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*Cg*SOCS6 negatively regulates the expression of *Cg*IL17s and *Cg*Defh1 in the pacific oyster *Crassostrea gigas*

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ABSTRACT

As a family of negatively feedback regulating factors, the suppressor of cytokine signaling (SOCS) can depress cytokine signal transduction, and eventually modulate growth, development, differentiation, and immune response. In the present study, a SOCS homologue (designated as *Cg*SOCS6) was identified from oyster *Crassostrea gigas*. The open reading frame of *Cg*SOCS6 cDNA was of 1167 bp encoding a peptide of 388 amino acid residues with a central Src homology 2 (SH2) domain, a conserved C-terminal SOCS box, and a nucleus localization sequence (NLS) in its N-terminus. The deduced amino acid sequence of *Cg*SOCS6 shared 37.9–45.5% similarity with other SOCS6/7 family members. In the unrooted phylogenetic tree, *Cg*SOCS6 was clustered with *Es*SOCS6 from Chinese mitten crab *Eriocheir sinensis* and assigned into the SOCS6/7 group. The mRNA transcripts of *Cg*SOCS6 were constitutively distributed in all the tested tissues, with the highest level in hemocytes. After lipopolysaccharide (LPS) stimulation, the mRNA expression of *Cg*SOCS6 in hemocytes was significantly up-regulated to the highest level at 6 h (8.48-fold compared to the control group, $p < 0.01$), and then kept at a relatively higher level from 12 h to 72 h. *Cg*SOCS6 protein could be translocated into the hemocyte nucleus after LPS stimulation. The mRNA expressions of interleukin 17-4 (*Cg*IL17-4), *Cg*IL17-5, and defensin (*Cg*Defh1) in the hemocytes of *Cg*SOCS6-knockdown oysters increased significantly (2.55-fold, 2.68-fold, 4.68-fold of that in EGFP-RNAi oysters, $p < 0.05$, $p < 0.05$, $p < 0.001$, respectively) after LPS stimulation. These findings suggested that *Cg*SOCS6 was involved in the oyster immune response by regulating the expressions of *Cg*IL17-4, *Cg*IL17-5, and *Cg*Defh1.

1. Introduction

Suppressor of cytokine signaling (SOCS), as the feedback inhibitor of cytokine signaling, is a key physiological regulator of both innate and adaptive immunity [1]. There are eight SOCS related subfamilies, SOCS 1–7 and CIS (cytokine-inducible SH2-containing protein) identified in vertebrates. The main function of SOCS members is to negatively regulate janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway. SOCS members regulate cytokine signaling by blocking the interaction of STAT with its receptor or inhibiting the catalytic activity of JAK through the kinase inhibitory region (KIR) [2–4].

The SOCS family members commonly contain an N-terminal domain of variable length and sequence, a central conserved Src homology 2 (SH2) domain, and a SOCS box [5,6]. Based on their residues, the mammalian SOCS family members can be subdivided structurally into type I with the longest N-terminal region (SOCS4-7) and type II with the shortest N-terminal region (CIS, SOCS1-3) [7,8]. SOCS1 and SOCS3 have a unique architecture domain of KIR in N-terminus [9], which is essential for JAK inhibition [10–12]. SOCS2 and CIS commonly contain an N-terminal extended SH2 subdomain (N-ESS) [13], which plays an important role in blocking STAT recruitment to the phosphorylated receptor [14]. There is a unique SOCS domain in the N-terminus of SOCS4 and SOCS5 [15]. Whereas, SOCS6 and SOCS7 with a long N-terminal region can be translocated from cytoplasm into the nucleus, and SOCS7 is also identified to have a putative N-terminus nuclear

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export sequence (NES) [16]. Recently, some members of SOCS family have also been characterized in invertebrates, and they all contain a SH2 domain and a SOCS box, which is consistent with the counterparts in mammalian. For example, three SOCS homologs were found in *Drosophila melanogaster*, among which SOCS36E shared high similarity with mammalian SOCS5, and SOCS44A and SOCS16D were similar to mammalian SOCS6 and SOCS7, respectively [17–19]. Several SOCSs have also been identified from other species, such as *Es*SOCS2 and *Es*SOCS6 from Chinese mitten crab *Eriocheir sinensis* [20,21], *Mj*SOCS2 from kuruma shrimp *Marsupenaeus japonicus* [22], *Cg*SOCS2, *Cg*SOCS5 and *Cg*SOCS7 from pacific oyster *Crassostrea gigas* [23], and SOCS2 from pearl oyster *Pinctada fucata*, manila clam *Ruditapes philippinarum* and disk abalone *Haliotis discus discus* [24–26]. SOCS proteins interact directly or indirectly with JAKs or their specific cytokine receptors to inhibit the downstream cascades [1]. The conserved domain structure of SOCS family members indicates the conservative property of their functions in vertebrates and invertebrates.

Increasing evidences have favored that CIS and SOCS1-3 act as negative regulators in mediating the cytokine signaling, whereas, SOCS4-7 mainly regulate the growth factor receptor signaling [27–29]. SOCS1-3 and CIS are the most intensively studied members of SOCS family, which are involved in the negative regulation of various immune effectors [30], such as cytokines and antibacterial peptides (AMPs) in the immune response of vertebrates and invertebrates. In murine embryonic fibroblast, SOCS1 could suppress the expression of tumor necrosis factor-α (TNF-α) by inhibiting the activation of JAK [31], and it could also inhibit the IL13-induced STAT6 activation and function as a negative regulator of IL13 [32]. SOCS3 could inhibit IL6 production by attenuating the phosphorylation of STAT1 and STAT3 in liver cells of mouse [33]. SOCS3 was also characterized as an essential negative regulator for inhibiting IL23 secretion in helper T17 (Th17) cells [34]. In invertebrates, SOCS members are found to inhibit the expression of antibacterial peptides (AMPs). *Bm*SOCS2-12 from Dazao *Bombyx mori* could negatively regulate the production of cecropinD, gloverin2, moricin, and attacin [35]. *Mj*SOCS2 in the shrimp *M. japonicus* could negatively mediate the expressions of AMPs, such as antilipopolysaccharide factors C1, C2 and D1, and Crustin I [22]. SOCS1-3 and CIS proteins have been implicated in regulating immune homeostasis and determining cell fate because their obstruction or imbalance causes a broad range of diseases. To date, the information about SOCS4-7 proteins in mediating the immune response in vertebrates and invertebrates is still very limited.

