



## Full length article

# A novel calreticulin-related molecule that interacts with bacteria and enhances host resistance against bacterial infection in black rockfish, *Sebastes schlegeli*

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## ABSTRACT

Calreticulin (CRT) is a highly conserved and multi-functional protein with diverse localizations. CRT has lectin-like properties and possesses important immunological activities in mammalian. In teleost, very limited studies on CRT immunologic function have been documented. In the present study, a CRT homologue (SsCRT) was cloned, identified and characterized from black rockfish, *Sebastes schlegeli*, an important aquaculture species in East Asia. The full length of SsCRT cDNA is 2180 bp and encoded a polypeptide of 425 amino acids. SsCRT contains a signal peptide, three distinct structural and functional domains (N-, P- and C-domains), and an endoplasmic reticulum (ER) retrieval signal sequence (KDEL). The deduced amino acid sequence of SsCRT shares 89–92% overall sequence identities with the CRT proteins of several fish species. SsCRT was distributed ubiquitously in all the detected tissues and was highly expressed in the spleen, muscle and liver. After the infection of fish extracellular bacterial pathogen *Vibrio anguillarum* and intracellular bacterial pathogen *Edwardsiella tarda*, the mRNA transcripts of SsCRT in spleen, liver, and head kidney were significantly up-regulated. The expression patterns were time-dependent and tissue-dependent. Recombinant SsCRT (rSsCRT) exhibited apparent binding activities against different bacteria and PAMPs. *In vivo* studies showed that the expressions of multiple immune-related genes such as TNF13B, IL-1 $\beta$ , IL-8, SAA, Hsp70, and ISG15 in head kidney were significantly enhanced when black rockfish were treated with rSsCRT. Furthermore, rSsCRT reduced pathogen dissemination and replication in fish kidney and spleen. These results indicated that SsCRT served as an immune receptor to recognize and eliminate the invading pathogens, which played a vital role in the immune response of *Sebastes schlegeli*. These findings provide new insights into understanding the roles of CRT proteins in immune response and pathogen infection in teleost.

## 1. Introduction

As a Ca<sup>2+</sup>-binding protein, calreticulin (CRT) was firstly identified from rabbit skeletal muscle by Ostwald and MacLennan in 1974 [1]. Since then, CRT has been abundantly studied in vertebrates, invertebrates, and higher plants [2]. CRT can be divided into three domains: the amino-terminal N domain, the flexible mid proline-rich P domain, and the highly acidic carboxyl-terminal C domain [3]. The N-domain is a highly folded globular structure, which plays important roles in protein folding [4]. The P-domain has two typical proline-rich

motifs, which are essential for Ca<sup>2+</sup> binding of CRT with high capacity [5]. The C-domain terminates with the KDEL endoplasmic reticulum (ER) retrieval sequence and is responsible for the Ca<sup>2+</sup>-buffering activity [6]. Various studies verified that CRT is a highly conserved and multi-functional protein with diverse localizations. In the ER lumen, CRT performs two major functions: chaperonin and regulation of Ca<sup>2+</sup> homeostasis [7,8]. Outside the ER, CRT modulates many physiological/pathological processes, including integrin-dependent Ca<sup>2+</sup> signaling, steroid-sensitive gene expression and cell adhesion [9–11].

CRT possesses lectin-like properties and important immunological

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activities. The functions of CRT in mammalian species were intensively investigated and well characterized. CRT was considered to be the dominant pro-phagocytic signal on the surface of multiple human or murine cancer cells in the immune evasion [12,13]. It was found that increased CRT expression was an adverse prognostic factor in diverse tumors including neuroblastoma, bladder cancer, and non-Hodgkin's lymphoma [14]. Additionally, extracellular CRT has been observed in a number of physiological and pathological contexts [15,16]. In mice, peritoneal macrophages and splenic dendritic cells are shown to mediate CRT-dependent uptake of dying cells [17,18]. Compared with intensive studies of CRT in mammals, studies about CRT in teleost fish to date was still limited. CRT has been identified and characterized in the amphioxus, Asian seabass, channel catfish, rainbow trout, and tongue sole [19–25]. However, functional studies of CRT in teleost fish have not been well characterized.

*Sebastes schlegeli*, commonly known as black rockfish, is an important aquaculture species in Korea, Japan and China. However, prevalence of infectious diseases is resulting in great economic losses to the industry of black rockfish. In order to improve the quality and quantity of black rockfish in aquaculture production, it is vital to study the immune mechanism of black rockfish thoroughly. In this study, a CRT homologue from *Sebastes schlegeli* (*SsCRT*) was identified and characterized. The mRNA expression level in different tissues and the expression pattern by immune challenge were investigated. Moreover, the immunological activities of *SsCRT* was examined *in vitro* and *in vivo*.

## 2. Materials and methods

### 2.1. Fish

Black rockfish (average 26.3 g) were obtained from a commercial fish farm (Qingdao, Shandong Province, China) and maintained at 20 °C in culture tanks for two weeks. Before the experiment, fish were randomly sampled and verified to be clinically healthy as reported previously [26]. Fish were euthanized with tricaine methanesulfonate

**Table 1**  
PCR primers used in the study.

Primers name	Sequence(5'–3')
cDNA conserved regions primers	
CRT-F	GGAAARTTTTATGGKATGCTGA (R = A or G K = G or T)
CRT-R	ATCAATYTTCTCACGSTCATCCCA (Y = C or T S = C or G)
Race primers	
<i>SsCRT</i> -5'GSP outer	TGAACGACCAAAAGGCTTGCCCT
<i>SsCRT</i> -5'GSP inner	CTGGCTTGTTGACAGACCTTTGTC
<i>SsCRT</i> -3'GSP outer	CGGAAGACATTGCAATGGAGACAT
<i>SsCRT</i> -3'GSP inner	GAGCCAGAGAGGAAAATGAAACAG
qRT-PCR primers	
EF1A-F	AACCTGACCACCTGAGGTGAAGTCTG
EF1A-R	TCCTTGACGGACACGTTCTTGATGTT
<i>SsCRT</i> -F	AGCCTTTGGTCGTTCAAGTTAC
<i>SsCRT</i> -R	CTGCCAGATTCTACCTTCTCGTTA
TNF13B-F	GGAAAACCTTCAGGAAAGAATACA
TNF13B-R	TGAGGCTCGTCTGCCACC
IL-1β-F	GCATCCGAGGACAAAATCC
IL-1β-R	ACACCCGCTCCACTCAACAG
IL-8-F	CCTCATTTTAATACCACAGG
IL-8-R	ACAAACAAGCACAGACTTCT
Hp-F	GGCAGGAAAAGAGGGAATAG
Hp-R	GGAAAGTGTGGATGGAGAAAAA
SAA-F	CTTCCCGGTGAAGCCTTTA
SAA-R	CCATGCTCATTGCTCTCTGAT
HSP70-F	CTGTTTGAAGCAATTGAGGGC
HSP70-R	CAGGAGTTTCTGGATTTAGGGA
ISG15-F	CTACGGCCTGCAGCAAGGAGC
ISG15-R	CCCTGGTCTTGAAGTTGGCCA
Recombinant primers	
r <i>SsCRT</i> -F	AAGGTTTACTTTCGGGAGGAGTTT
r <i>SsCRT</i> -R	TCACTTCTCCATCGTCCGT

