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RESEARCH REPORT

# Molecular cloning and functional characterization of a calreticulin gene from the sea cucumber *Apostichopus japonicus*

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## Abstract

Calreticulin (CRT) plays crucial roles in the regulation of $Ca^{2+}$ homeostasis, immune responses and molecular chaperon. In this study, a new member of the *CRT* gene (denoted as *AjCRT*) was cloned and characterized from the sea cucumber *Apostichopus japonicus*. The full-length of the gene was of 3316 bp, which consisted of 204 bp 5' untranslated region (UTR), 1870 bp of 3'-UTR and a putative 1242-bp open reading frame encoding a 48-kDa polypeptide (*AjCRT*) with a theoretical pI of 4.19. *AjCRT* had 59 % - 65 % sequence identity with CRTs from other species. It contained two CRT domains (residues 94 - 109 and 126 - 134) with a RING finger domain. *AjCRT* was ubiquitously expressed in all tissues examined, but was highly expressed in the coelomocytes. Temporal transcriptional levels in the coelomocytes revealed significant upregulation of *AjCRT* after the animal was challenged with *Vibrio splendidus*, reaching 4.97-fold the level of control at 4 h, but then decreased to 2.56-fold the level of control at 72 h. *AjCRT* knockdown decreased the expression level of the binding of $Ca^{2+}$ to protein gene about 50 %. At the same time, intracellular concentration of $Ca^{2+}$ also increased by 1.86-fold and 1.94-fold compared to that of the control. Taken together, the results suggested that *AjCRT* may be associated with the immune response against bacterial infection, probably through participating in the regulation of intracellular $Ca^{2+}$ homeostasis in sea cucumber.

**Key Words**: calreticulin; *Apostichopus japonicus*; spatial expression; time-course expression; AjCRT knockdown; $Ca^{2+}$ concentration

## Introduction

*Apostichopus japonicus* is a sea cucumber that belongs to the phylum Echinodermata. It is widely distributed in China, Japan, Korea and Russia, and is one of the most important aquaculture animals in North China (Chang *et al*., 2004). However, increasing demand for sea cucumber and over-farming have resulted in the frequent occurrence of epidemic diseases in both juvenile and adult sea cucumber during the last decade, especially skin ulceration syndrome (SUS), which has caused massive mortality for cultured sea cucumbers (Deng *et al*., 2009; Wang *et al*., 2009; Ma *et al*., 2013), resulting in serious economic losses and limiting the sustainable development of this industry. To control epidemic diseases in sea cucumber, antibiotics and chemicals are frequently used in aquaculture. However**,** indiscriminate use of these drugs has resulted in drug residues in the animals and environment, which is considered as a form of water pollution (Bonnie and Stuart, 2011; Yang *et al*., 2015), further limiting the healthy and sustainable development of sea cucumber industry.

As a marine invertebrate which lacks adaptive immune system, *A. japonicus* completely relies on innate immunity to combat pathogen infection (Iwanaga and Lee, 2005). In recent years, several immune-related genes such have been found to play crucial roles in the defense against bacterial, fungal and viral pathogens. Some of these genes (Wang *et al*., 2011, 2015a, b; Lu *et al*., 2013; Sun *et al*., 2013; Ji *et al*., 2014; Jiang *et al*., 2014; Zhang *et al*., 2015; Shao *et al*., 2015a, b, 2016; Yang *et al*., 2015, 2016; Lv *et al*., 2016) are listed in this study. Therefore, identifying and characterizing more immune-related genes in *A. japonicus* will enable us to better understand the immune responses of sea cucumber and find a new way to ensure the development of a more robust and healthier aquaculture industry.