The release of pacific oyster *C. gigas* genome provides great convenience for the research of molluscan innate immunity [36]. As invertebrates, oysters rely on innate immunity to defend the invasion of verified pathogens. So far, various immune effectors have been identified in oyster. For instance, the IL-17 homologs, which were designated as *Cg*IL17-1 to *Cg*IL17-6, were found to be involved in innate immune responses in oyster [37,38]. Two Defensins (designed as *Cg*Defh1 and *Cg*Defh2) were also characterized from the oyster *C. gigas*, which belonged to the AMPs involving in oyster immune response [39]. The activation of immune system must be strictly regulated and controlled to avoid damage caused by excessive immune response. The members of SOCS family play important roles in negatively regulating immune response. The knowledge about the mechanism of SOCS to induce downstream responses is not only necessary to better understand the immune system of oyster, but also helpful to develop strategy to control disease. In the present study, a novel SOCS homologue (named *Cg*SOCS6) was identified from oyster *C. gigas*. There was a long N-terminal region in *Cg*SOCS6, which was consistent with the SOCS6 in vertebrates and different from previously identified SOCS2, SOCS5 and SOCS7 in *C. gigas*. The sequence characteristics, subcellular localization of *Cg*SOCS6, and the mRNA expression profiles of *Cg*SOCS6, as well as the expressions of CgIL17s and CgDefs after RNA interference of CgSOCS6 were investigated with the objectives to explore the function of *Cg*SOCS6 in inhibiting the immune response. The results would offer more information to cognize the immune functions of SOCS family members in molluscs.

2. Material and methods

2.1. Animals, treatment and sample collection

Adult oysters *C. gigas* (about 13.0 cm in shell length) were collected from a local farm in Dalian, Liaoning Province, China. They were cultured in laboratory aquarium tanks with aerated seawater at 15–20 °C for one week before processing. A total of 140 oysters were equally separated into two groups for immune treatment. The oysters in the treatment group received individually an injection with 0.1 mL of lipopolysaccharide (LPS, from *Escherichia coli* 0111:B4, Sigma Aldrich, USA) dissolved in PBS (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.42 g/L, KH_2PO_4 0.27 g/L) at a concentration of 100 mg/mL. The oysters received an injection with 0.1 mL PBS were employed as control group. Afterwards, nine oysters were sampled from each group at 0, 3, 6, 12, 24, 48 and 72 h post-stimulation, and the hemolymph collected from three individuals were pooled together as one sample. The hemocytes were harvested by centrifugation at 1500 rpm, 4 °C for 8 min. There were three duplicates for each time point. Different tissues including gonad, adductor muscle, mantle, gills, hemocytes, labial palps and hepatopancreas were collected from other nine untreated oysters as parallel samples. All the samples were stored at −80 °C for subsequent RNA extraction by using Trizol reagent (Thermo Fisher Scientific, USA).

2.2. RNA extraction and cDNA synthesis

The total RNA was extracted with the Trizol reagent following the manufacture's introduction. The quality and quantity of extracted RNA were evaluated by Nanodrop 2000 and electrophoresis on 1% agarose gel measurement, respectively. The cDNA synthesis was conducted with the total RNA as template according to the direction of manufacturer (Takara, China). The building-up reaction was executed at 42 °C for 30 min, and aborted by heating at 85 °C for 5 s. The cDNA mixture was diluted to 1:20 and stored at −80 °C for the subsequent experiments.

2.3. Sequence analysis of CgSOCS6

BLASTx (http://www.ncbi.nlm.nih.gov/) was used for homology analysis, and ExPASy (http://www.expasy.org/) was employed to predict the sequence characteristics of *Cg*SOCSs. The structure domains of SOCS proteins were predicted by SMART (http://smart.embl-heidelberg.de/). The nucleus localization sequence (NLS) was identified by using the online nls-mapper program at the website of http://nls-mapper.iab.keio.ac.jp. MEGA 6.0 program (Sudhir K, 2004) was used for phylogenetic analysis, and Clustal X was used for multiple sequence alignment.

2.4. Analysis of mRNA expression level

The distribution of *Cg*SOCS6 mRNA in different tissues was examined by quantitative real-time PCR (qRT-PCR), and the reactions were performed with the SYBR premix ExTap (RR420, Takara, Japan) on ABI PRISM 7500 Sequence Detection System (Thermo Fisher, USA). The primers of *Cg*SOCS6-RT-F and –R were used to amplify a fragment of 189 bp (Table 1). Elongation Factor (*Cg*EF, GenBank accession NM_001305313) fragment, amplified with primers *Cg*EF-RT-F and –R (Table 1), was used as internal control. The expression profile of *Cg*SOCS6 in the hemocytes of oysters was determined by qRT-PCR at 0, 3, 6, 12, 24, 48 and 72 h after LPS stimulation. The reactions were programmed at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 45 s. Melting analysis was conducted at the end of each PCR to confirm the specificity of products. The obtained data was analyzed by comparative Ct method ($2^{-\Delta\Delta Ct}$ method) [40].

Table 1
Sequences of the primers used in this study.

Primer	Sequence (5′-3′)
Clone primers	
*Cg*SOCS6-F	ATGAAGCGTATCACGTTTAAGAGC
*Cg*SOCS6-R	GTACTGATTTTCTTCGATCCATCC
M13-47	CGCCAGGGTTTTCCCAGTCACGAC
M13-RV	GAGCGGATAACAATTTCACACAGG
*Cg*SOCS6-DF	CATCGAAGCCCACTATAT
*Cg*SOCS6-DR	CTAGTACTGATTTTCTTCGAT
RT-PCR primers	
*Cg*SOCS6-RT-F	CTTACACCGTCGTAGGCGAA
*Cg*SOCS6-RT-R	CTTGTGTCGTTTGGAGTCGC
*Cg*EF-RT-F	AGTCACCAAGGCTGCACAGAAAG
*Cg*EF-RT-R	TCCGACGTATTTCTTTGCGATGT
RNA interference	
*Cg*SOCS6-Fi	GCGTAATACGACTCACTATAGGACCGAAGAAGGGACAGGAATA
*Cg*SOCS6-Ri	GCGTAATACGACTCACTATAGGGTTCAATCCGTGTATGCCAAG
EGFP-Fi	GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC
EGFP-Ri	GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC
*Cg*IL17-4-RT-F	ACTTGTCCCTGGGTTATGTGTAG
*Cg*IL17-4-RT-R	TCCAAGAGGAACACGGAGAC
*Cg*IL17-5-RT-F	TCTGGCTGACTCTCGTCCTTG
*Cg*IL17-5-RT-R	GACCCTGTCGTTGTCCTCTACC
*Cg*Defh1-RT-F	ATTAGCCGTTCTTCTGATGG
*Cg*Defh1-RT-R	GCTCTACAACCGATGGACCT
*Cg*Defh2-RT-F	TGGTCGTTCTCCTGATGGTTT
*Cg*Defh2-RT-R	CTGCGTCACAGTAGCCCG