(Sigma, St. Louis, USA) as reported previously before tissue collection [27].

### 2.2. Bacterial and viral strains

The fish pathogen *Edwardsiella tarda* and *Vibrio anguillarum* were reported previously [28]. *Escherichia coli* DH5α and *Staphylococcus aureus* were purchased from Transgene (Beijing, China). All strains were cultured in Luria-Bertani broth (LB) medium at 28 °C (for *E. tarda* and *V. anguillarum*) or 37 °C (for *E. coli* DH5α and *S. aureus*).

### 2.3. Cloning of the full-length cDNA of *SsCRT*

The full-length cDNA sequence of *SsCRT* was obtained by homologous cloning and rapid amplification of cDNA ends (RACE) [29]. RNAPrep pure Tissue Kit (Tiangen, Beijing, China) was used to extract total RNA from the spleen tissue following the manufacturer's protocols. The first-strand cDNA was synthesized with Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). The degenerate primers, CRT-F and CRT-R (Table 1) designed according to the conserved sequences of CRT nucleotide sequences, were used to amplify the conserved fragment of *SsCRT*. The amplifications of 5' and 3' RACE reactions were performed using a SMART™ RACE cDNA amplification kit (Clontech, Indianapolis, IN, USA), primers and nested primers were shown in Table 1. The PCR products were gel-purified and sub-cloned. The positive clones were sequenced using an ABI 3730 DNA Analyzer at Tsingke Biotechnology Company.

### 2.4. Sequence analysis of *SsCRT*

Homology comparisons were performed with BLAST program at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/blast>). The information of the deduced amino acid sequence was analyzed with the DNAMAN software package (Lynnon Biosoft, Quebec, Canada). The signal peptide and protein domains were analyzed using the SMART program (<http://smart.embl-heidelberg.de>). The multiple alignment and phylogenetic tree was constructed with the ClustalW Multiple Alignment program (<http://www.ebi.ac.uk/clustalw/>) and MEGA 6.0 software package considering 1000 bootstrap hits. The presumed 3D protein structural model was established using protein homology/analogy recognition engine V 2.0 (Phyre2).

### 2.5. Quantitative real time reverse transcription-PCR (qRT-PCR) analysis of *SsCRT* expression under normal conditions

qRT-PCR analysis of *SsCRT* expression under normal conditions was determined as reported previously [30]. Total RNA from the spleen, liver, kidney, blood, intestine, muscle, gills, heart, and brain, aseptically collected from five black rockfish, was extracted using RNAPrep pure Tissue Kit (Tiangen, Beijing, China). RNA was digested with DNaseI. One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Roche, Mannheim, Germany). qRT-PCR was performed using an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript QRT-PCR Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) [31]. The PCR reaction was performed in a 20 μl volume containing 10 μl SYBR® premix Ex Taq™ (Tli RNaseH Plus). The primers for qRT-PCR were listed in Table 1. The PCR conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 20 s. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected. The expression level of *SsCRT* was analyzed using the comparative threshold cycle method ( $2^{-\Delta\Delta CT}$ ) with beta-actin as an internal reference [32].

### 2.6. qRT-PCR analysis of *SsCRT* expression during pathogens infection

qRT-PCR analysis of *SsCRT* expression during bacterial infection

was performed as reported previously [30]. *V. anguillarum* and *E. tarda* were cultured in LB broth at 28 °C to an optical density at 600 nm ( $OD_{600}$ ) of 0.8. Then, the cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS to a concentration of  $1 \times 10^6$  CFU/ml. Three groups of black rockfish were injected intraperitoneally (i.p.) with 100  $\mu$ l *V. anguillarum*, *E. tarda* or PBS. At 0 h, 4 h, 8 h, 12 h, 24 h and 48 h post-injection, five individuals from each group were randomly sampled. *SsCRT* expression in liver, spleen and head kidney of the fish was determined by qRT-PCR as above. The experiment was repeated three times.

## 2.7. Expression and purification of recombinant *SsCRT* protein

To construct pBSsCRT, which expresses His-tagged recombinant *SsCRT* (rSsCRT), the coding sequence of *SsCRT* without signal peptide sequence was amplified by PCR with primers *SsCRT*-F and *SsCRT*-R (Table 1). The PCR products were ligated with the expression vector pEASY-Blunt E1 with His-tag (Transgen, Beijing, China), resulting in pBSsCRT. The recombinant plasmid pBSsCRT was transformed into Transetta (DE3) (Transgen, Beijing, China), and the expression and purification of rSsCRT was performed as according to a previous description [33]. During the protein purification, 1% Triton X-114 was added to the nickel-nitrilotriacetic acid column to remove endotoxin as reported previously [33]. The purified protein was subjected to sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie brilliant blue. The concentration of the protein was determined using the Bradford method with bovine serum albumin as a standard.

