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Short communication

Molecular characterization and expression analysis of signal transducer and activator of transcription 1 (STAT1) in Japanese eel *Anguilla japonica*

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ABSTRACT

Signal transducer and activator of transcription 1 (STAT1) is one of critical signal transduction proteins of interferon (IFN) pathway and the structure and function of this protein have been well identified in mammals, but the information about the STAT1 is still limited in teleost fishes. In the present study, the full-length cDNA sequence of *STAT1* (*Aj*STAT1) in Japanese eel (*Anguilla japonica*) was identified and characterized. Multiple alignment of the amino acid sequence showed that the *Aj*STAT1 protein has the typical conserved domains including the amino-terminal, coiled-coil, DNA-binding, linker, Src homology 2 (SH2), transcriptional activation domains (TAD). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis revealed a broad expression for *Aj*STAT1 in a wide range of tissues, with the predominant expression in liver, followed by the spleen, intestine, gills, skin, kidney, and the very low expression in heart and muscle. The *Aj*STAT1 expressions in liver, spleen and kidney were significantly induced following injection with LPS, the viral mimic poly I:C, and *Aeromonas hydrophila* infection. *In vitro*, the *Aj*STAT1 transcripts of Japanese eel liver cells were significantly enhanced by the treatment of poly I:C or the stimulation of the high concentration of *Aeromonas hydrophila* (1×10^7 cfu/mL and 1×10^8 cfu/mL). Subcellular localization showed that in the natural state *Aj*STAT1was uniformly distributed in the cytoplasm, but *Aj*STAT1 was found to aggregated in the cytoplasm as well as partly in the nucleus after the stimulation of LPS and poly I:C. These results collectively suggested *Aj*STAT1 is an important transcription factor possibly involved in Japanese eel defense against viral and bacterial infection.

1. Introduction

The interferons (IFNs) are a family of multifunctional cytokines as the first line against virus infections and can be induced through different signaling pathways in response to pathogen infection or pathogen associated molecular patterns (PAMPs) stimulation [1,2]. The Jak/Stats pathway plays very import role in the mediation of the signaling by interferons. When the IFNs are bond with their receptors, the Janus kinases (JAKs) are activated and subsequently phosphorylate signal transducer and transcription activators (STATs), which in turn moves to the nucleus, and directly activates hundreds of IFN-stimulated genes to ultimately exert the antiviral functions [3,4]. The seven members of the STAT family, named as STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 have been identified in mammals and all the STAT proteins contain the conserved domains with the amino-terminal, coiled-coil, DNA-binding, Linker, Src homology 2 (SH2), and transcriptional activation domains (TAD) [5]. Among the STATs family, STAT1 is well known for its essential role in mediating both type I interferons (IFN-α/β) and type II interferons (IFN-γ) during the host responses to stress, especially the pathogens infection [4,6]. The activation of STAT1 during the JAK-STAT signal transduction is slightly different in response to type I and type II IFNs. Following IFN-α stimulation, two separated receptors named IFNAR1 and IFNAR2, combined with IFNAR1 and IFNAR2 respectively, form a heterodimer and this conformational change causes the receptor-associated JAK1 and JAK2 activated. Then the two monomeric STAT1 are bound to a phosphotyrosine in the receptors via its SH2 domain, and the C-terminal tyrosine is phosphorylated by Jak1/Jak2 leading to the formation of STAT1 homodimers. Similarly, upon binding of IFN-γ, JAK1 and TYK2 associated with IFNAR1 and IFNAR2 respectively are activated

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and then phosphorylate STAT1 and STAT2 on tyrosine forming the predominantly STAT1-STAT2 heterodimers or STAT1 homodimers. After translocating to the cell nucleus, STAT1-STAT2 heterodimers companied with IRF9 bind type I IFN-stimulated response element ISRE to promote the expression of target genes whereas STAT1 homodimers activate gene expression by binding to gamma activated sequence (GAS) element [4,7].

In mammals, STAT1 is the essential cellular component to connect the innate and adaptive immune response and regulates several signaling pathways, such as the JAK/STAT signaling pathways involving in the antiviral and antibacterial activities [7–9], the apoptotic and non-apoptotic pathways promoting cell death [10,11], and the TLR signaling pathways leading to the inflammatory response against pathogen infections [12]. Recently, Meissl et al. [13] reviewed studies based on the data in mice and from human patients, and highlighted the functions of STAT1 as both a tumour suppressor and a tumour promoter indicating that the better knowledge of the complex underlying mechanisms or pathways might be used in cancer therapy.

In teleost, STAT1 genes have been cloned and analyzed in various species, such as orange-spotted grouper (*Epinephelus coioides*) [14], rock bream (*Oplegnathus fasciatus*) [15], Malabar groupers (*Epinephelus malabaricus*) [16], zebrafish (*Danio rerio*) [17,18], crucian carp (*Carassius auratus*) [19,20], and Atlantic salmon (*Salmo salar*) [21,22]. Similar to mammals, the expression of STAT1 could be strongly induced by stimulation with various virus such as nervous necrosis virus [14,16], iridovirus [14,15], hemorrhagic virus [20], or the synthetic dsRNA analogue poly I:C [15,16,18,19,23], which suggested its important role in the antiviral immune response. Yu et al. [19] found that the overexpression of STAT1 in crucian carp blastulae embryonic (CAB) cells could increase the effects of IFN on both the expression of ISGs and the inhibition of virus infection, and believed that the STAT1 of fish possessed similar functions like that in mammals. Recently, the significant up-regulations of STAT1 expression were noted following bacterial infections of *Edwardsiella tarda* and *Streptococcus iniae* in rock bream, or *Edwardsiella ictalurid* in channel catfish (*Ictalurus punctatus*) [24], which revealed that the STAT1 genes were involved in anti-bacterial immune response. However, the immune mechanism of the STAT1 in anti-bacterial, as well as in antiviral are still limited in fishes and are waiting for further studied.

Japanese eel (*Anguilla japonica*) is the commercially important species for aquaculture in Asia [25]. The eel industry has suffered huge economic losses caused by virus, parasite, and especially by a number of bacterial pathogens, such as *Aeromonas hydrophila*, *E. tarda* and *Vibrio vulnificus* [26,27]. Better understanding of the antiviral and the antibacterial immune mechanisms may contribute to the development of management strategies for disease control and long term sustainability of eel farming. This study is the first to describe the Japanese eel's full-length cDNA of *Aj*STAT1 along with its expression pattern in various organs. Next, we evaluated the temporal expression profiles of the gene in liver, spleen, and kidney after stimulation with LPS, poly I:C, and *Aeromonas hydrophila* infection at 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h. Furthermore, the expression pattern of *Aj*STAT1 was examined in Japanese eel liver cells in response to the different PAMPs, including poly I:C, LPS, CpG-DNA and PGN and different concentration of *A. hydrophila* infection at 0 h, 3 h, 6 h, 12 h, 24 h, and 48 h. Finally, subcelluar localization of *Aj*STAT1 following LPS and poly I:C stimulation was observed in HEK293T cells. The information presented in this study will provide more information to insight the function of STAT1 protein in the innate immune responses upon both virus and bacterial infection.

