparus aurata* (Funkenstein et al., 2009).

Prominent expression levels of *FST* differed among species under physiological conditions. In *Sparus aurata*, higher expression levels were detected in the gill filaments, eye, and brain tissues (Funkenstein et al., 2009). Although broad tissue distribution profiles of FST have been detected in mammals (Michel et al., 1991; Tuuri et al., 1994) and chickens (Davis and Johnson, 1998), only a few fish species exhibiting a wide range of *FST* tissue-specific expression have been documented, including sea bream (Funkenstein et al., 2009). Moreover, prominent expression of *FST* has been detected in mammalian muscle tissue (Tuuri et al., 1994) as well as in chickens and other fish species, including sea bream (Funkenstein et al., 2009) and zebrafish (Bauer et al., 1998). Studies conducted on the ability of FST to bind with myostatin (MSTN) have shown considerable levels of *FST* expression in muscle tissues from mammals and chickens. Evidence has been gathered on the possible association between FST and MSTN, similar to that observed in mammalian and chicken muscles, in fish muscles from studies on sea bream FST (Funkenstein et al., 2009).

Transcriptional modulation of *RbFST* upon pathogen injection

In both vertebrates and invertebrates, *FST* is known to be

Table 2. Amino acid identity and similarity of RbFST entire sequence to other homologues

Species	GenBank accession number	Amino acids	Identity %	Similarity %
Siniperca chuatsi	AIB10410	312	98.7	99.4
Stegastes partitus	XP_008301591	310	95.8	96.8
Pundamilia nyererei	XP_005747258	312	93.6	96.5
Oryzias latipes	XP_004082017	311	89.1	93.6
Salmo salar	ACI33002	310	87.1	93.6
Danio rerio	NP_001034710	310	84.3	90.1
Homo sapiens	NP_009016	308	71.4	81.3
Bos Taurus	NP_001017950	307	71.3	81.2
Pongo abelii	NP_001125838	307	71	80.9
Mus musculus	NP_032073	306	70	79.9
Gallus gallus	NP_989969	315	68.4	79.7
Columba livia	EMC84573	316	67.3	76.4
Silurana tropicalis	CAJ82730.1	299	65.8	78.9
Xenopus laevis	NP_001089049	300	64.1	77.8
Tupaia chinensis	ELW67561	456	43.5	48.7
Saccoglossus kowalevskii	NP_001158432	408	36.8	24.6
Schistoso mamansoni	AGB14644.1	456	9.9	16.9
Drosophila melanogaster	NP_652376	767	3.1	4.8

involved in growth and immunological pathways (Murakami et al., 2012). The liver is a key component of both the adaptive immune and innate immune systems (Racanelli and Rehermann, 2006). The components of the innate immune system play a major role since they act as the first line of defense against infectious pathogens. In order to examine the immune responsive expression of *FST* under pathogenic conditions, liver tissue was collected from immune-challenged rock bream fish.

As shown in Fig. 5A, after healthy rock breams were challenged with *S. iniae*, the transcript levels of *RbFST* at 6 h post injection (p.i.) were significantly up-regulated ($P < 0.05$) (fold ~ 2.5) in the liver. At the 12 h p.i. time-point, there was not a significant ($P > 0.05$) elevation in the expression levels from the basal level. A considerable augmentation in *RbFST* mRNA levels was observed at 24 h and 48 h p.i. ($P < 0.05$), reflecting the positive regulation of *RbFST* upon *S. iniae* infection. After injection with *E. tarda*, *RbFST* expression levels were up-regulated (fold ~ 3.2) at 6 h p.i. Following a significant down-regulation at 24 h p.i., *RbFST* mRNA levels reached the basal level. These data suggest the plausible involvement of *RbFST* in immune-related mechanisms. Recent studies on FST-like proteins have also confirmed the role of FST in innate immunity via CD14 and toll-like, receptor 4-related signaling pathways (Murakami et al., 2012). Collectively, these observations may further confirm the importance of FST in innate immunity and possibly in the expressional modulation of *FST* mRNA levels upon pathological challenges.

We have characterized a member of the FST-family of proteins from rock bream (RbFST) at the molecular level and determined its expression levels in different tissues under physiological conditions, revealing ubiquitous expression. Moreover, *RbFST* expression was modulated upon pathogen stress mounted by two different live pathogens (*S. iniae* and *E. tarda*). Altogether, findings from this study suggest the putative involvement of *RbFST* in immune regulation in rock bream, further validating its important role in rock bream physiology. Nevertheless, further studies are needed to confirm the involvement of FST in the immune regulation of teleosts.

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