## 2.8. Binding assay of rSsCRT with bacteria in vitro

Enzyme-linked immunosorbent assay (ELISA) was used to examine the binding activity of rSsCRT to pathogen-associated molecular patterns (PAMPs) and bacteria cells according to previous description [34]. Briefly, 40  $\mu$ g of lipopolysaccharide (LPS), peptidoglycan (PGN) (Sigma–Aldrich), which are the major constituents of the outer membranes of Gram-negative and Gram-positive bacteria, respectively, *V. anguillarum*, or *E. tarda* ( $5 \times 10^7$  CFU/ml) in 100  $\mu$ l carbonate-bicarbonate buffer (50 mM, pH 9.6) were added into a 96-well plate, respectively, and incubated at 4 °C overnight. After fixing the cells with 50  $\mu$ l 0.05% glutaraldehyde and blocking with 100  $\mu$ l of 3% bovine serum albumin (BSA) in PBS, rSsCRT (50  $\mu$ l at a concentration of 0.16 mg/mL) was added to each well (three wells as parallel samples) and incubated at room temperature for 4 h. A His-tagged inactive recombinant suppressor of cytokine signaling 3 (rSmSOCS3) [35] from turbot was used as a negative control. PBS was used as a blank. After incubation with anti-His antibody (Solarbio, Beijing, China) (diluted 1:1000 in carbonate-bicarbonate) and horseradish peroxidase conjugated goat anti-mouse IgG (Solarbio) (diluted 1:1000 in carbonate-bicarbonate), the plate was washed by PBST, then 100  $\mu$ l of tetramethylbenzidine was added to each well and incubated at 37 °C for 15 min. The reaction was stopped by adding 50  $\mu$ l 2 M  $H_2SO_4$ , then the plate was read at 450 nm with an ELISA reader (Molecular Devices). Samples with P/N value [P (sample)-B (blank)/N (negative)-B (blank)] > 2.1 were considered positive. The binding assays were repeated for three times under the same procedures for statistical analysis. The experiments were performed three times. The purified rSsCRT (1 mg/ml) and rSmSOCS3 (1 mg/ml) were labeled with fluorescein isothiocyanate (FITC) using a ReadILink™ Antibody Labeling Kit (Solarbio) according to the manufacturer's instructions [22]. FITC-labeled rSsCRT was respectively mixed with *V. anguillarum*, *E. coli*, and *S. aureus* at room temperature for 1 h. The microbes were washed three times with PBS (pH 7.4) and resuspended in PBS (pH 7.4). Aliquots were applied to microscope slides and observed under a LEICA DM2500 imager fluorescence microscope. The same concentration of rSmSOCS3 was used as a negative control.

## 2.9. In vivo effect of rSsCRT on bacterial infection

Three groups of black rockfish were injected intraperitoneally (i.p.) with 15  $\mu$ g/fish of rSsCRT or PBS (control). At 2 h post-injection, the fish were infected with 100  $\mu$ l *V. anguillarum* ( $10^7$  CFU/ml) or *E. tarda* ( $10^7$  CFU/ml). The kidney and spleen of fish were taken under aseptic conditions at 12 h and 24 h post-infection. After the tissues were homogenized in PBS, the homogenates were diluted serially in PBS and plated in triplicate on LB agar plates. The plates were incubated at 28 °C for 48 h, and the colonies that emerged on the plates were counted. The experiments were performed three times.

## 2.10. In vivo effect of rSsCRT on expression of immune genes

To determine the expression of immune genes including interleukin (IL) -1 $\beta$ , IL-8, tissue necrosis factor (TNF) 13B, haptoglobin (Hp), serum amyloid A (SAA), heat shock protein 70 (HSP70), and ISG15, black rockfish were administrated with rSsCRT or PBS (control) for 12 h. Total RNA was extracted from the head kidney tissue after rSsCRT treatment and qRT-PCR was used to analyze the expression of immune genes as described above. All experiments were performed three times. The PCR primers of the immune genes are listed in Table 1.

## 2.11. Statistical analysis

All statistical analyses were performed with SPSS version 17.0 software (Chicago, IL, USA). Data were analyzed with one-way analysis of variance, and statistical significance was defined as  $P < 0.05$ .

## 3. Results

### 3.1. Sequence analysis of *SsCRT*

The full-length cDNA of *SsCRT* contains a 5'-UTR of 58 bp, an open reading frame (ORF) of 1278 bp, and a 3'-UTR of 844 bp (Fig. 1A). The ORF encodes a polypeptide of 425 amino acids with a theoretical pI of 4.14 and predicted molecular mass of 49.0 kDa. *SsCRT* contains a signal peptide composed of 21 amino acids at the N-terminus and an ER-retrieval sequence KDEL at the C-terminus. Three distinct structural and functional domains (N-, P- and C-domains) are observed in *SsCRT*, including two CRT signature motifs, KHEQKIDCGGGYVK and IMFGPD-ICG, which are highly conserved in CRTs of different species (Fig. 1 and Fig. S1). Two triplicate repeats (A repeat-1, 2, 3 and B repeat-1, 2, 3) are at the proline-rich region of *SsCRT* (Fig. 1A). The 3D protein structural model of *SsCRT* is 76% identical to the single highest scoring template c1jhnA with 100% confidence (Fig. S1). These results indicated *SsCRT* is a member of CRT family. BLAST analysis showed that *SsCRT* shares high sequence similarity (73.9–89.5%) with CRT proteins from *Poecilia reticulata*, *Oryzias latipes*, *Epinephelus coioides*, *Gallus gallus*, *Mus musculus* and *Homo sapiens* (Fig. 2). Phylogenetic analyses showed that CRTs derived from fish are clustered into a branch that is distinct from those formed by the CRTs of Amphibian, Avian, and Mammalian. In fish branch, two groups are formed, CRTs from *Sebastes schlegeli*, *Takifugu rubripes*, *Epinephelus coioides*, *Oreochromis niloticus*, and *Onchorhynchus mykiss* form a separate group, CRTs from other fish species form another group (Fig. 3).

### 3.2. Expression of *SsCRT* under normal physiological conditions

Under normal physiological conditions, qRT-PCR analysis showed that *SsCRT* was distributed ubiquitously in all the detected tissues with a higher expression level in the spleen, muscle and a lower expression level in the brain and gills (Fig. 4). The difference in expression level between liver and blood was 12.4-fold.