2. Materials and methods

2.1. Fish collection and immune challenge

Healthy Japanese eels, weighing 45–50 g, were purchased from an eel farm (Fuqing, China). They were kept in a 1000-L tank at 25 °C with recirculated and aerated water for a week to acclimate to laboratory conditions. The fish were first anesthetized by immersion in water containing 100 ppm Eugenol (The Second Reagent Factory of Shanghai) and then Liver, spleen, gills, kidney, intestine, heart, skin, and muscle were harvested and frozen in liquid nitrogen and then stored at −80 °C for RNA extraction.

A. hydrophila, isolated from diseased eels in Fuqing, China, were inoculated in Tryptone soya broth (TSB) and incubated on a shaker at 28 °C for 24 h [26]. The bacteria were collected and diluted to the concentration of 4×10^4 cfu/mL in 0.01 mmol/L PBS (pH = 7.4). Fish immune stimulation was performed by intraperitoneal injection of 250 μL LPS (Sigma, 4 mg/mL, USA) in phosphate buffered saline (PBS), 250 μL poly I:C (Sigma, 2 mg/Ml, USA) in PBS, and 250 μL 4×10^4 cfu/mL *A. hydrophila* in PBS, respectively. Fish injected with 250 μL PBS were used as controls. Four fish were sacrificed for the control group and experimental group for each time point. Liver, spleen and kidney of each group were collected at 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h after injection and preserved for quantitative real-time polymerase chain reaction (qRT-PCR).

2.2. Cell culture and treatments

For *in vitro* studies, Japanese eel liver cell line was cultured as described in our previous study [28]. Cells were then treated with 30 μg/mL LPS (Sigma, USA), 50 μg/mL poly I:C (Sigma), 30 μg/mL CpG-DNA (Sangon Biotech, Shanghai, China), 30 μg/mL peptidoglycan (PGN, Sigma, USA), three different concentration of *A. hydrophila* (1×10^6 cfu/mL, 1×10^7 cfu/mL, and 1×10^8 cfu/mL, respectively), and the untreated cells were served as control. Four parallel samples were included of each group at 0 h, 3 h, 6 h, 12 h, 24 h, and 48 h after treatment. Total RNA from cells was isolated using E.Z.N.A.™ Total RNA Kit II (Omega, USA) following manufacturer's instructions.

2.3. Cloning of full-length cDNA of AjSTAT1

Total RNA from tissues was isolated using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol. Total RNA from cells was isolated using E.Z.N.A.™ Total RNA Kit II (Omega) following manufacturer's instructions. Total RNA from the liver of Japanese eel was used to synthesize the first-strand cDNA for the RACE reaction using the SMART RACE cDNA Amplification Kit (Takara, China) according to the manufacturer's instructions. Primers were designed according to the partial sequence of STAT1 from the Japanese eel transcriptome database in our lab with a local version of the Primer Premier 5.0 design software tool (http://www.premierbiosoft.com/primerdesign/index.html) (Supplementary Table 1) and PCR was performed to amplify the partial cDNA sequences of eel STAT1 gene. The purified PCR product was inserted into the pMD19-T vector (Takara, China) and transformed into JM109 competent cells. The plasmids from positive clones were subjected to DNA sequencing by Sangon Biotech Corp (Shanghai, China). Based on the partial gene sequence of STAT1, the full-length cDNA sequence was pulled out using 5′and 3′ RACE System for Rapid Amplification of cDNA Ends (Takara, China) with gene-specific primers as listed in Supplementary Table 1. The RACE PCR products were gel-purified, cloned, and sequenced as described above.

2.4. Bioinformatics analysis

Sequence similarity analysis was performed using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The full-length cDNA sequence of *Aj*STAT1 was analyzed with ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The deduced amino acid sequence was analyzed using the ExPASy Molecular Biology server (http://www.us.expasy.org/tools/). Multiple sequence alignment was performed using the CLUSTALW program (http://www.ebi.ac.uk/clustaw/). The protein domain features were predicted using NCBI CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/docs/cdd_search.html). The phylogenetic tree was constructed using MEGA 5 software based on Neighbour-Joining method with the bootstrapping of 1000 repetitions.

2.5. Expression analysis of *Aj*STAT1 by qRT-PCR

First strand cDNA was synthesized from total RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, China) following the manufacturer's instructions, and the genomic DNA was ruled out by the gDNA Eraser (with highly efficient DNAase activity for the gDNA degrading) provided by the Kit. Then, synthesized cDNA was diluted with nuclease-free water by 10-fold and stored at −20 °C until use. Primers for *Aj*STAT1 and β-actin (endogenous control gene) were designed using Primer 5.0 software (Supplementary Table 1) and tested to ensure the amplification of a single discrete band with no primer-dimers. The PCR product was also sequenced to verify the specificity of RT-PCR. The PCR reactions were set up in a total volume of 20 μL, containing 10 μL of 2 × AceQ® qPCR SYBR® Green Master Mix (VazymeTM, Q111-02), 1 μL of the diluted cDNA, 0.5 μL of each primer (10 μM), and 8 μL of nuclease-free water. The amplification was performed on a Roche Light Cycler 480 machine (Roche, Sussex, UK), and the qRT-PCR conditions were as follows: an incubation at 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Dissociation analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The relative quantification of the target and reference genes was evaluated using standard curves. The comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to determine the relative mRNA expression level of *Aj*STAT1 as described in our previous studies [29]. All data were given in terms of relative mRNA expression as arithmetic means ± standard error of the mean (SEM) of four separate individuals, each assayed in triplicate.

2.6. Subcellular localization

To examine the subcellular localization of *Aj*STAT1 *in vitro*, the ORFs of *Aj*STAT1 was cloned and inserted into pEGFP-N1 vector using corresponding primers (Supplementary Table 1). The constructed recombinant plasmid was confirmed by sequencing as described above. HEK-293T cells were seeded in 6-well plate and transfected with purified plasmids using Lipofectamine 2000 Reagent (Invitrogen, USA) according to the manufacturer's recommendations, with the pEGFP-N1 vector as a control. After transfection for 12 h, cells were then treated with 30 μg/mL LPS (Sigma), 50 μg/mL poly I:C (Sigma), and the PBS treatment as a control. After 24 h, cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with 6-diamidino-2-phenylindole (DAPI) (1 mg/mL) as described in Ref. [30]. Samples were observed under confocal fluorescence microscopy (Leica).