2.5. Recombinant expression and purification of CgSOCS6

The open reading frame (ORF) of *Cg*SOCS6 (1167 bp) was amplified from oyster hemocytes by the primers of *Cg*SOCS6-F and *Cg*SOCS6-R (Table 1). After gel-purification with MiniBest Agarose Gel DNA Extraction Kit Ver.4.0 (Takara, Japan), the products were inserted into pMD19-T vector and sequenced in both directions with M13–47 and M13-RV (Table 1). The cDNA fragment (912 bp) encoding the polypeptide containing a SH2 domain and SOCS box of *Cg*SOCS6 was amplified with the specific primers of *Cg*SOCS6-DF and *Cg*SOCS6-DR (Table 1), and the targeted fragments were inserted into the expression vector PEASY-Blunt E1 (Trans Gen Biotech, China). The recombinant plasmid PEASY-Blunt E1-*Cg*SOCS6 was verified by sequencing, and transformed into *Escherichia coli* Transetta (DE3) (Trans Gen Biotech, China). The positive strain of *E. coli* Transetta (DE3) with PEASY-Blunt E1-*Cg*SOCS6 was incubated in LB medium (containing 50 mg/mL kanamycin) at 37 °C with shaking at 180 rpm for about 4 h. The cells were incubated for additional 5 h with the induction of Isopropyl β-D-Thiogalactoside (IPTG) at the final concentration of 1 mM after the OD_{600} of culture medium reached 0.4–0.6. Then the bacteria were collected by centrifuging at 12000 rpm for 10 min. The recombinant *Cg*SOCS6 protein (r*Cg*SOCS6) was purified by His-tag purification resin (Sangon Biotech, China), pooled by elution with 400 mmol/L imidazole under denatured condition (8 mol/L urea). The purified proteins were renatured for 12 h in gradient urea-TBS glycerol buffer (50 mmol/L Tris-HCl, 50 mmol/L NaCl, 15% glycerol, 2 mmol/L reduced glutathione, 0.2 mmol/L oxide glutathione, a gradient urea concentration of 6, 4, 3, 2, 1, 0 mol/L, pH 7.6) at 4 °C for each gradient. The purified protein was examined by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and visualized with Coomassie bright blue R250 [41]. The purified protein was quantified with bicinchoninic acid (BCA) method [42], and then stored at −80 °C.

2.6. RNA interference and LPS stimulation

T7 promoter linked primers, *Cg*SOCS6-Fi and -Ri, EGFP-Fi and -Ri (Table 1), were used to amplify the DNA fragment of *Cg*SOCS6 (556 bp) from oyster genomic DNA and enhanced green fluorescent protein (EGFP) DNA (657 bp) from pEGFP vector, respectively. The dsRNAs of *Cg*SOCS6 and EGFP were synthesized by using T7 polymerase according to the instruction of manufacture (Takara, China). Eighteen oysters were employed and equally divided into two groups (one experiment group and one control group). The oysters in experiment group received an injection with dsRNA of *Cg*SOCS6, and the oysters received an injection with dsRNA of EGFP were employed as control [43]. The dsRNAs (100 μg) for *Cg*SOCS6 and EGFP were injected into oyster adductor muscle with a syringe, respectively. In order to strengthen the effect of RNA interference, the second injection was carried out at 12 h after the first injection. The hemocytes were collected from the oysters in the experiment and control groups at 24 h after the second injection.

Other twenty-seven oysters were used to detect the expressions of *Cg*IL17s and *Cg*Defs in *Cg*SOCS6-RNAi oysters after LPS stimulation. The oysters were separated into three groups averagely, including one experiment group, one control group (EGFP group), and one Blank group. The oysters in experiment and EGFP groups were stimulated twice with dsRNAs of *Cg*SOCS6 and dsRNA of EGFP according to the above description, respectively. They were stimulated by LPS (100 μg) at 24 h after the second injection with dsRNAs. The total RNA was collected from hemocytes at 24 h after LPS stimulation as mentioned above. The mRNA expression of *Cg*IL17-4 (GenBank accession KJ531895), *Cg*IL17-5 (GenBank accession KJ531896), *Cg*Defh1 (GenBank accession DQ400101) and *Cg*Defh2 (GenBank accession DQ400102) were examined by qRT-PCR according to the description above. The primers for *Cg*IL17-4, *Cg*IL17-5, *Cg*Defh1 and *Cg*Defh2 were listed in Table 1.

2.7. Preparation of polyclonal antibody and western blot analysis

The mice of 6-week old were treated by the injection of recombinant *Cg*SOCS6 to acquire polyclonal antibody as previous description [41]. The specificity of *Cg*SOCS6 polyclonal antibody was examined by Western blot. The protein samples extracted from oyster hemocytes were analyzed by 12% SDS-polyacrylamide gel electrophoresis and then diverted onto nitrocellulose membranes. Subsequently, 5% no-fat milk in TBST (20 mM Tris-HCl, 150 mM NaCl, 1% Tween-20, pH 7.5) was used to block the membranes for 2 h. The membranes were incubated with prepared anti-*Cg*SOCS6 antibody at a ratio of 1:1000 (v/v) for 4 h. After washed with TBST to wipe off the free nonspecifically binding antiserum, the membranes were incubated with the alkaline phosphatase (AP)-labeled goat anti-mouse IgG (Beyotime, China) at a ratio of 1:2000 (v/v) for 2 h. After washed for three times, the membranes were incubated in the reaction system (10 mL of TBS with 45 μL and 35 μL of NBT and BCIP, respectively, Sangon Biotech, China) in dark for 5 min to visualize the signals.

2.8. Immunocytochemical assay

The translocation of *Cg*SOCS6 in hemocytes of oysters after LPS stimulation was detected by immunocytochemical assay with PBS as control. The hemolymph was collected from oysters at 1 h after LPS stimulation, and mixed with an anticoagulant solution (510 mM NaCl, 100 mM glucose, 200 mM citric acid, 30 mM sodium citrate, 10 mM EDTA·2Na, pH 7.4) and 4% paraformaldehyde (1:1 in volume), and then centrifuged at 800 *g* at 4 °C for 10 min. After washed with PBS for three times, the collected hemocytes were resuspended in L15 cell culture media and deposited on dishes pre-coated with poly-L-lysine, and then incubated at 37 °C for 2 h to adhere to the glass slides. After washed for three times with PBS, the hemocytes on the glass slides were permeabilized with 1% Triton X-100 at 37 °C for 10 min. After washed for three times with PBS, the hemocytes were blocked with 3% (w/v)

Fetal bovine serum albumin (BSA) diluted in PBS at 37 °C for 30 min, and then incubated with the antibody of anti-*Cg*SOCS6 (diluted 1:500 (v/v) in 3% BSA) at 37 °C for 1 h. After washed for three times with PBS, the samples were incubated with Alexa Fluer 488-labeled goat-anti-mouse antibody (Solarbio life sciences, China, diluted 1:1000 (v/v) in 3% BSA) at 37 °C in dark for 1 h. A volume of 20 μL 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, Solarbio life sciences, China) was directly added to stain the nucleus for 5 min. After washed for three times, coverslips were fixed on slides containing a volume of 20 fluorescent mounting media. The oyster hemocytes were observed by using the oil immersion lens of Fluorescence microscope (Axio Imager A2, ZEISS).