**A**

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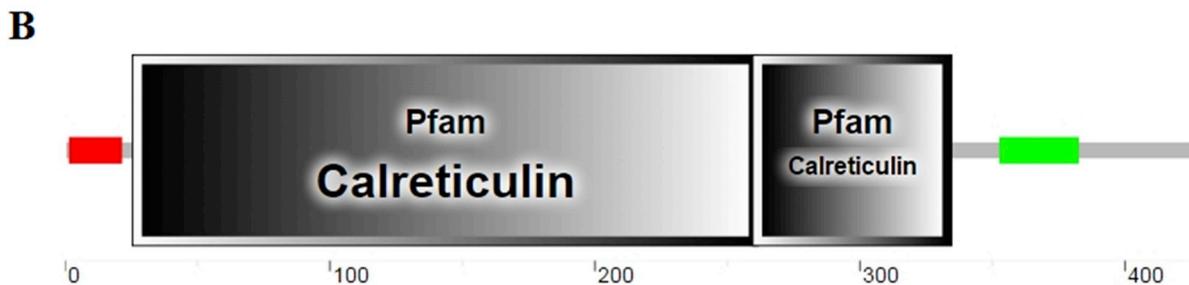
-58      gatactcagcgaggacgttagtcagcagcagcagcagaaagtgaagcagagagagagag
1  ATGGGGAAAATGCAAGTCTGGGTGTAGTAGCGGTGATATTAGCAGTGTGCTGCGTTTCATGCGAAGGTTTACTTTCGGGAGGAGTTCG
1  M G K M Q V L G V V A V I L A V C C V H A K V Y F R E E F L
91  GACCGTGTGAATGGAGAAGTCTGGTGGTGAACCTCCAAACAAGCTCTGACTACGGAGAGTGGAACTAACAGCTGGCAGCTTCTATGGA
31  D G D E W R S R W N S K H K K S D C Y G E W K L T A G S F Y G
181  GATGCTGAGAAAGACAAAGTCTGCAAAACAGCCAGGATCGTTCGTTCTATGCCACCTCTGCCCGCTTTGAGCCTTCAGCAACGAGGGC
61  D A E K D K G L Q T S Q D A R F Y A T S A R F E P F S N E G
271  AAGCCTTTGGTCTGTTTACAGTTAAG CATGAGCAGAAGATCGACTGTGGTGGTGGCTATGGAAGTCTTCCCACTGATTGGAT
91  K P L V V Q F T V [K.H.E.Q.K.I.D.C.G.G.G.Y.V.K] V F P T D L D
361  CAGACTGCAATGCATGGAGACTCCTCATACTACATCATGTTTGGCCCTGATATCTGTGGCTACAGCACCAGAAAGTCCACGTCATCTTC
121  Q T A M H G D S S Y Y [I.M.F.G.P.D.I.C.G] Y S T K K V H V I F
451  AATTACAAGGCAAGAATCACCTCATCAAGAAAGATTAAAGTCAAGGATGATGAGCTGACCCACATGTACAGCTGATCCTGAATCCT
151  N Y K G K N H L I K K E I K C K D D E L T H M Y T L I L N P
541  GATCAGACCTATGAGGTGAAGATCGATAACGAGAAGGTAGAATCTGGCAGTCTGGAGGAAGACTGGGACTTCTGCCTCCTAAGACAATT
181  D Q T Y E V K I D N E K V E S G S L E E D W D F L P P K T I
631  AAGGACCCCAAGCAAGAAGCCAGAGGACTGGGATGATCGTCCAAGATTGATGATGATGCTGACACCAAGCCCGAGGACTGGGACAAA
211  K D P E A K K P E D W D D R A K I D D D A D T K P E D W D K

          A1                      A2
721  GCTGAAAACATTCCAGACCCTGACGCTAAA AAGCCTGAAGACTGGGAGGTGGATATGGATGGAGAGTGGGAGCCACCCATGATCCCTAAC
241  A E N I P D P D A K K P E D W E V D M D G E W E P P M I P N

          A3                      B1
811  CCAGAGTACAAGGGA GAATGGAAACCCAAACAGATTGAAAACCCCAACTACAAGGAACATGGGTGCATCCTGAGATCGACAACTCCTGAA
271  P E Y K G E W K P K Q I E N P N Y K G T W V H P E I D N P E

          B2                      B3
901  TACAGTGTGATTCAAACATCTACAAGTTTGACAAAATTGCTGTTTTAGGCCTTGATCTTTGGCAGGTGAAATCT GGTACCATCTTTGAC
301  Y S A D S N I Y K F D K I A V L G L D L W Q V K S G T I F D
991  AACTTCTGTATCAGCGCAGATGTGAAGGAAGCGGAAGACATTGCAATGGAGACATGGGGT GTGACAAAGGAGCCAGAGAGGAAAATGAAA
331  N F L I S D D V K E A E D I A M E T W G V T K E P E R K M K
1081  CAGGAGCAAGATGACCTGAACCGAAAAGGAGGCGAAGACCAAGAACAGACACCGAAGTGCATGATGACGGTGCAGGTGAAGAT
361  Q E Q D D L N R K E E E A K T K E Q D T E V D D D G D G E D
1171  GATGATGAAGACATAGAGGATGAGGAAGAA ACAACGGCAGATGGAGAGAAGGCCTTTCAGAAAGAGATGAAGAGGAAGCAAATCTTCAA
391  D D E D I E D E E E T T D D G E K A S S E R D E E E A N L Q
1261  GATAAAGATGAACCTTGA
421  D K D E L *

1279  ggaggtgtggccagagatttctctgtccctcctttagaaaactctgttttatttccaggggtattgtggctgtgtgcatcctttcc
1369  ctgagattcggcagcgaatctaatattgggggtgcaaaaatggtgaaaggggtcaagtggtatgtaattggacttagttttcttaattttgg
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2089  aaaagattataaccgtaaaaaaaaaaaaaaaaaaaaa
    
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**Fig. 1.** Sequence analysis of SsCRT. (A) The nucleotide and deduced amino acid sequences of SsCRT. CRT repeat motifs A and B are shaded in grey. The two CRT signature motifs are indicated by boxes. The stop codon is marked by an asterisk. The signal peptide and the polyadenylation signal are underlined. (B) The schematic of protein motifs of SsCRT. The two CRT signature motifs are indicated by boxes. The signal peptide is showed with red rectangle. The coiled coil region is marked by green rectangle. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**3.3. Expression of SsCRT upon pathogens challenge**

To examine the effect of pathogens infection on SsCRT expression, black rockfish were challenged with the fish extracellular pathogens *V. anguillarum* and intracellular pathogen *E. tarda*. SsCRT expressions in liver, spleen, and head kidney were analyzed by qRT-PCR at 0, 4, 8, 12, 24, and 48 h post infection (hpi). The results showed that after *V. anguillarum* infection, SsCRT expressions in liver, spleen, and head kidney

were significantly enhanced at 4, 8, 12, 24, and 48 hpi, with the highest level occurring at 12 hpi (21.9-fold), 8 hpi (17.6-fold), and 12 hpi (13.7-fold), respectively (Fig. 5A). The results in Fig. 5B showed that SsCRT expressions in liver, spleen, and head kidney induced by *E. tarda* infection were also remarkably enhanced at all the examined time points, peaked at 8 hpi (16.1-fold), 12 hpi (26.9-fold), and 12 hpi (18.4-fold), respectively.