2.7. Statistical analysis

Data from all experiments were analyzed with SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). Significance of differences was determined by Student's *t*-test. *P* values smaller than 0.05 were considered as statistically significant.

3. Results

3.1. cDNA sequence of *Aj*STAT1

The full-length cDNA sequence of *Aj*STAT1 (GenBank accession No: KT156976) is composed of 2978 bp with a 5′-untranslated region (UTR) of 259 bp, an open reading frame (ORF) of 2244 bp encoding, and a 3′-UTR of 475 bp within a eukaryotic polyadenylation signal (AATAAA) (Fig. 1). The ORF encodes a 747 amino acid protein with a calculated molecular mass of 86.6 kDa and a theoretical isoelectric point of 5.47. Sequence analysis by using NCBI CDD revealed this protein contained N-terminal domain and SH2 conserved domains. Within the SH2 domain, the highly conserved sequence “GTFLLRFS(E/D)S” was found (Fig. 1).

3.2. Structural analysis of *Aj*STAT1

Multiple alignments and amino acid sequence similarity comparison of Japanese eel *Aj*STAT1 with STAT1 in Asian arowana (*Scleropages formosus*), turbot (*Scophthalmus maximus*), zebrafish, African clawed frog (*Xenopus laevis*), Chicken (*Gallus gallus*), Mouse (*Mus musculus*) and Human were shown in Supplementary Fig. 1. Similar domain organization of STAT1 proteins was as follows: the amino-terminal, coiled-coil, DNA-binding, linker, Src homology 2 (SH2), transcriptional activation domains (TAD). The highly conserved sequence “GTFLLRFS(E/D)S” was found in the SH2 domain. As shown in Table 1, the putative protein of *Aj*STAT1 has an identity of 81.7% with Asian arowana STAT1, 73.1% with STAT1 of rainbow trout (*Oncorhynchus mykiss*), 72.0% with turbot, 71.8% with chicken, 71.1% with Atlantic salmon, 70.7% with human, 69.2% with African clawed frog, 69.1% with mouse, 68.8% with medaka (*Oryzias latipes*), 63.4% with zebrafish, and 61.1% with channel catfish.

3.3. Phylogenetic analysis

To determine the evolutionary position of *Aj*STAT1, phylogenetic trees were constructed using the NJ method (Fig. 2). All the sequences were clustered into two main branches belonging to the mammals and teleost, and the bird and amphibian were the transition types. The Japanese eel with Asian arowana formed a separated branch.

3.4. Expression of *Aj*STAT1 mRNAs in tissues

The constitutive expressions of *Aj*STAT1 in the tissues of healthy Japanese eels were determined by qRT-PCR. The *Aj*STAT1 transcript was broadly expressed in liver, spleen, intestine, gills, skin, kidney, heart, and muscle. The high expression was observed in the liver, followed by the spleen, intestine, gills, skin, kidney, and the very low expression in heart and muscle (Fig. 3).

3.5. The temporary expression pattern of *Aj*STAT1 in liver, spleen and kidney after immune activation

To determine the effects of *Aj*STAT1 on the antibacterial and antiviral response, the mRNA expression levels of *Aj*STAT1 in liver, spleen and kidney of Japanese eels at 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h after LPS, poly I:C, and *A. hydrophila* challenge were quantified by qRT-PCR.

After injection with LPS, the *Aj*STAT1 expression levels were shown to increase significantly in all the three tissues (Fig. 4A). For liver tissue, the expression was significantly up-regulated to the peak at 6 h (13.3 folds, $P < 0.01$), then decreased at 12 h (8.9 folds, $P < 0.01$) and 24 h (4.4 folds, $P < 0.01$) and returned to control level from 48 h to 72 h ($P > 0.05$). For kidney tissue, the expression was significantly up-regulated at 6 h (7.7 folds, $P < 0.01$), 12 h (4.4 folds, $P < 0.01$) and 48 h (6.7 folds, $P < 0.01$) post-injection whereas no significant change of *Aj*STAT1 mRNA expression was found in all other time points ($P > 0.05$). For spleen tissue, the expression was significantly enhanced to the peak at 6 h (2.8 folds, $P < 0.01$), then decreased at 12 h (2.4 folds, $P < 0.01$) and 24 h (2.1 folds, $P < 0.01$), and returned to control level at 48 h, whereas a significant down-regulation was found at 72 h (1.5 down folds, $P < 0.05$).

After stimulation with poly I:C, the up-regulation of *Aj*STAT1 gene expression was observed in all these tissues especially in the liver (Fig. 4B). For liver tissue, the expression was sharply up regulated and peaked at 6 h (119.8 folds, $P < 0.01$), then decreased at 12 h (20.9

