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Short communication

# Cloning and gene expression of signal transducers and activators of transcription (STAT) homologue provide new insights into the immune response and nucleus graft of the pearl oyster *Pinctada fucata*

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ABSTRACT

The signal transducers and activators of the transcription (STAT) family play an important role in regulatory and cellular functions by regulating the expression of a variety of genes, including cytokines and growth factors. In the present study, a *Pinctada fucata* STAT protein, termed PfSTAT, was described. The deduced amino acid sequence of PfSTAT contains the conserved STAT_bind domain and the SH2 domain, and the additional Bin/Amphiphysin/Rvs (BAR) domain, but does not have STAT_alpha and STAT_int domains. Multiple sequence alignments revealed that PfSTAT showed relatively low identity with vertebrate and other invertebrate STATs, and phylogenetic analysis indicated that the evolution of STAT may have been more complex and ancient. Gene expression analysis revealed that PfSTAT is involved in the immune response to polyinosinic–polycytidylic acid (poly I:C) stimulation and in the nucleus insertion operation. This study contributes to a better understanding of PfSTAT in protecting the pearl oyster from disease or injury caused by grafting.



## 1. Introduction

In vertebrates, cytokines and growth factors play a central role in regulation and coordination of the immune response, growth and development [1,2]. These factors are activated and their functions are mediated by members of the STAT family (signal transducers and activators of transcription). In humans, the STAT family contains seven known members: STAT1, STAT2, STAT3, STAT4, STAT5 (STAT5A and STAT5B) and STAT6, and shares several redundant functions. STAT family members have similar structural domains, an N-terminal domain, a coiled coil domain, a DNA binding domain, a linker domain, an Src homology 2 (SH2) domain and a transcription activation domain. The SH2 domain is involved in signal transduction, and the DNA binding domain has an immunoglobulin (Ig)-like fold. After activation and phosphorylation by Janus kinases (JAKs), STATs dimerize and translocate into the cell nucleus; they then more specifically promote or induce gene expression [3].

Among insects, *Drosophila* d-STAT or STAT 92E has been identified [4,5], and it has been revealed that it is involved in embryogenesis, hematopoiesis and anti-bacterial/viral mechanisms [6,7]. The *Drosophila* JAK/STAT pathway has also been shown to regulate various biological processes, especially in immunity [6,8]. Similar functions also are found in the mosquito STATs such as *Aedes albopictus* STAT (AaSTAT) and *Culex tritaeniorhynchus* STAT (CtSTAT). These have been observed to become activated in response to bacterial challenge, revealing that the JAK/STAT pathway participates in immune response regulation [9–11]. In the protozoan *Dictyostelium*, several STAT proteins have been identified to regulate gene expression changes and are required for cell growth, differentiation and chemotaxis [12–15]. However, the function of STAT in other invertebrates remains unclear. To provide insight into the role of STAT in invertebrate animals, it is necessary to isolate and characterize STAT homologues from other invertebrate species.

The pearl oyster *Pinctada fucata* is one of the most important bivalve mollusks for seawater pearl production. In recent years,

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disease outbreaks in pearl oysters, especially those caused by bacteria and viruses, have become severe [16–18]. A combination of disease outbreaks and the injury arising from the nucleus grafting operation has resulted in mass mortality of pearl oysters and brought heavy economic losses [19,20]. We have previously characterized some immune-related genes such as cytokine-induced suppressor 2 (SOCS2), interferon regulatory factor 2 (IRF-2) homologues and nuclear factor of activated T cells (NFAT) in the pearl oyster [21–23]. Herein, we describe a *P. fucata* STAT protein, termed PfSTAT, which shares significant structural and functional homology with other STAT family members. The results of this study indicate that PfSTAT participates in the immune response to infection in mollusks, and is involved in the nucleus grafting operation.

## 2. Materials and methods

### 2.1. Experimental animal

Pearl oysters (shell length 4.5–5.5 cm) were collected from Daya Bay in Shenzhen and Xuwen in Zhanjiang, Guang-dong Province, China. The immune stimulation experiments were performed in Daya Bay and pearl oysters were acclimated in indoor cement ponds at ambient seawater temperature for one week before experimentation. The nucleus grafting operation experiments were performed in Xuwen and pearl oysters were collected directly from the sea.