2.9. Statistical analysis

All the data were presented as mean ± standard deviation, and analyzed by Statistical Package for Social Sciences (SPSS) 18.0. The significant differences among groups were tested by one-way analysis of variance (Duncan) and multiple comparisons. Significant differences across controls were indicated with an asterisk for significant different at $p < 0.05$, with two asterisks for extreme significant different at $p < 0.01$, and with three asterisks at $p < 0.001$.

3. Results

3.1. Sequence characters of CgSOCS6 cDNA

The full length cDNA of *Cg*SOCS6 was of 1440 bp with an open reading frame of 1167 bp encoding a peptide containing 388 amino acid residues with the predicted molecular mass of 44.66 kDa (Fig. 1A). There were a central SH2 domain and a SOCS box in the C-terminal, and an NLS domain in the N-terminal of *Cg*SOCS6 (Fig. 1B).

The previously reported amino acid sequences of SOCSs from *Homo sapiens* and *D. melanogaster* were selected to analyze the domain architectures of SOCS members. They commonly contained a SH2 domain and a SOCS-box in the C-terminus (Fig. 1C). *Cg*SOCS6 contained an additional NLS domain, which was similar to the counterpart of SOCS6 in *H. sapiens* (Fig. 1C). According to the multiple alignment, the deduced amino acid sequence of *Cg*SOCS6 shared high conservative property ranging from 37.9% to 45.5% with that of other SOCS6/7 family members (Fig. 2). A phylogenetic tree was constructed with the deduced amino acid sequences of *Cg*SOCS6 and other SOCSs from several species (*Eriocheir sinensis*, *Bombyx mori*, *Drosophila melanogaster*, *Danio rerio*, *Rattus norvegicus*, *Mus musculus*, and *H. sapiens*). In the phylogenetic tree, all the SOCS family members were separated into seven groups (CIS, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, and SOCS6/7), which were divided into two types, type I and type II. *Cg*SOCS6 was firstly clustered with *Es*SOCS6 from *E. sinensis* and *Dr*SOCS6 from *D. rerio*, and then clustered with vertebrate SOCS6 members, which belonged to type I (Fig. 3).

3.2. Tissue distribution of CgSOCS6 mRNA

The mRNA transcripts of *Cg*SOCS6 could be detected in all the tested tissues, including gonad, mantle, hepatopancreas, gills, adductor muscle, labial palps and hemocytes with qRT-PCR. The highest level of *Cg*SOCS6 mRNA transcripts was detected in hemocytes, which was 2.38-fold ($p < 0.05$) of that in gonad. There were no significant differences between gonad and other tissues (Fig. 4A).

3.3. Expression profile of CgSOCS6 after LPS stimulation

The mRNA expressions of *Cg*SOCS6 in hemocytes after LPS stimulation were examined by qRT-PCR with *Cg*EF as internal control. After LPS stimulation, the mRNA expression of *Cg*SOCS6 was significantly up-regulated at 3 h (3.81-fold, $p < 0.05$), reached the highest level at 6 h (8.48-fold, $p < 0.01$), and kept a higher level from 12 h to 72 h (2.18-fold, 4.83-fold, 4.21-fold, 4.57-fold, $p < 0.05$, respectively), compared with that in PBS group (Fig. 4B). The mRNA expressions of *Cg*SOCS6 in PBS group did not change significantly during the whole experiment.

3.4. The recombinant protein and polyclonal antibody of CgSOCS6

The r*Cg*SOCS6 protein was purified by using the Ni-NATA affinity chromatography and examined by 15% SDS-PAGE. An evident band with a molecular weight about 35 kDa was observed (lane 3 in Fig. 5A), which was consistent with the predicted molecular mass of r*Cg*SOCS6 with a His-tag.

Preparation of polyclonal antibody was programmed with the purified r*Cg*SOCS6 protein. The specificity of polyclonal antibody against *Cg*SOCS6 was examined with the hemocyte protein from oysters by Western blot. A single band about 45 kDa with the high specificity was revealed (lane 1 in Fig. 5B), which was identical to the prediction of molecular mass of *Cg*SOCS6.

3.5. The subcellular localization of CgSOCS6 protein in oyster hemocytes after LPS stimulation

The immunocytochemical assay was conducted by fluorescence microscope to detect the subcellular localization of *Cg*SOCS6 protein in hemocytes at 1 h after LPS stimulation with PBS as control. The *Cg*SOCS6 with anti-*Cg*SOCS6 antibody were observed in green signals and the nucleus stained by DAPI were in blue signals. In the PBS control group, the green signals of *Cg*SOCS6 mainly distributed in the cytoplasm. The green signals of *Cg*SOCS6 in the nucleus of hemocytes became stronger after LPS stimulation compared to that in PBS group, indicating that *Cg*SOCS6 could be translocated into the hemocyte nucleus after LPS stimulation (Fig. 6).

3.6. The mRNA expressions of CgIL17-4, CgIL17-5, CgDefh1 and CgDefh2 after the RNA interference of CgSOCS6

In the *Cg*SOCS6 dsRNA-injected oysters, the mRNA expression of *Cg*SOCS6 was examined by qRT-PCR. The *Cg*SOCS6 mRNA expression in hemocytes was down-regulated significantly (0.38-fold of that in EGFP group, $p < 0.05$) (Fig. 7A) after the injection with dsRNA of *Cg*SOCS6. In order to demonstrate the immune functions of *Cg*SOCS6, the mRNA expressions of *Cg*IL17-4, *Cg*IL17-5, *Cg*Defh1 and *Cg*Defh2 were detected in hemocytes of *Cg*SOCS6-RNAi oysters after LPS stimulation (Fig. 7B–E). EGFP-RNAi was used as control. The mRNA expressions of *Cg*IL17-4 and *Cg*IL17-5 in *Cg*SOCS6-RNAi oysters increased significantly after LPS stimulation, which was 2.55-fold ($p < 0.05$) and 2.68-fold ($p < 0.05$) of that in the EGFP-RNAi group, respectively. The mRNA expression of *Cg*Defh1 in *Cg*SOCS6-RNAi oysters increased extreme significantly (4.68-fold, $p < 0.0001$) after LPS stimulation, compared with that in EGFP control group. However, there was no significant difference of *Cg*Defh2 mRNA between the *Cg*SOCS6-RNAi and the EGFP-RNAi group.

4. Discussion

SOCS family members, especially SOCS1-3, participate in host immune responses in mammals by negatively regulating the production of cytokines and AMPs [44,45]. Recently, several SOCS1-3 proteins have been identified in invertebrates, and some of them also function as negative regulators to inhibit AMP expression [22]. However, there is less report about the negative regulation of SOCS4-7 on the expressions of cytokines and AMPs in both vertebrates and invertebrates. In the present study, a homologue of vertebrate SOCS6 was identified from oyster *C. gigas*, and it played important role in the regulation of immune effector production in molluscs.