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   1 GAGAGGCGTTGATTGGTTTTTCACGAAAGCGGAAGTAGTTTACAGAACGTTCTCGATTTTTATTGGAGAAAACAAGTGTCACTCTTACAGTGTAGACAGT 100
 101 AGAAGAAATTAGTAGTGCTTGTTGTATTTTTGCAGTCTTCATGAAGTGCAGATAGCGGGTAGTGTCGAGAGAAGTGCGAAGTCGGATTCAGAAGTTGGAT 200
 201 TCACTTTAGCCTAACACCAGCTTTCTAGTTTTTCGGTTTGTTCGTAGTTCTAACGGAAAatggctcagtggtaccgtttgcagcagatggactctaagta 300
   1                                                            M A Q W Y R L Q Q M D S K Y 14
 301 cctggagcaggttgaccagctgtacgatgacaccttccccatggaggtccgtcagtacctcagccagtggatcgaaagccaggactgggagcttgtggca 400
  15 L E Q V D Q L Y D D T F P M E V R Q Y L S Q W I E S Q D W E L V A 47
 401 aataacgattccttggcgaccgtgcggttccatgacctcttgggccagctggatgaccagtacagccgattcaccctggagaacaacttcctgcagcaac 500
  48 N N D S L A T V R F H D L L G Q L D D Q Y S R F T L E N N F L Q Q H 81
 501 acaacatccgcaagatcaaacgcaacctacaggaccacttccaggaggacccggtgcacatggccatgatcatcagcaagtgcctgcaagaggagcagaa 600
  82 N I R K I K R N L Q D H F Q E D P V H M A M I I S K C L Q E E Q K 114
 601 gatcctggcgtcggcggagaaaaccgaggacaacatcgggagcacacagaccagcatggtggtggagaagcagagggagctggacggcaaagtgaaggac 700
 115 I L A S A E K T E D N I G S T Q T S M V V E K Q R E L D G K V K D 147
 701 atcaagaacagagtccaggaaatagagcaaaagataaaatctctggaggacctgcaagacgagcacgacttcaagtacaagtccctgcagagcagagagc 800
 148 I K N R V Q E I E Q K I K S L E D L Q D E H D F K Y K S L Q S R E H 181
 801 acgatgtgaacggggcgaaccagaaggaggtgaaacgtgaggagatgctgattcgagaaatgttcatcaggctgaatatccacagagaggaggtggtgcg 900
 182 D V N G A N Q K E V K R E E M L I R E M F I R L N I H R E E V V R 214
 901 tcagatggcggacgtgctgaacgcggtggagcagatccagtacgccctggtcacggacgagctgcccgactggaaacggcgccagcagattgcctgcatc 1000
 215 Q M A D V L N A V E Q I Q Y A L V T D E L P D W K R R Q Q I A C I 247
1001 ggcggcccaccaaacacctgcctggaccagctgcagacctggttcacaacggtggccgagagcctccagcaggtgcggcagcaggtaaggaagcttctgg 1100
 248 G G P P N T C L D Q L Q T W F T T V A E S L Q Q V R Q Q V R K L L E 281
1101 aactggagcagcagtacacgttcgagaacgaccccattacccagaagaagagcttcctggaggaccgggcgctgaacctgttccggaacctcgtcatcaa 1200
 282 L E Q Q Y T F E N D P I T Q K K S F L E D R A L N L F R N L V I N 314
1201 ctccatcgtggtggagaggcagccctgcatgcccacgcacccgcagcgacccctggtgctgaagacgggcgtgcagttcaccgtcaagctcaggctgttg 1300
 315 S I V V E R Q P C M P T H P Q R P L V L K T G V Q F T V K L R L L 347
1301 gtgaagctgcaggaatttaactaccagctccgagtcaaagcatcttttgacaaggatgtccctgaaaaaaacaagttgaaaggcttccgcaagttcaaca 1400
 348 V K L Q E F N Y Q L R V K A S F D K D V P E K N K L K G F R K F N I 381
1401 tcctgggcaccagcaccaaggtgatgaacatggaggagtccaacggcagcctggccgccgagtttcggcatctgcaattgaaagaacagaaggctgctgg 1500
 382 L G T S T K V M N M E E S N G S L A A E F R H L Q L K E Q K A A G 414
1501 taacaggacaaatgaggggccgctcatcgtgacggaggagctgcactccatcagctttgagacacagctctgccagccgggactcatcatcgacctggag 1600
 415 N R T N E G P L I V T E E L H S I S F E T Q L C Q P G L I I D L E 447
1601 ataacctccctcccgattgtggtgatctccaacgtgagccagctccctagcggctgggcctctatcctgtggtacaacatgctgtgcaccgagccaaaga 1700
 448 I T S L P I V V I S N V S Q L P S G W A S I L W Y N M L C T E P K N 481
1701 acctgtccttcttcctcagtgcccccaggcccgctggtgccagctgtccgaagtgctgagctggcagttctcctccgtcaccaagcgggggctgagcga 1800
 482 L S F F L S A P Q A R W C Q L S E V L S W Q F S S V T K R G L S E 514
1801 ggagcagctcagcatgctgggagacaaactgctgggtcgagaggcgggcggaaaccccgaagggctcatcccctggaccaagttctgtaagagtgagaag 1900
 515 E Q L S M L G D K L L G R E A G G N P E G L I P W T K F C K S E K 547
1901 accttccccttctggctgtggattgagggaattctggagctgatcaagagacacctgctggcactctggaacgatgggcacattatgggtttcgtgagta 2000
 548 T F P F W L W I E G I L E L I K R H L L A L W N D G H I M G F V S K 581
2001 aggaccgcgagcgggcgctacttaaggacatgaactccgggaccttcctgctgcggttcagcgagagcagccgtgaaggagccatcaccttcacctgggt 2100
 582 D R E R A L L K D M N S G T F L L R F S E S S R E G A I T F T W V 614
2101 ggagttctcccccaccggcgagccccagttccatgcggtggagccgtacaccaaaaaggagctcacggccgtctccttccccgacatcatccgcaactac 2200
 615 E F S P T G E P Q F H A V E P Y T K K E L T A V S F P D I I R N Y 647
2201 aaggtcatggcggaagagaacattccagaaaacccgctgcgcttcctgtaccccaacatccccaaagacagtgccttcgggaagtactacagccggcaga 2300
 648 K V M A E E N I P E N P L R F L Y P N I P K D S A F G K Y Y S R Q T 681
2301 ctgaacaatcggagcccatggatgtggagagccccagcggaccggggtacataaagacagagctgatctccgtgtcggaagttcatccttctagatttca 2400
 682 E Q S E P M D V E S P S G P G Y I K T E L I S V S E V H P S R F Q 714
2401 agacaccatgctgcccttgtccccagaagacttcggagcactcacaaagttcatcaaccccgcggagatcgagaccgtgatgtgcagcgcgttcccaaat 2500
 715 D T M L P L S P E D F G A L T K F I N P A E I E T V M C S A F P N 747
2501 tagACGGACTCCTATCCGAACCAGGATCCACCGGGCAAGGAGAGGTTCCTCATCTTTTCGTTGATGCTTTGCGTGTGACTCCTGCTGAAAGCATTCGCTG 2600
     *
2601 TATTAGCCTTCTCTGCAGTGACTTATGTCTGCTTGTATTACTGATGTTACATTATTTGCAAATGACGCATGAGTCTTGTACAGATACTTGTTTTAAGTGT 2700
2701 GCTCAATCAAAATCATTTGCTTGTTGTTTTTTTTTAAATGTTTGAAATCCAACAGTTCAGTTCGGTTCAATATGTTTGTTTGGTTATTTTTTTTTCCTCT 2800
2801 GAAAAATCCACATTGTCAAACGTTATTTTTAACTTATCATGACGTACTGTAGCAAGAGCAGTAACGGACACAGGCGCCTAGGGGGCGCTGCTCCACATTC 2900
2901 AGGTGTGTGAATATCACTACGTATTCAGTGCAAATAAATTCATTTGTTCGTGCCTCTGAAAAAAAAAAAAAAAAAAAA 2978
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Fig. 1. Nucleotide and deduced amino acid sequences of *Aj*STAT1. The nucleotide and amino acid sequences were numbered on the left. The start codon (ATG) was under line and stop codon (TAG) was marked with an asterisk. The polyadenylation signals AATAAA were under double line. In the deduced amino acid sequence, N-terminal domain was indicated as shaded residues (2–136 aa) and the SH2 domain was shaded in bold (575–677 aa). The highly conserved sequence "GTFLLRFS(E/D)S" was boxed in the SH2 domain.