### 2.2. Obtaining full-length cDNA of PfSTAT

The annotation sequences (ESTs) from our lab were analyzed [24] and an approximately 3700 bp fragment of the STAT homologue sequence was obtained. Sequence analysis showed that the PfSTAT cDNA had a 5′-terminal but lacked a 3′-terminal. To obtain full-length cDNA of the PfSTAT homologue, rapid amplification of cDNA ends (RACE) was performed. Total RNA was extracted from the digestive gland and gills of the pearl oysters using a Mollusc RNA Kit (Omega Bio-Tek, USA), following the manufacturer's instructions. 3′-RACE was performed using a SMART RACE cDNA Amplification Kit (Clontech, Japan) following the manufacturer's instructions. Specific primers (3RPSTATseq3750 and 3RPSTATseq3800) and Universal Primer A Mix/Nest Universal Primer (Table S1) were used. Nested or semi-nested PCR was employed. The PCR products were purified with a Gel Extraction Kit (Omega, USA), following the manufacturer's instructions, and sequenced. Other primers (RT5STATseq146 and RT3NFATseq4159) were used to verify the full-length cDNA by performing reverse transcription PCR (RT-PCR) (Table S1).

### 2.3. Sequence analysis and amino acid alignment

Sequence similarities were analyzed using the BLAST algorithm at the NCBI web site (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequences were analyzed using SMART (Simple modular architecture research tool, http://smart.embl-heidelberg.de) and the NCBI CDS program (Conserved Domain Search, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Based on amino acid sequences, the comparison and phylogenetic analysis were performed with Clustal omega –Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) and MEGA software 6.06 [25], using the neighbor-joining algorithm and performing 10,000 bootstrap replications.

### 2.4. Sample treatment and collection

For analyses of gene expression in different tissues, the digestive gland, gills, adductor muscle, hemocytes, heart, mantle, foot, intestine and gonad were collected from pearl oysters. To harvest hemocytes, the hemolymph was collected from the pericardial cavity through the adductor muscle using a syringe (1 ml), and was centrifuged at 3000 *g* for 2 min.

The immune stimulation experiment involved three groups: the lipopolysaccharide (LPS) (Sigma, Germany), polyinosinic–polycytidylic acid (poly I:C) (Invivogene, USA) and phosphate-buffered saline (PBS) (the negative control) stimulation groups. Each pearl oyster was injected in the adductor muscle with 100 μl of LPS dissolved in sterile PBS (LPS 1 μg/μl), 100 μl poly I:C dissolved in sterile PBS (poly I:C 1 μg/μl), or 100 μl sterile PBS, respectively. The untreated pearl oysters were used as the blank group (0 h). A total of five time points—6, 12, 24, 48 and 72 h after injection, respectively—were selected. For the nucleus grafting operation experiment, a total of six points—1, 3, 5, 7, 15 and 30 days after the nucleus grafting operation—were selected, and day 0 was recorded as the blank group. The hemocytes from pearl oysters were collected for qPCR assays. Nine pearl oysters were randomly selected at each time point. Each group of nine individuals was randomly divided into three replicates, and the hemocytes were obtained from each replicate and pooled as one sample. All samples were stored in Sample Protector (TaKaRa, Japan) until used.

### 2.5. RT-PCR and quantitative PCR (qPCR) analysis

cDNA was prepared using the PrimeScript™ reagent kit with gDNA Eraser (TaKaRa, Japan) following the manufacturer's instructions, and the mRNA expression of PfSTAT and the internal control 18S (GI:62549260), were analyzed. To investigate PfSTAT distribution in different tissues, RT-PCR was performed to detect the expression in different tissues. The PCR used the following conditions for both PfSTAT and 18S: one cycle at 94 °C for 3 min, and 34 cycles of 94 °C for 35 s, 59 °C for 40 s and 72 °C for 30 s, followed by one cycle at 72 °C for 5 min.

To analyze the changes in gene expression in the challenge and nucleus grafting operation experiments, qPCR assays were performed using the Master SYBR Green I system (TaKaRa) with the Roche LightCycler480 (Roche, Switzerland), following the manufacturer's instructions. The amplifications were performed in a total volume of 10 μl containing 5 μl of SYBR Premix ExTaqTM (TaKaRa), 1 μl of diluted cDNA, 0.4 μl of each primer (10 mM) and 3.2 μl of double-distilled water. The qPCR reactions were performed under the following conditions: one cycle at 95 °C for 3 min, and 45 cycles of 95 °C for 5 s, 59 °C for 25 s. In a 384-well plate, each hemocyte sample was tested in three replicates. After the qPCR program, the data were analyzed by following the LightCycler480 instrument operator's manual. The primers used for RT-PCR and qPCR are listed in Table S1. The efficiencies for 18S and PfSTAT were 1.915 and 1.834, respectively. The relative standard curve method was used for the calculation of fold changes in gene expression [26]. Statistical analysis was performed with the Student t-test in Microsoft EXCEL. Differences were considered significant at $P < 0.05$. All experiments were repeated at least three times.

## 3. Results and discussion

### 3.1. Sequence analysis of PfSTAT

Using RACE, a full-length cDNA of the *P. fucata* STAT (PfSTAT) was obtained (GenBank accession No. KT159188). The PfSTAT cDNA consisted of 4298 bp, with an open reading frame of 3597 bp