The structures of SOCS family members are highly conservative in

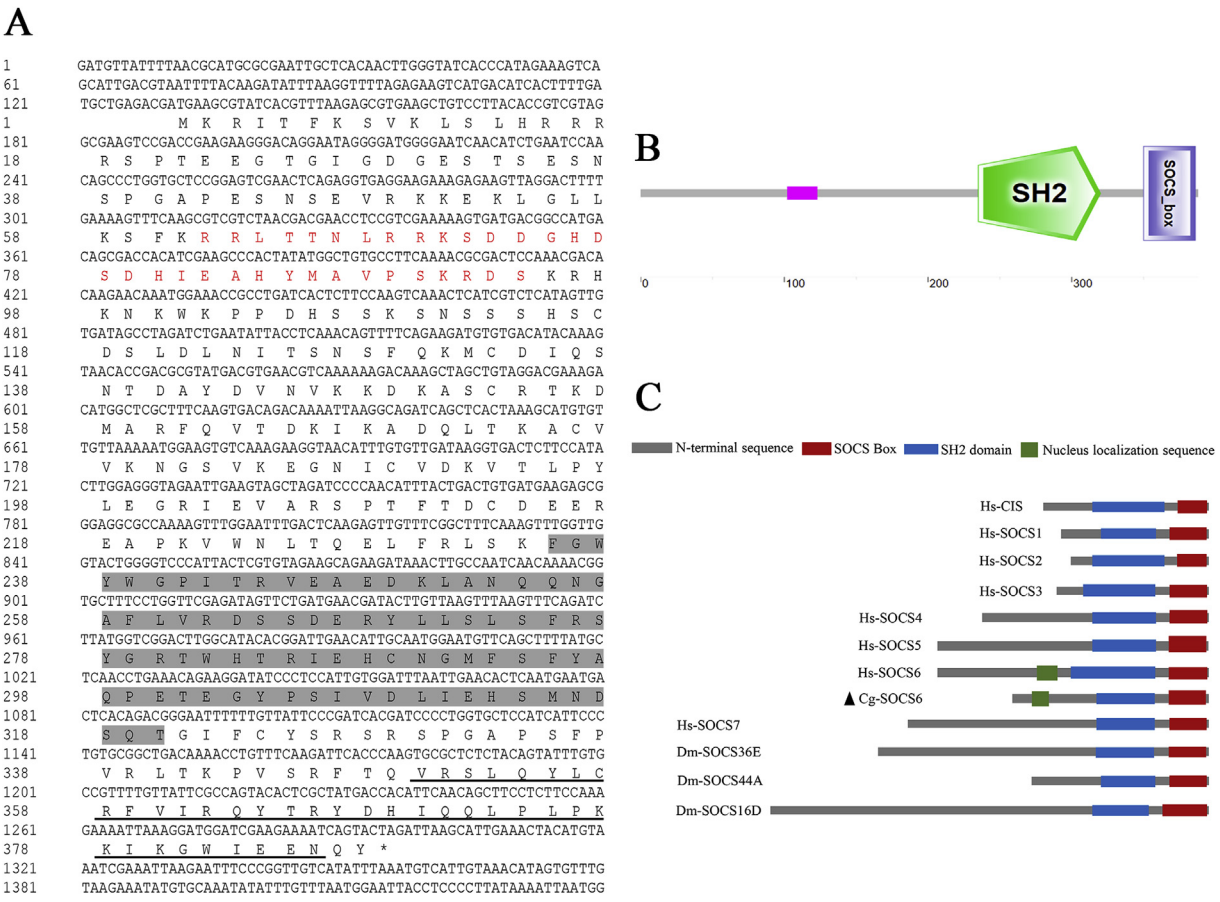

Fig. 1. Complete nucleotide sequence and the deduced structural domains of SOCS proteins. A. The NLS is labeled in red, the Src homology 2 (SH2) domain is labeled in gray, the SOCS box is underlined. B. The predicted structural domains of *Cg*SOCS6 by SMART (http://www.smart.embl-heidelberg.de/). C. The domain architectures of SOCSs from *Crassostrea gigas*, *Drosophila melanogaster* and *Homo sapiens* were predicted by SMART (http://www.smart.embl-heidelberg.de/). *Cg*SOCS6, XP_011452437.1; *Dm*SOCS36E, NP_523593.5; *Dm*SOCS 44A, NP_523659.1; *Dm*SOCS16D, AGB95504.1; *Hs*SOCS1, NP_003736.1; *Hs*SOCS2, NP_001257400.1; *Hs*SOCS3, NP_003946.3; HsSOCS4, NP_543143.1; *Hs*SOCS5, NP_054730.1; *Hs*SOCS6, NP_004223.2; *Hs*SOCS7, NP_055413.1; *Hs*CIS, NP_037456.5. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

both vertebrates and invertebrates, including an N-terminal domain with variable length and sequence, a central SH2 domain, and a C-terminal 40 amino-acid region named SOCS box [5,6]. These conserved architectures and properties are the most significant features of the SOCS family, which are crucial for SOCS proteins to maintain the physiological functions. For instance, SOCS2, SOCS5 and SOCS7 from oyster *C. gigas* commonly contain a SH2 domain and a SOCS box, which is consistent with the counterparts of mammalian SOCS members [23]. In the present study, one homologue of vertebrate SOCS6 was identified from oyster *C. gigas*, named as *Cg*SOCS6. The typical features of SOCSs [46], including a conservative SH2 domain in the middle of the entire sequence and a reserved C-terminal SOCS box, were identified in *Cg*SOCS6, suggesting that it was a representative SOCS family member. Apart from this, *Cg*SOCS6 was found to contain a variable NLS in the N-terminus, which was consistent with the counterpart of mammalian SOCS6, implying that it might be one of SOCS6 homologs in oyster. In vertebrates, it was reported that the NLS in the N-terminus was capable of driving the transport of SOCS6 into the nucleus in the HEK293T, HeLa, and breast cancer cell lines [47]. The NLS in *Cg*SOCS6 might be responsible for the localization of *Cg*SOCS6 in the nucleus. Apart from this, *Cg*SOCS6 shared high similarity with SOCS6 proteins from other species. In the phylogeny tree, *Cg*SOCS6 was firstly clustered with *Es*-SOCS6, and then grouped with other SOCS6 members, which finally assigned into the type I group. In addition to containing a SH2 domain and a SOCS-box, *Cg*SOCS6 also contained an additional NLS in the N-terminus, suggesting that it was a novel member of SOCS6 in molluscs.