Table 1
Pair wise comparison of the amino acid sequences of *Aj*STAT1 with those of selected STAT1.

Common name	Amino acid identity (%)	Accession No.
Asian arowana	81.7%	KPP63821.1
Rainbow trout	73.1%	AAB60924.1
Turbot	72.0%	AHV91021.1
Chicken	71.8%	NP_001012932.1
Atlantic Salmon	71.1%	ACI33829.1
Human	70.7%	ADA59516.1
African clawed frog	69.2%	AAM51552.1
Mouse	69.1%	AAA19454.1
Medaka	68.8%	XP_020563406.1
Zebrafish	63.4%	NP_571555.1
Channel catfish.	61.1%	AHH40051.1

folds, $P < 0.01$), 24 h (10.5 folds, $P < 0.01$), 48 h (5.4 folds, $P < 0.01$) and 72 h (5.6 folds, $P < 0.01$). For kidney tissue, the expression was significantly up-regulated to the peak at 6 h post-injection (20.0 folds, $P < 0.01$), decreased at 12 h (5.1 folds, $P < 0.01$) and then returned to control level from 24 h to 72 h ($P > 0.05$). For spleen tissue, the expression was significantly up-regulated to the peak at 6 h (13.3 folds, $P < 0.01$), decreased at 12 h (7.7 folds, $P < 0.05$) and at 24 h (4.9 folds, $P < 0.01$) and then returned to control level from 48 h to 72 h ($P > 0.05$). With the infection of *A. hydrophila*, the increase of the mRNA levels of *Aj*STAT1 was found in all the three tissues especially in the liver (Fig. 4C). For liver tissue, significant up-regulation of *Aj*STAT1 gene expression was shown at 6 h (24.1 folds, $P < 0.01$), 12 h (2.3 folds, $P < 0.01$), 24 h (2.5 folds, $P < 0.01$), and 72 h (1.5 folds, $P < 0.05$), and no significant change was found at 48 h ($P > 0.05$). For kidney tissue, the expression was significantly up-regulated at 6 h (3.3 folds, $P < 0.01$), 24 h (3.0 folds, $P < 0.01$), 48 h (7.0 folds, $P < 0.01$) and 72 h (5.9 folds, $P < 0.01$), and no significant difference in *Aj*STAT1 mRNA expression was found at 12 h ($P > 0.05$). For spleen tissue, the expression was significantly up-regulated only at 24 h (1.9 folds, $P < 0.05$) and 72 h (1.6 folds, $P < 0.05$), and no significant difference in *Aj*STAT1 mRNA expression in all other time points ($P > 0.05$).

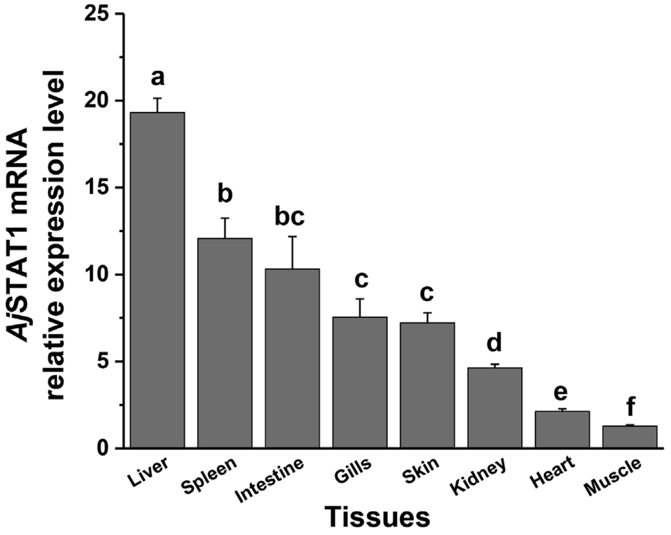

Fig. 3. Relative expression of *Aj*STAT1 in different tissues of healthy Japanese eel, include liver, spleen, intestine, gills, skin, kidney, heart, and muscle, respectively. The mean values marked by the different letter indicated significant difference ($P < 0.05$).

3.6. The temporary expression pattern of *Aj*STAT1 in vitro after viral/bacterial PAMPs stimulation and different concentration of *A. hydrophila* infection

In order to understand the modulation of *Aj*STAT1 expression in response to different pathogen associated molecular patterns (PAMPs), the transcriptional levels of *Aj*STAT1 in Japanese eel liver cells after treatment with poly I:C, LPS, CpG-DNA, PGN (Fig. 5A) and different concentration of *A. hydrophila* infection (Fig. 5B) at 0 h, 3 h, 6 h, 12 h, 24 h and 48 h were assessed by qRT-PCR.

As shown in Fig. 5A, the significant up-regulation of *Aj*STAT1 expression was found at 3 h (1.7 folds, $P < 0.01$), 6 h (2.3 folds, $P < 0.01$), 12 h (1.5 folds, $P < 0.01$), and 24 h (1.2 folds, $P < 0.01$) with the treatment of poly I:C while no significant increase of *Aj*STAT1 was detected following stimulation with LPS and PGN ($P > 0.05$).

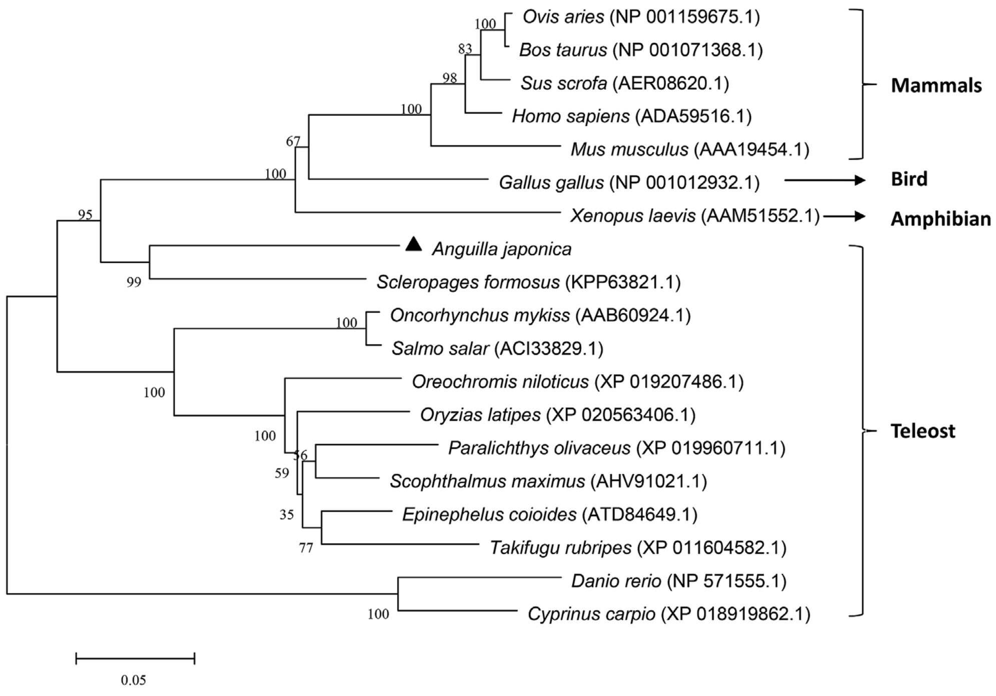

Fig. 2. Phylogenetic tree of *Aj*STAT1 and other vertebrates STAT1s. The phylogram was constructed with the MEGA5 program for the entire amino acid sequence by using the neighbour-joining (NJ) method. The numbers at the relevant branches refer to bootstrap values from 1000 replications. The accession numbers of the STAT1 sequences are indicated in parentheses after the species names.