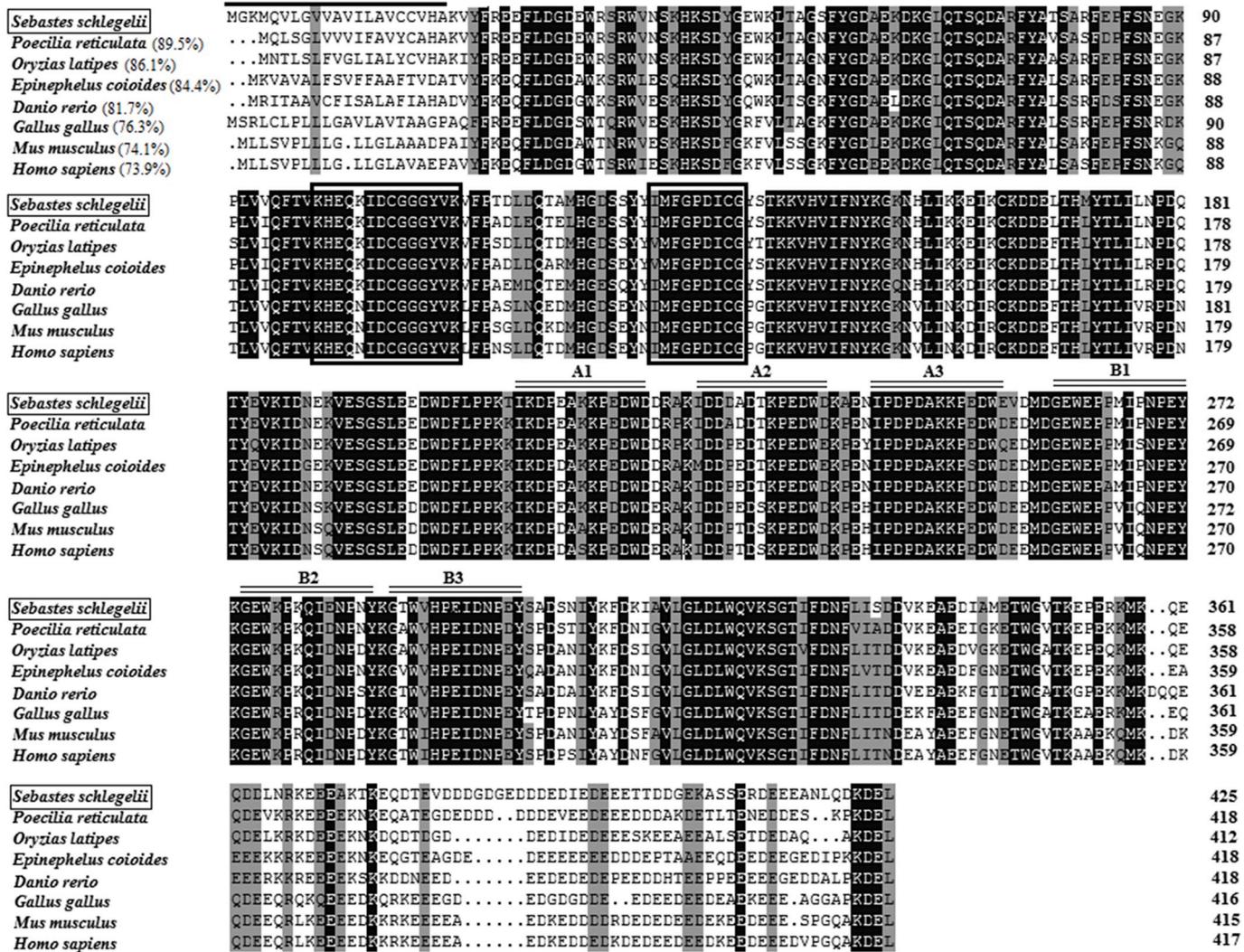


Fig. 2. The multiple sequence alignment of SsCRT homologue. The two CRT signature motifs are marked by boxes. The signal peptide is marked by a single thick line. CRT repeat motifs A and B are marked by double thin lines. Amino acid residues that are highly conserved in 100% of the sequences are indicated by dark shading, and the residues that are  $\geq 75\%$  identical among the aligned sequences are in grey. SsCRT shared 73.9–89.5% similarity with CRT proteins from *Poecilia reticulata* (XM\_008434438.1), *Oryzias latipes* (XP\_004079512.1), *Epinephelus coioides* (KC460314.1), *Danio rerio* (AAH68336.1), *Gallus gallus* (XP\_015155688.1), *Mus musculus* (AAH03453.1) and *Homo sapiens* (AAB51176.1).

3.4. The binding activity of rSsCRT to different PAMPs and bacteria

His-tagged rSsCRT was purified from *E. coli* (Fig. S2) and used to measure the binding of rSsCRT to LPS and PGN. The result showed that rSsCRT was able to bind to LPS and PGN. In contrast, no apparent binding was observed when control protein rSmSOCS3 was used (Fig. 6A). Next, we examined the binding activity of rSsCRT to pathogens *V. anguillarum* and *E. tarda*. The result showed that rSsCRT was also able to bind with *V. anguillarum* and *E. tarda* (Fig. 6B). The value of P/N of rSsCRT for *V. anguillarum* and *E. tarda* were all higher than 2.1. Under a LEICA DM2500 imager fluorescence microscope, FITC-labeled rSsCRT was observed to bind to *V. anguillarum*, *E. coli*, and *S. aureus* (Fig. 7). By contrast, no visible binding was detected in the negative control. These data indicated the binding specificity of rSsCRT to those different bacteria.