In mammals, SOCSs mainly exist in Th17 cell, T cells, and NK cells. One of the most important functions of SOCSs is the inhibition for the immune responses [34,43]. In the present study, *Cg*SOCS6 was distributed in all the examined tissues with highest mRNA transcripts in hemocytes. Similarly, *Po*SOCS6 in *Paralichthys olivaceus* was detected in all the tested tissues with higher expression level in immune related organizations [48]. The higher expression of *Cg*SOCS6 in hemocytes might suggest its important role in mediating the immune response. Furthermore, numerous researches demonstrated that SOCS expression level could be induced after pathogen challenge. For instance, the expressions of SOCS1 and SOCS3 in macrophages were found to be up-regulated significantly after LPS stimulation, which was due to the activation of JAK/STAT signaling pathway [49]. In the present study, the mRNA expression of *Cg*SOCS6 in hemocytes was significantly up-regulated from 3 to 72 h after LPS stimulation. It was reported that, the expression level of SOCS6 mRNA in *B. mori* was up-regulated remarkably with the challenge of microbe [50]. The *Es*SOCS6 from *E. sinensis* could be induced by bacteria invasion, and possible to regulate the activation of NF-κB signaling pathway in the innate immune responses [21]. These results suggested that *Cg*SOCS6 might be involved in immune responses in the oyster hemocytes against LPS stimulation. In immunocytochemical assay, *Cg*SOCS6 protein could be translocated into the nucleus after LPS stimulation, which was consistent with the previous reports [47]. The translocation of *Cg*SOCS6 could be due to the existence of the additional N-terminal NLS. In vertebrates, SOCS6 could enter into the nucleus dependent on its N-terminal region, and its C-

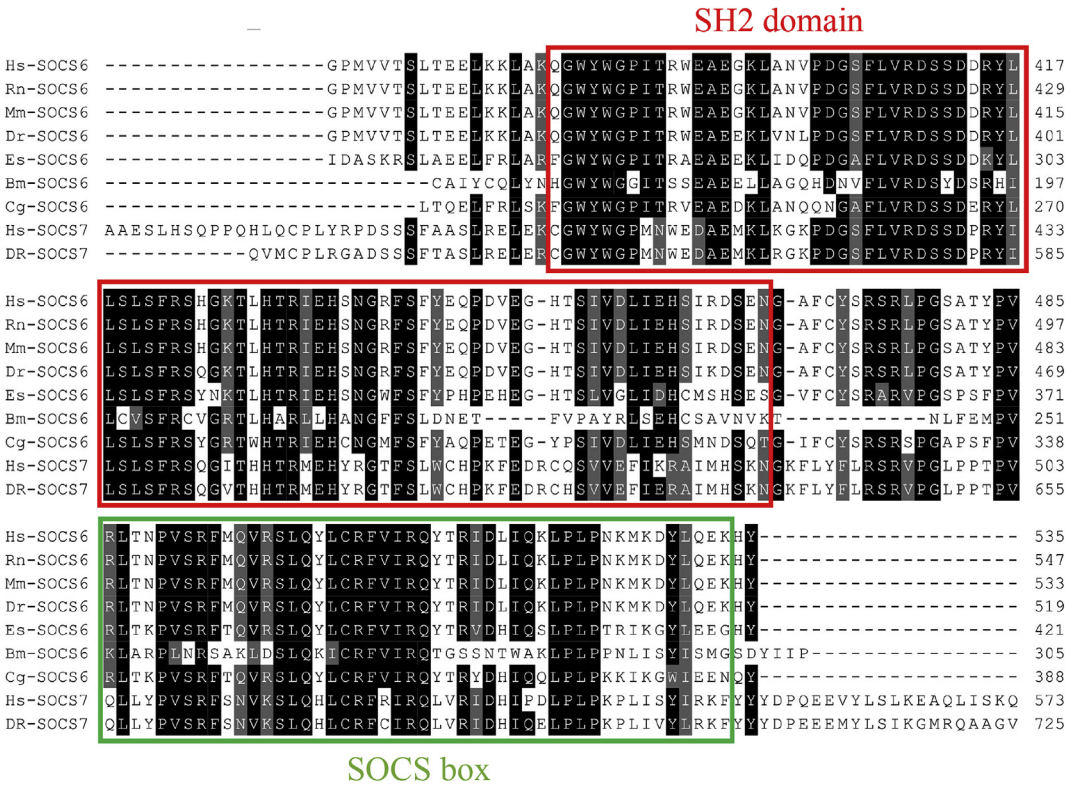

Fig. 2. Multiple sequence alignment of *Cg*SOCS6 with other SOCS6/7 family members. *Rn*, *Rattus norvegicus*; *Mm*, *Mus musculus*; *Dr*, *Danio rerio*; *Es*, *Eriocheir sinensis*; *Bm*, *Bombyx mori*. The SH2 domain is labeled with red frame, the SOCS box is labeled with green frame. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

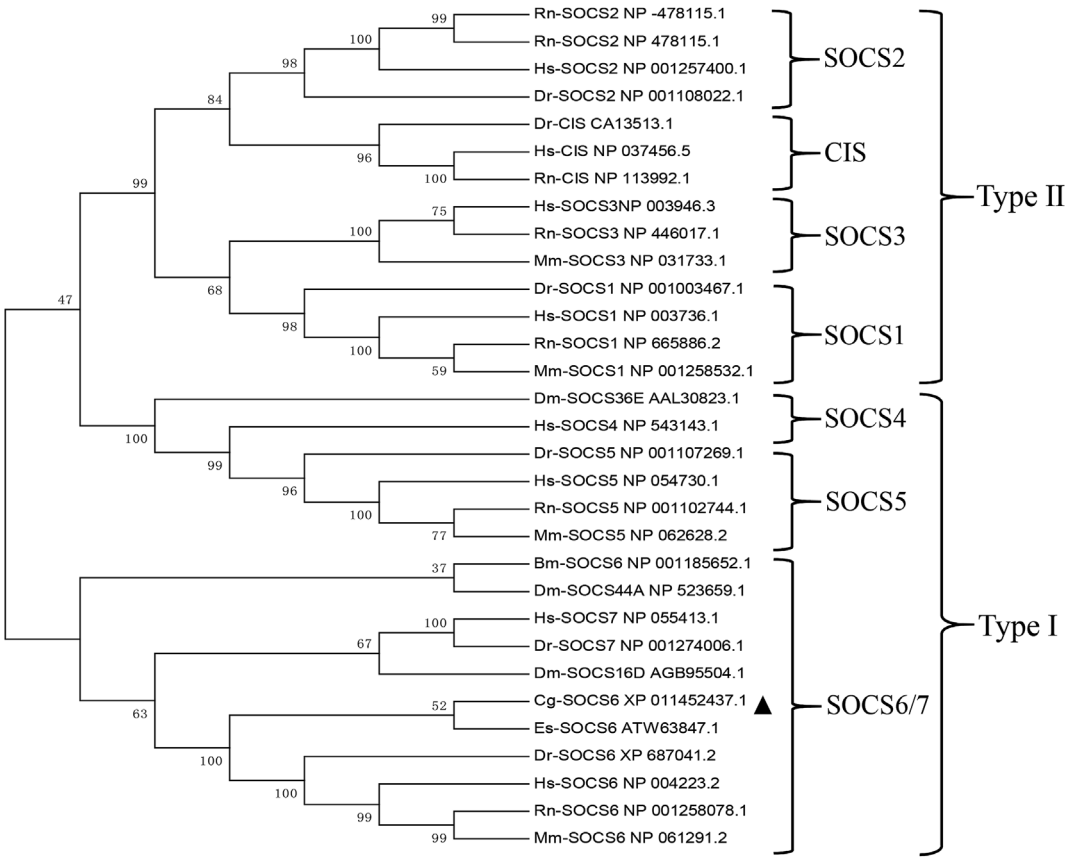

Fig. 3. Phylogenetic analysis of *Cg*SOCS6 with other SOCS family members from different animals.