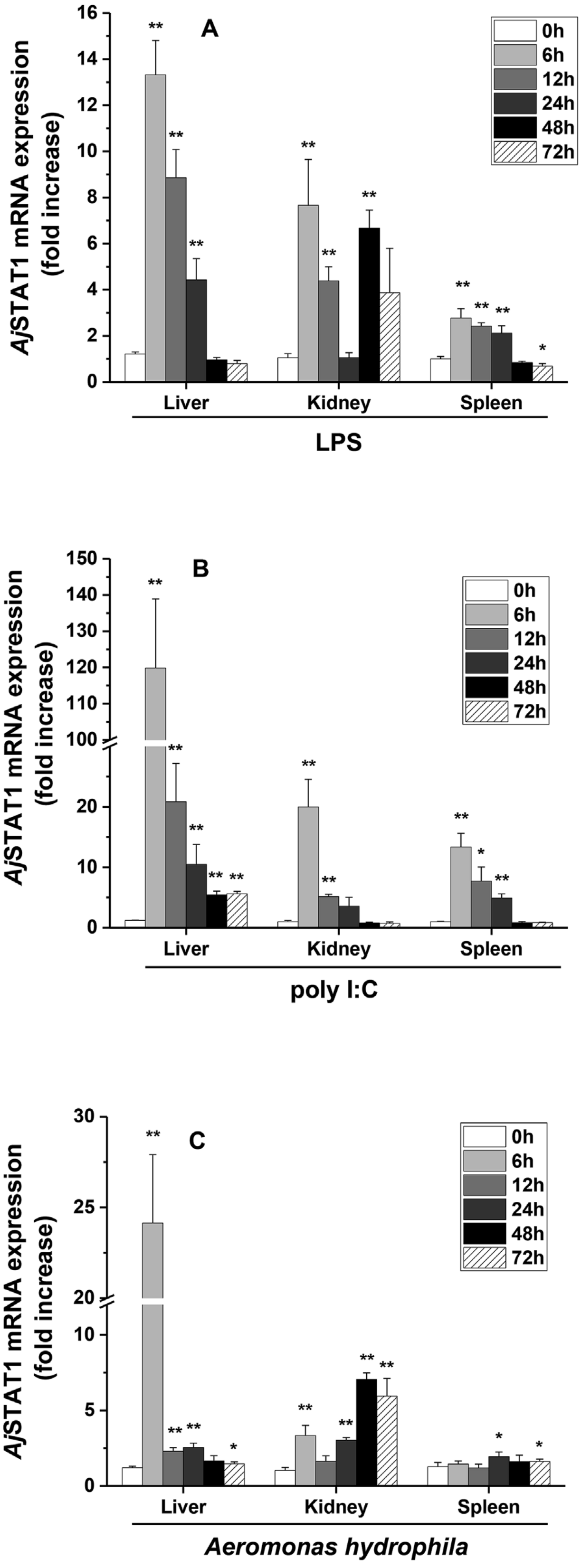

Fig. 4. Temporal expression profile of the *Aj*STAT1 transcripts in the liver, kidney and spleen of Japanese eel at 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h after LPS (Fig. 4A), poly I:C (Fig. 4B), and *A. hydrophila* (Fig. 4C) challenge. The *Aj*STAT1 expression level in LPS, poly I:C, and *A. hydrophila* treated samples was normalized to that in the PBS control group. Data are expressed as means ± SEM of four separate individuals, each assayed in triplicate. An asterisk (*) indicates a significant difference ($P < 0.05$) in expression level relative to the PBS treatment at the same sampling time and two asterisks (**) indicates

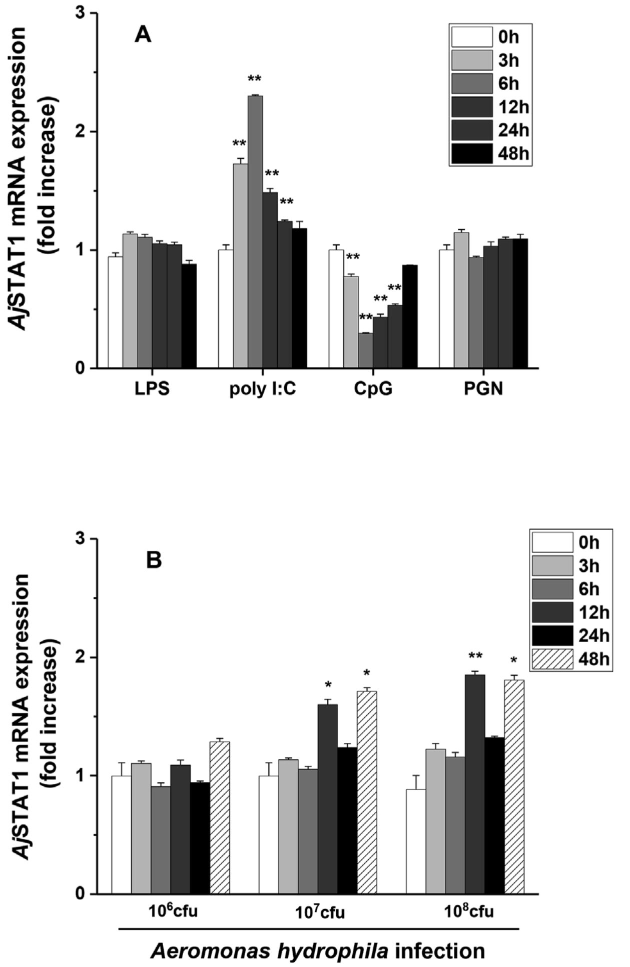

Fig. 5. Temporal expression profile of the *Aj*STAT1 transcripts in Japanese eel liver cells at 0 h, 3 h, 6 h, 12 h, 24 h and 48 h after treatment with LPS, poly I:C, CpG-DNA and PGN (Fig. 5A) or three different concentration of *A. hydrophila* (1×10^6 cfu/mL, 1×10^7 cfu/mL, and 1×10^8 cfu/mL) infection (Fig. 5B). The *Aj*STAT1 expression level in LPS, poly I:C, CpG-DNA and PGN treated samples, and different concentration of *A. hydrophila* infection samples were normalized to that in the PBS control group. Data are expressed as means ± SEM of four separate individuals, each assayed in triplicate. An asterisk (*) indicates a significant difference ($P < 0.05$) in expression level relative to the PBS treatment at the same sampling time and two asterisks (**) indicates ($P < 0.01$).