3.5. In vivo effect of rSsCRT on bacterial infection

To investigate the *in vivo* effect of rSsCRT on bacterial infection, black rockfish were administered with rSsCRT before being inoculated with *V. anguillarum*. Bacterial counts in the head kidney and spleen of

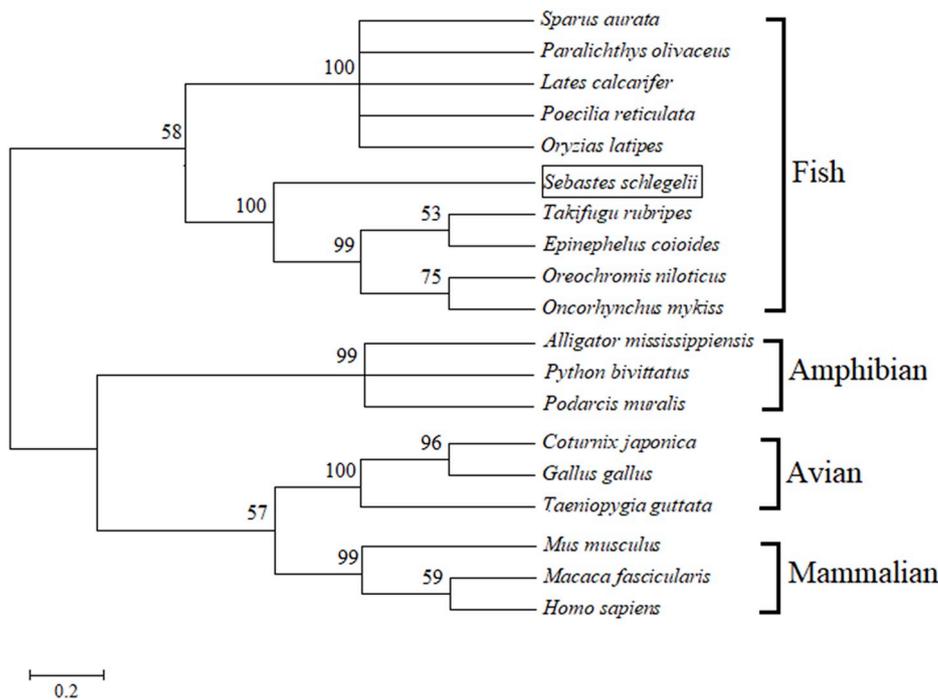
the infected fish were determined at different times post-infection. The results indicated that at 12 h and 24 h post-infection, the numbers of *V. anguillarum* recovered from the head kidney and spleen of rSsCRT-treated fish were significantly lower than those from control fish (Fig. 8).

3.6. Effect of rSsCRT on gene expression

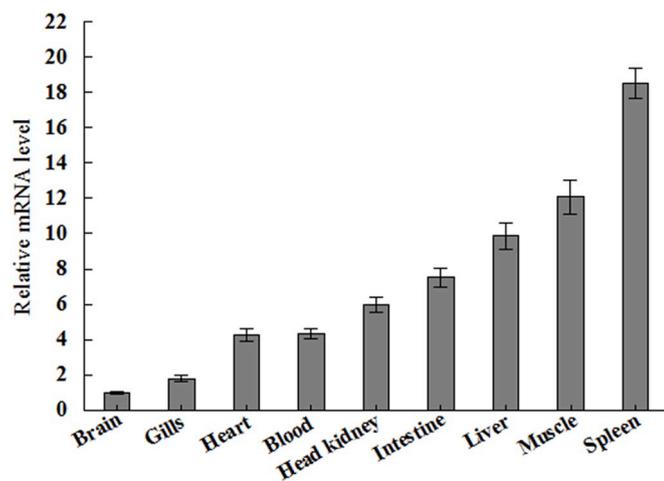
To examine whether rSsCRT had any effect on the expression of immune genes, black rockfish were treated with rSsCRT or PBS, and the expression of TNF13B, IL-1 $\beta$ , IL-8, Hp, SAA, HSP70, and ISG15 in head kidney were determined by qRT-PCR. The results showed that, compared to control, rSsCRT-treated fish exhibited significantly increased inductions of all the examined genes except Hp (Fig. 9).

4. Discussion

As a multi-functional protein with diverse sub-cellular localizations, CRT has been identified in vertebrates and invertebrates abundantly. In this study, a CRT homologue, SsCRT, was isolated and characterized from black rockfish by homologous cloning and RACE. SsCRT shares



**Fig. 3.** Phylogenetic analysis of SsCRT and other CRT proteins. The phylogenetic tree was constructed with MEGA 6.0 software (<http://www.megasoftware.net/>) using the neighbor-joining method. Numbers beside the internal branches indicate bootstrap values based on 1000 replications. The accession numbers of the analyzed sequences are as follows: *Poecilia reticulata* (XM\_008434438.1), *Oncorhynchus mykiss* (NP\_001117950.1), *Oreochromis niloticus* (XP\_013129008.1), *Oryzias latipes* (XP\_004079512.1), *Epinephelus coioides* (KC460314.1), *Takifugu rubripes* (XP\_003974218.1), *Sparus aurata* (KF857313.1), *Lates calcarifer* (HM597712.1), *Paralichthys olivaceus* (DQ535486.1), *Eisenia andrei* (ABI74618.1), *Alligator mississippiensis* (XP\_006262375.2), *Coturnix japonica* (XP\_015706103.1), *Taeniopygia guttata* (XP\_012431483.1), *Podarcis muralis* (XP\_028589341.1), *Python bivittatus* (XP\_007432164.1), *Gallus gallus* (XP\_015155688.1), *Mus musculus* (AAH03453.1), *Macaca fascicularis* (NP\_001274539.1) and *Homo sapiens* (AAB51176.1).



**Fig. 4.** The mRNA expression of SsCRT in different tissues under normal conditions. SsCRT expression in the blood, brain, muscle, head kidney, spleen, intestine, heart, gills, and liver of black rockfish was determined by quantitative real time RT-PCR. The expression level of SsCRT in brain was set as 1. Values are shown as means  $\pm$  SEM (N = 3). N, the number of times the experiment was performed.