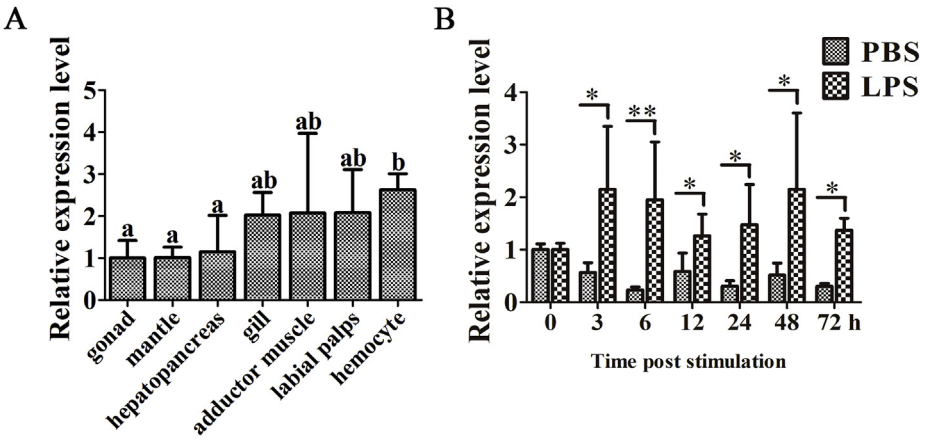

Fig. 4. The tissues distribution and temporal expression of the *Cg*SOCS6 are detected by qRT-PCR. A. The tissues distribution of the *Cg*SOCS6. *Cg*EF is used as control. The transcript levels of *Cg*SOCS6 mRNA in mantle, hepatopancreas, gills, adductor muscle, labial palps and hemocytes are normalized to that of gonad. B. The temporal expression of the *Cg*SOCS6. The mRNA expression in oyster hemocytes at 0, 3, 6, 12, 24, 48, and 72 h after LPS stimulation. The PBS group is used as control. Comparison of the mRNA level of *Cg*SOCS6 is normalized to that of control group at 0 h. Vertical bars represent the mean ± S.D. (N = 3). The different letters show that there exist significant differences comparing with other groups ($p < 0.05$, Duncan). *: $p < 0.05$, **: $p < 0.01$.

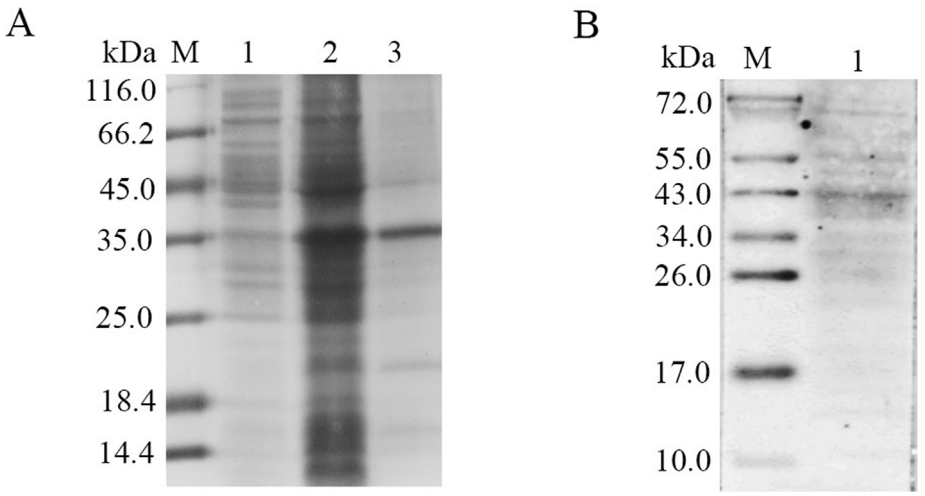

Fig. 5. The recombination protein of *Cg*SOCS6 and specificity detection for its polyclonal antibodies. SDS-PAGE is used to analyze r*Cg*SOCS6. A. Lane M: standard protein molecular weight marker; Lane 1: negative control (without induction); Lane 2: induced r*Cg*SOCS6; Lane 3: purified r*Cg*SOCS6. B. Western-blot with anti-*Cg*SOCS6-antibody in the hemocytes of *C. gigas*.

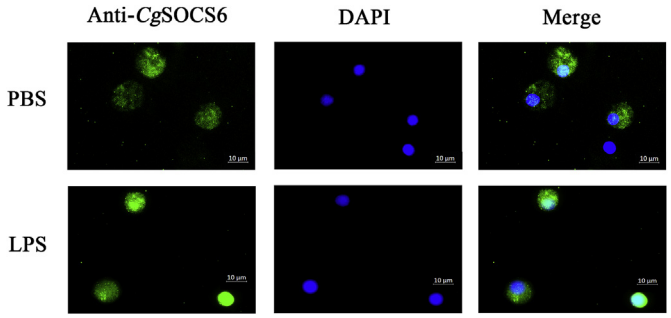

Fig. 6. *Cg*SOCS6 translocate into hemocyte nucleus after LPS stimulation. PBS is used as the control. Nucleus staining with DAPI is shown in blue signal; anti-*Cg*SOCS6 conjugated to Alexa-fluor 488 is shown in green signal. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

terminal region could reduce the activity of STAT3 [47]. All these results collectively suggested that *Cg*SOCS6 might play an important role in negatively regulating the activity of STAT in the hemocyte nucleus to participate in the antibacterial immune response. SOCS proteins are central regulators of microbial pathogen-induced signaling of cytokines in vertebrates, principally through the inhibition of the activation of JAK/STAT signaling cascades.