CpG-DNA was shown to significantly down-regulate the *Aj*STAT1 expression from 3 h to 24 h with the down folds 1.3-, 3.4-, 2.3-, and 1.9-, respectively ($P < 0.01$).

As illustrated in Fig. 5B, no significant change was found in *Aj*STAT1 mRNA expression in all time points under the 1×10^6 cfu/mL *A. hydrophila* infection ($P > 0.05$). When the concentration of *A. hydrophila* was up to 1×10^7 cfu/mL, *Aj*STAT1 expression was significantly induced at 12 h (1.6 folds, $P < 0.05$) and 48 h (1.7 folds, $P < 0.05$). Under the highest concentration of *A. hydrophila* (1×10^8 cfu/mL) treatment, the expression was significantly up-regulated at 12 h (1.9 folds, $P < 0.01$) and 48 h (1.8 folds, $P < 0.05$) whereas no significant difference in *Aj*STAT1 mRNA expression in all other time points ($P > 0.05$).

3.7. Subcelluar localization of AjSTAT1 after viral/bacterial PAMPs stimulation

Protein subcellular localization is a key functional characteristic and tightly concerned with its function. To determine the localization of *Aj*STAT1, the recombinant plasmid pEGFP-*Aj*STAT1 was constructed

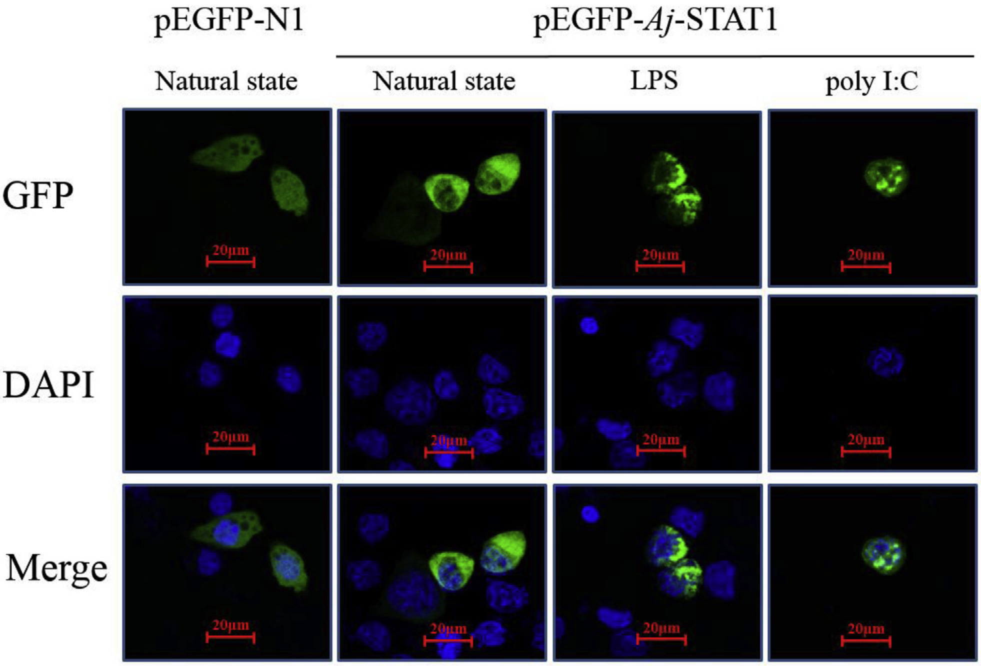

Fig. 6. Subcellular localization of Japanese eel *Aj*STAT1 in HEK293T cells. The nucleus was stained with DAPI.

and transfected in HEK293T cells and then the cells were treated with LPS and poly I:C. As shown in Fig. 6, *Aj*STAT1 was found to uniformly distribute throughout the cytoplasm under natural condition. When stimulated with LPS and poly I:C, *Aj*STAT1 was found to aggregated in the cytoplasm as well as partly in the nucleus.

4. Discussion

The alignment analysis of *Aj*STAT1 with its homologs from other species showed that *Aj*STAT1 contained conserved domains including the amino-terminal, coiled-coil, DNA-binding, linker, Src homology 2 (SH2), transcriptional activation domains (TAD) which were consistent with the structures of human, mouse, African clawed frog, and other teleost fish. The highly conserved sequence "GTFLLRFS(E/D)S" in the SH2 domain was found to bind with tyrosine kinase and promote to form of homologous or heterogeneous dimerization for the STAT signal transduction [21] indicating the immunomodulatory function of *Aj*-STAT1 in Japanese eel is conserved and similar to the mammalian STAT1.

The constitutive expression of the *Aj*STAT1 was observed in a wide range of healthy organs examined in the Japanese eels (Fig. 3). This is consistent with many reports from bony fish, such as rock bream [15], Atlantic salmon [21], mandarin fish [23], Malabar grouper [16], and olive flounder [31], suggesting that the constitutive expression pattern of STAT1 is prevalent in teleost fish. The highest abundance of *Aj*STAT1 expression was observed in the liver which conformed to the results from Malabar grouper [16]. The high *Aj*STAT1 expression was also observed in the spleen, intestine and gills of Japanese eels, and the similar expression patterns were present in mandarin fish and olive flounder [23,31]. However, the even expression of STAT1 was found in different tissues of Atlantic salmon [21], which might due to its high species-specificity. Furthermore, a high expression level of *Aj*STAT1 in gills and skin were detected suggesting its function as a barrier between the organism and environment to take part in the innate immune response.

STAT1 is characterized for involving in type I and type II IFNs-induced signaling pathways in mammals, which are essential components of the cellular antiviral response [32]. Viral PAMPs mainly include double strand RNA (dsRNA), single strand RNA (ssRNA), and the synthetic dsRNA (poly I:C). In the present study, *Aj*STAT1 expression in the liver was found to have a strong and specific response to poly I:C at 3 h, 6 h, 12 h, 24 h and 48 h post induction (Fig. 4B), which was in accordance with the findings in rock bream [15]. Collet et al. [22] reported that the STAT1 gene expressions in kidney of Atlantic salmon was strongly induced by the infection of virus ISAV and IPNV, and the significant up-regulation of *Aj*STAT1 gene expression was also observed in kidney and the spleen at 3 h, 6 h, or 12 h (Fig. 4B). Notably, the expression peak of *Aj*STAT1 in liver was up to 123.6 folds far higher than that in spleen and kidney with 10.5 folds and 18.9 folds, respectively, indicating the critical role of liver to take part in the antiviral immune response [28].