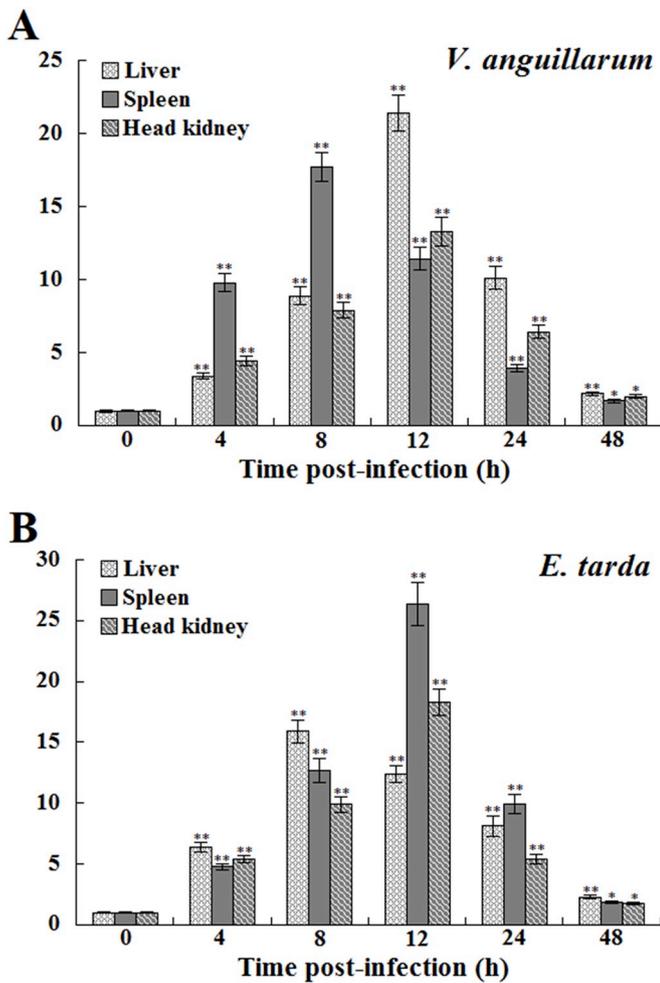
over 81.4%–91.2% sequence identify with teleost CRT and 73.9% sequence identify with human CRT, and contains a signal peptide and an ER-retrieval sequence KDEL that is responsible for retention in the ER. Three distinct structural and functional domains (N-, P- and C-domains) were observed in SsCRT including two CRT signature motifs. A phylogenetic analysis indicated that SsCRT formed a separate cluster and was clustered most closely with *B. japonicum*. The high sequence identity, together with the phylogenetic analysis and the structural features, indicated that SsCRT is a new member of the vertebrate CRT subfamily.

Besides its main location in ER [36], CRT has been found to reside in the cell surface [37], the plasmodesmata [38], and the nuclear envelope [39], which indicate that CRT is essential for normal cell function. In rainbow trout, the CRT expression was distributed in all the examined tissues with highest expression in liver [25]. In tongue sole, the CRT expression was widely distributed with higher expression in immune

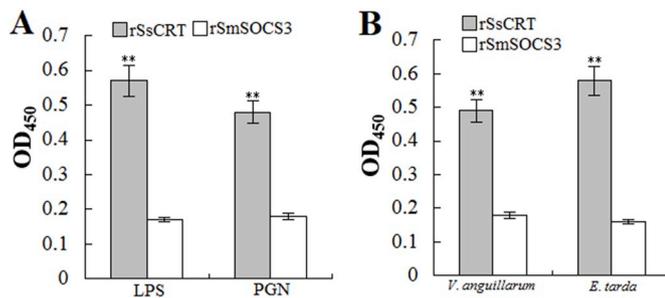
organs including liver, spleen, and head kidney [22]. Similarly, in this study, we found that SsCRT expression was ubiquitously distributed in all examined tissues with a higher expression in spleen, muscle, and liver, which maybe play vital roles in the immune system of teleost. Furthermore, the expression of SsCRT in head kidney, spleen, and liver was significantly induced by fish extracellular pathogen *V. anguillarum* and intracellular *E. tarda*, which is similar to the results of CRT in in other aquatic animals. In tongue sole, the expression of two CRT was significantly induced by infection of *V. anguillarum* [22]. In channel catfish, CRT expression was enhanced by pathogen *E. ictaluri* [21], a specie that belongs to the same genus with *E. tarda*. These results suggest that SsCRT is probably involved in host immune response against microbial pathogens.

The immune-related regulatory functions of CRT have received extensive attention in mammalia. In human, pre-apoptotic exposure of calreticulin has been linked to enhanced immunogenicity of dying tumor cells, which is suggested to relate to the immunogenicity of extracellular calreticulin [40]. In mice, peritoneal macrophages and splenic dendritic cells are shown to mediate calreticulin-dependent uptake of dying cells [18,41]. As a potential receptor for an altered conformation of C1q, CRT may extend from the ER to the topologically equivalent cell surface, where it contributed to the elimination of immune complexes and apoptotic cells [42].

In contrast to mammalian, the studies about immunoregulatory function of CRT proteins in teleost fish are very limited. The recombinant protein rCsCRT in the half-smooth tongue sole *Cynoglossus semilaevis* could bound to different pathogen-associated molecular patterns (PAMPs) and to different bacteria. Moreover, rCsCRT significantly enhanced the killing of *V. anguillarum* by tongue sole macrophages [22]. The recombinant rBjCRT from the amphioxus *Branichostoma japonicum* was able to bind the Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Staphylococcus aureus*. Meanwhile, both rBjCRT as well as human recombinant calreticulin were able to promote the phagocytosis of *E. coli* and *S. aureus* by sea bass macrophages [19]. Similar results have been observed in this study. *In vitro*, rSsCRT can also bind different PAMPs and bacteria. The findings suggested that SsCRT could participate in the resistance to pathogenic microbes. *In vivo*, rSsCRT augmented the expression of multiple immune related genes, including IL-1 $\beta$ , IL-8, TNF13B, SAA and

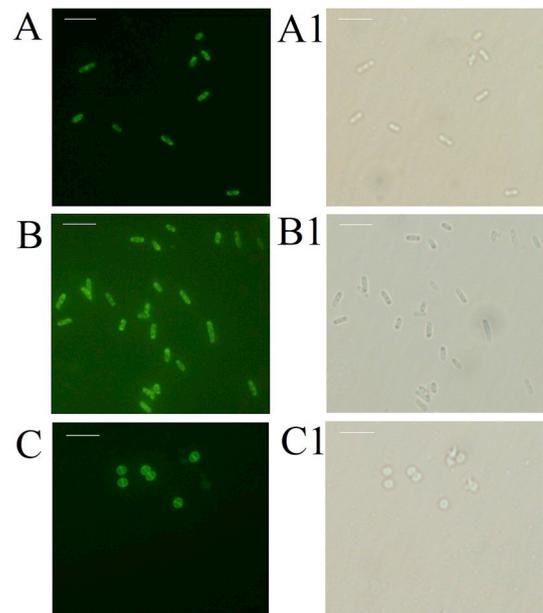


**Fig. 5.** SsCRT expression in response to pathogens challenge. Black rockfish were infected with *Vibrio anguillarum* (A) or *Edwardsiella tarda* (B), and SsCRT expressions in liver, spleen, and head kidney were determined by quantitative real time RT-PCR at 4 h, 8 h, 12 h, 24 h, and 48 h post infection. In each case, the expression level of the control fish was set as 1. Values are shown as means  $\pm$  SEM (N = 3). N, the number of times the experiment was performed. \*\* $P < 0.01$ , \*  $P < 0.05$ .