Mammalian SOCS can regulate different ILs by mediating the activity of JAK and the phosphorylation of STAT. For instance, SOCS1 negatively regulated the expression of IL13 in murine embryonic fibroblast by suppressing the activity of JAK via its KIR domain [31,32]. SOCS3 was reported to inhibit the expression of IL6 by restraining the phosphorylation of STAT1 and STAT3 in liver cells of mouse [33]. Recently, there were six primitive interleukin family members (IL17-1 to IL17-6) found in *C. gigas*, and they might play diversified roles in innate immune response of oyster [37,38]. In the present study, the mRNA expressions of *Cg*IL17-4 and *Cg*IL17-5 in hemocytes of *Cg*SOCS6-RNAi oysters increased significantly after LPS stimulation, but the expressions of IL17-1, IL17-2, IL17-3 and IL17-6 were not changed significantly (data not shown). Previous reports indicated that *Cg*IL17-1 participated in regulating the glucose homeostasis in serum, and could respond to the stress of air exposure in oysters *C. gigas* [37,38]. *Cg*IL17-5 played an important role in activating signal transduction for releasing other cytokines and modulating the bacteria clearance process in oysters [51]. The difference in activation of *Cg*IL17s modulated by *Cg*SOCS6 might be attributed to the different functions of *Cg*IL17s in the immune response of oysters. The mRNA expressions of *Cg*IL17-4 and *Cg*IL17-5 in hemocytes of the *Cg*SOCS6 RNAi-oysters increased significantly, indicating that *Cg*SOCS6 could inhibit the expression of *Cg*IL17-4 and *Cg*IL17-5, which was possibly by regulating the kinase activity of JAK and the phosphorylation of STAT in oysters, but the detailed mechanism still requires further investigation.

SOCSs also function as a negative regulators in mediating the production of AMPs in vertebrates and invertebrates. In mammals, SOCS1 and SOCS3 could down-regulate the production of human β-defensins (HBD-2 and HBD-3) by inhibiting the STAT1 and NF-κB signaling [45]. Recently, *Mj*SOCS2 in shrimp *M. japonicus* was reported to negatively regulate the secretions of AMPs (anti-lipopolysaccharide factors C1, C2 and D1, and Crustin I) via inhibiting the phosphorylation of *Mj*STAT [22]. In the present study, in order to certify whether *Cg*SOCS6 could

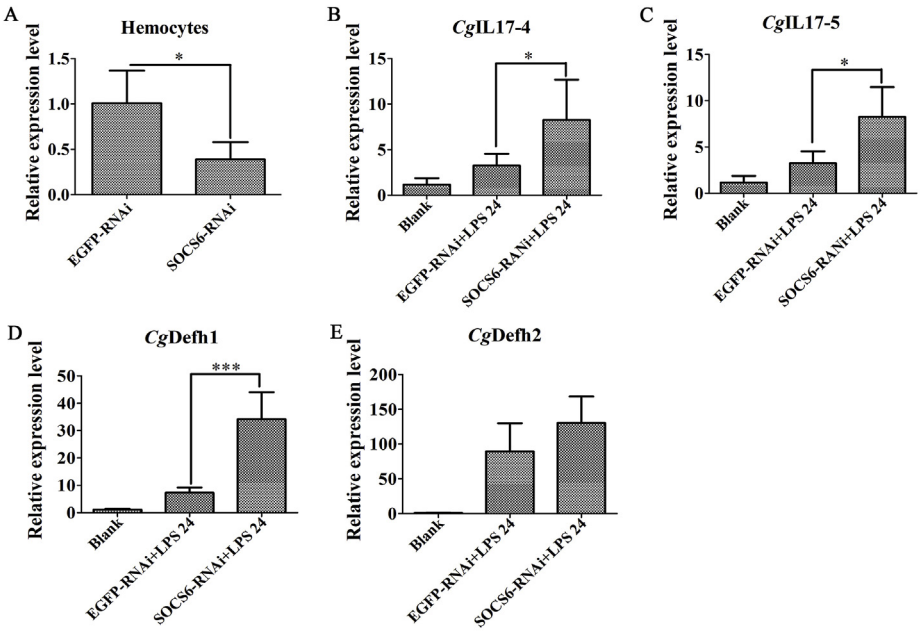

Fig. 7. The mRNA expressions of *Cg*IL17-4, *Cg*IL17-5, *Cg*Defh1 and *Cg*Defh2 in the hemocytes of *Cg*SOCS6-RNAi oysters after LPS stimulation. A. The efficiency of *Cg*SOCS6-RNAi in hemocytes, and EGFP-RNAi is used as control. B-E. The mRNA expressions of *Cg*IL17-4, *Cg*IL17-5, *Cg*Defh1 and *Cg*Defh2 in *Cg*SOCS6-RNAi oysters after LPS stimulation are detected by qRT-PCR. Vertical bars represent the mean ± S.D. (N = 3). *: $p < 0.05$, ***: $p < 0.001$.

regulate the production of AMP, the mRNA expressions of *Cg*Defh1 and *Cg*Defh2 were examined after *Cg*SOCS6 was knocked down by RNA interference. The mRNA expression of *Cg*Defh1 in the hemocytes of *Cg*SOCS6-RNAi oysters increased significantly after LPS stimulation. Interestingly, there was no significant difference of *Cg*Defh2 mRNA between the *Cg*SOCS6-RNAi and the EGFP-RNAi group. It was previously found that *Cg*Defh1 could play an important role in the defense against the microbial invasion in hemocytes, especially the gram-negative bacteria of *Vibrio splendidus* and *V. anguillarum*. *Cg*Defh2 was also reported to participate in defensing the bacteria invasion [39]. SOCS family members play different functions in inhibiting the expression of AMPs through selectively reducing the activation of JAK/STAT, NF-κB, and RTK pathways [2–4,52]. Previous reports demonstrated that most mollusc defensins had the broad-spectrum anti-bacterial activity, but some defensins exhibited a relatively violent killing ability on different pathogens. In addition to this, it was suspected that *Cg*Defh1 and *Cg*Defh2 were synthesized and regulated by different signaling pathways. These results indicated that *Cg*SOCS6 might negatively regulate the expression of *Cg*Defh1 to inhibit the immune response. Although SOCS6/7 proteins were rarely evidenced in regulating the production of cytokines and AMPs in vertebrates, there was raising evidence for the involvement of SOCS6/7 proteins in regulating receptor tyrosine kinase (RTK) signaling [52]. The results collectively suggested that *Cg*SOCS6 might be involved in inhibiting the expression of *Cg*Defh1 through regulating the RTK signaling in the hemocytes of oyster.

In conclusion, a novel SOCS family member (*Cg*SOCS6) was identified from oyster *C. gigas*, which contained a classical central SH2 domain, a C-terminal SOCS box, and an additional NLS domain in the N-terminus. The mRNA expression of *Cg*SOCS6 could be induced after LPS stimulation. *Cg*SOCS6 protein could be translocated into the hemocyte nucleus after LPS stimulation due to the existence of NLS. *Cg*SOCS6 also negatively regulated the mRNA expressions of *Cg*IL17-4, *Cg*IL17-5 and *Cg*Defh1 in oyster hemocytes. These results would be helpful for the understanding of SOCS functions in regulating the expression of cytokines and AMPs in oysters.

Acknowledgements

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