In vitro, the transcriptional levels of *Aj*STAT1 in Japanese eel liver cells were significantly enhanced at 3 h, 6 h, 12 h and 24 h after treatment with poly I:C (Fig. 5A) and this modulation of *Aj*STAT1 expression pattern was coincident with the reports in Mandarin fish fry (MFF-1) cells [23]and crucian carp (*Carassius auratus*) blastulae embryonic (CAB) cells [20]. Oates et al. found that zebrafish STAT1 protein could rescue interferon-signaling in a STAT1^{-} deficient human cell line and suggested that the STAT1 protein was conserved from the teleost to mammal [18]. Yu et al. provided strong evidence that the over-expression of wild-type STAT1 increases the effects of IFN on both the expression of ISGs and the inhibition of grass carp hemorrhagic virus (GCHV) infection [19]. Recently, the study in orange-spotted groupers has demonstrated that the expression levels of *Ec*STAT1a in grouper cells was significantly up-regulated under the infection of red-spotted grouper nervous necrosis virus (RGNNV) or Singapore grouper iridovirus (SGIV) and the further overexpression of EcSTAT1a was found to significantly inhibit both SGIV and RGNNV replication in grouper cells [14]. In mammals, Xu et al. reported that IFN regulatory factor 1 could effectively inhibit hepatitis E virus replication by activating STAT1 to induce antiviral IFN-stimulated genes in human cells [33]. Thus, these results collectively suggested that *Aj*STAT1 was an inducible protein both *in vivo* and *in vitro*, which involved in the antiviral innate immune response in Japanese eels, but its function in the antiviral immune response requires further investigation.

The JAK/STAT pathway has a crucial role in the control of immune responses in mammals [34]. This pathway has also been shown its involvement in anti-bacterial immune response. However, the knowledge of JAK/STAT signal pathway about anti-bacterial is still limited in teleost fish. In rock bream, two variants of STAT1 genes (RbSTAT1 and RbSTAT1L) in the liver were identified and reported to be strongly induced by the bacteria (*E. tarda* and *S. iniae*) and LPS [15]. Recently, Jin et al. [24] found that the STATb gene expression of the liver, gill and intestine tissues were significantly up-regulated in channel catfish after the Gram negative bacterium *E. ictaluri* infection. In this study, the *Aj*STAT1 gene expression in liver, spleen and kidney, and in the Japanese eel liver cells were significantly up-regulated after the infection of *A. hydrophila* infection (Figs. 4C and 5B), which further supported the regulatory function of STAT1 in the role of antibacterial responses, although much more detailed of the up-regulation of STAT1 expression needs further carried out. As the main outer-membrane component of gram-negative bacteria, LPS was found to induce the expression of *Aj*STAT1 in the liver, spleen, and kidney (Fig. 4A) which evidenced that gram-negative bacterial analogues could induce STAT1 signaling pathway similar as the report from rock bream [15]. However, the study in Malabar grouper (*Epinephelus malabaricus*) demonstrated that no obvious change of STAT1 expression in the head kidney, brain and spleen was observed following LPS stimulation [16], which indicated that the responses of STATS against LPS stimulation is various in fish species. Unlike the rock bream peripheral blood cells [15], no significant increase of *Aj*STAT1 expression was detected following stimulation with LPS in Japanese eel liver cells, which might due to the various species cell characteristic. Notably, the maximum up-regulation of *Aj*STAT1 expression in the liver of Japanese eel after the infection of *A. hydrophila* was shown at 6 h with 24.1 folds much higher than that of the kidney (7.0 folds) and spleen (1.9 folds) (Fig. 4C). This result highlighted the liver as a very important innate immune response organ of Japanese eel, which is consistent with our previous studies [26,28].

CpG-containing oligodeoxynucleotides (ODNs) are known to be immunostimulatory in vertebrate systems and can activate both innate and adaptive immune responses [35]. CpG DNA was found to significantly induce IFN gene expression both in the Japanese liver cells and Atlantic salmon kidney leukocytes [1,36]. However, in the present study CpG-DNA was shown to quickly down-regulate the *Aj*STAT1 expression from 3 h to 24 h Japanese eel liver cells (Fig. 5A). It has been reported that Atlantic salmon SOCS1 could suppress type I and II IFN signaling and inhibit STAT1 nuclear localization upon IFNγ-treatment [37], but the precise suppression mechanisms of STAT1 gene expression in Japanese eel liver cells by CpG-DNA are still remaining for further studied.

Protein subcellular localization is a key functional characteristic and tightly concerned with its function. In mammals as transcription factors, the phosphorylated STAT1 and STAT2 could be heterodimerized and together with interferon regulatory factor 9 to form a complex ISFG3, and then this complex migrates into the nucleus to activate the transcription of IFN-stimulated genes (ISGs) [38]. Recent report showed that when the HeLa cells were treated with type I IFN the STAT1 and STAT2 proteins were found to translocate to the nucleus [39]. In teleost, Sobhkhez et al. [40] demonstrated that STAT1a co-localized with STAT2 in the cytoplasm of unstimulated salmon cells, when stimulated with type I or type II IFN, both proteins were found to accumulate in the nucleus as well as in cytoplasm. In the present study, *Aj*STAT1 was found to uniformly distribute throughout the cytoplasm under natural condition, but this protein was found to aggregated in the cytoplasm as well as partly in the nucleus when stimulated with LPS or poly I:C (Fig. 6), which indicated that *Aj*STAT1 may involve in the JAK/STAT signal pathway transcription upon pathogens stimulation.

In conclusion, the full-length cDNA sequence of STAT1 was cloned in Japanese eel. *Aj*STAT1 transcripts were broadly expressed in all examined tissues with the highest level in liver and the lowest level in muscle and heart. *In vivo*, the expression of STAT1 in liver, spleen and kidney was significantly up-regulated in response to LPS, poly I:C, and *A. hydrophila* challenge. *In vitro*, its expression can be induced in liver cells treated with poly I:C and *A. hydrophila* infection. After stimulation of LPS and poly I:C, *Aj*STAT1 proteins were found to translocate to the nucleus. It is collectively indicated that *Aj*STAT1 plays an important role in fish defense against viral and bacterial infection. The information presented in this study will help better understanding the complex regulation network in fish JAK/STAT pathways.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.046.

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