**Fig. 6.** Binding of rSsCRT to PAMP and bacteria. LPS, PGN (A), or bacteria (B) was coated in 96-well plate and then incubated with PBS (control), rSsCRT, or rSmSOCS3. The binding of rSsCRT was determined by ELISA. Values are shown as means  $\pm$  SEM (N = 3). N, the number of times the experiment was performed. \*\* $P < 0.01$ .

HSP70, these genes play vital roles in innate immune system and participate in B-cell activation, regulating host immune defense against pathogen infections, mediating inflammatory reaction, thermal and oxidative tolerance, protecting healthy cells from being damaged, the signaling processes and elimination of invading pathogens, respectively [42–48]. These findings suggested that SsCRT played an important role



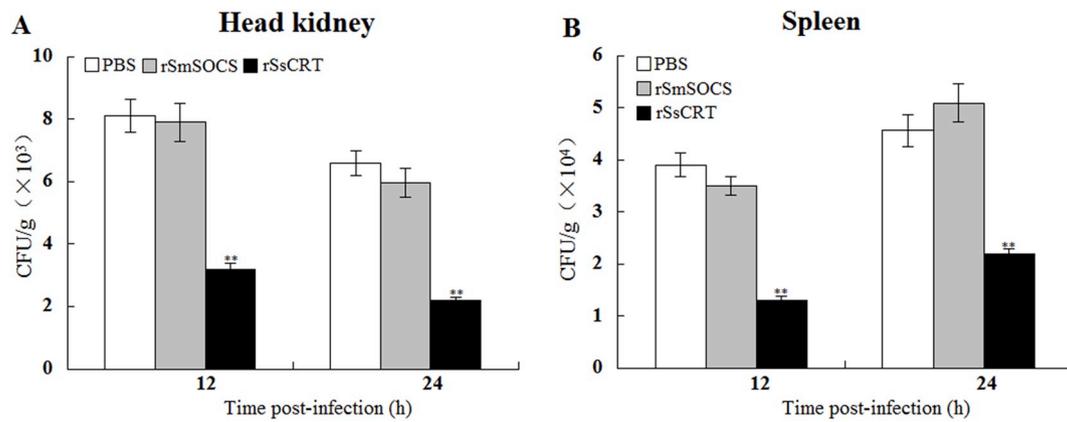
**Fig. 7.** Binding of FITC-labeled rSsCRT to bacteria. A: *V. anguillarum*, B: *E. coli*, C: *S. aureus*. A-C were FITC passage; A1-C1 were Bright Field. The results were observed under a LEICA DM2500 imager fluorescence microscope. Scale bar = 25  $\mu$ m.

in regulating the innate immune system. Moreover, the presence of rSsCRT significantly inhibited *V. anguillarum* infection. As mention above, CRT homologue was also found to localize on cell surface. It may remain associated with chaperone proteins such as MHC class 1, alternatively, its KDEL retrieval sequence may be cleaved [49,50]. rSsCRT on cell surface may directly interact with extracellular pathogens, or induce some immune related factors, which emerge the efficacy against extracellular pathogens. These results suggested SsCRT possessed immunological activities and played an important role in antibacterial immunity of black rockfish. It should be noted that this study has examined only immune-related activities of SsCRT *in vitro* and *in vivo*. However, the immune mechanism of CRT is still limited in teleost, further studies should be carried out to better characterize its detailed roles in teleost innate immunity.

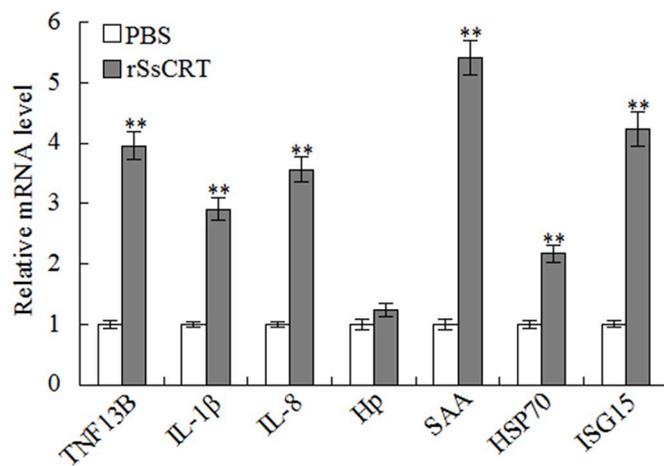
In summary, we demonstrated the sequence characteristics, tissue distribution, expression pattern after bacterial stimulation, and immune-related activities of SsCRT *in vitro* and *in vivo*. SsCRT was distributed ubiquitously in all the detected tissues and was highly expressed in the spleen, muscle and liver. The mRNA transcript of SsCRT was significantly up-regulated after bacterial stimulation. We also found that rSsCRT could bind to different bacteria and PAMPs *in vitro*. After rSsCRT-treated fish infected with *V. anguillarum*, the pathogen loads in fish tissues were significantly reduced and the mRNA expression of immune genes were remarkably upregulated. These results indicated that SsCRT could serve as an immune receptor to recognize and eliminate the invading pathogens. The study adds new insights into the immunoregulatory function of teleost CRTs.

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**Fig. 8.** Effect of rSsCRT on pathogen infection *in vivo*. Black rockfish were administrated with rSsCRT or PBS (control) before infected with *Vibrio anguillarum*. Bacterial loads in the head kidney (A) and spleen (B) of the fish were determined at 12 h and 24 h post-infection. Values are shown as means  $\pm$  SEM (N = 3). N, the number of times the experiment was performed. \*\* $P < 0.01$ .



**Fig. 9.** Effect of rSsCRT on expression of immune genes. Black rockfish were administrated with rSsCRT or PBS (control). Then total RNA was extracted from the head kidney tissue and qRT-PCR was used to analyze the expression of tissue necrosis factor (TNF) 13B, interleukin (IL) -1 $\beta$ , IL-8, haptoglobin (Hp), serum amyloid A (SAA), heat shock protein 70 (HSP70), and ISG15. Values are shown as means  $\pm$  SEM (N = 3). N, the number of times the experiment was performed. \*\* $P < 0.01$ .

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2019.08.043>.

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