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**Table 1** Primer sequences used in the cloning of the *AjCRT* gene

| Primers | Sequences (5'-3') | Application | Melting temperatures |
|---|---|---|---|
| *AjCRT*-5'-out | CTTCTGCTCGTGCTTGACTGTA | 5'-RACE | 56 °C |
| *AjCRT*-5'-in | GGTGGTAACACGCACTCCCTGG | 5'-RACE | 56 °C |
| *AjCRT*-3'-out | TAGCAGGTCCACAGTACATGCCCA | 3'-RACE | 56 °C |
| *AjCRT*-3'-in | ATCGCTTTGTTTACATAAGGAATA | 3'-RACE | 56 °C |
| UMP-1 | TAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT | RACE | 56 °C |
| UMP-2 | CTAATACGACTCACTATAGGGC | RACE | 56 °C |
| *AjCRT*-F | TCCTCCACTCTGACAACACCTACG | qPCR | 60 °C |
| *AjCRT*-R | AGTCCTCTGGTTTCTTGGCTTCGG | qPCR | 60 °C |
| Cytb-F | TGAGCCGCAACAGTAATC | Reference gene | 60 °C |
| Cytb-R | AAGGGAAAAGGAAGTGAAAG | Reference gene | 60 °C |
| *AjCRT* siRNA | GCAAGUGGGUUCAUCCUAUTT AUAGGAUGAACCCACUUGCTT | *AjCRT* silence | |
| Negative control (NC) siRNA | UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT | NC for *AjCRT* siRNA interference | |

Calreticulin (CRT) was first isolated from the sarcoplasmic reticulum (SR) of rabbit by Ostwald and MacLennan in 1974. It is a unique endoplasmic reticulum (ER) luminal resident protein, and it plays a crucial role in the regulation of $Ca^{2+}$ homeostasis, immune responses and molecular chaperoning (Thomas and David, 1974; Michalak *et al*., 1999). Structurally, CRT contains three functionally distinct domains: N-domain, P-domain and C-domain. The N-domain forms a stable core which is resistant to proteolysis in the presence of calcium. The P-domain contains a high-affinity calcium-binding site and participates in either substrate binding or protein-protein interactions. The C-domain has a large number of negatively charged residues (Ellgaard *et al*., 2001; Corbett *et al*., 2009; Michalak *et al*., 2009) CRT is also a long-existing and highly conserved protein that has a variety of functions and is found in a wide range of species (Wang *et al*., 2012). Initially known as a high capacity calcium-binding protein in ER that participates in the folding of newly synthesized SR proteins, CRT is now known to associate with many intracellular and extracellular processes, including lectin-like chaperone activity, $C_a^{2+}$ storage and signaling, wound healing, inhibition of tumor growth and C1q-dependent complement activation, as well as in the regulation of gene expression, cell adhesion, cancer and autoimmunity (Johnson *et al*., 2001; Gelebart *et al*., 2005; Michalak *et al*., 2009; Ayoola and Miodrag, 2010).

Calreticulin is also a major calcium-binding/storage chaperone residing in the ER lumen, where it plays important roles in molecular chaperoning function and in the response to viral infection (Michalak *et al*., 2002). Intracellular $Ca^{2+}$ is associated with various biological processes, such as apoptosis and phagocytosis (May *et al*., 2001). In addition, CRT and the binding of $Ca^{2+}$ protein gene are likely to be critical for host-pathogen interaction (Zhang *et al*., 2014). In human, CRT plays vital roles in T-cell development and in the initiation of immune cell response (Zhang *et al*., 1998; Bryce *et al*., 2007). In marine organisms, CRT has been identified in *Dicentrachus labrax* (Rute *et al*., 2007), *Exopalaemon carinicauda* (Duan *et al*., 2014), *Fenneropenaeus chinensis* (Luana *et al*., 2007) and *Trypanosoma carassii* (Zhao *et al*., 2011), where it can be induced by bacterial or viral challenge. However, no study on the *CRT* gene in *A. japonicus* has been reported. Furthermore, the connection between CRT and the binding of $Ca^{2+}$ to protein is still poorly understood (according to our knowledge). Therefore, by studying the CRT gene of *A. japonicas*, we could gain more insight into the role of this gene in sea cucumber and at the same time, obtain further information on its regulation of intracellular $Ca^{2+}$ homeostasis.

## Materials and Methods

*Animals preparation and samples collection*

Healthy sea cucumber (body weight 68 ± 4.59) were collected from Dalian Heshengfeng Marine Product Farm and maintained at 16 - 17 °C in our laboratory for one week. The intestine, respiratory tree, tube feet, coelomic fluid, body wall and longitudinal muscle were then carefully removed from three healthy animals. To harvest the coelomocytes, the collected coelomic fluid was immediately centrifuged at 1,000*g*/4 °C for 5 min. All of the other tissues were immediately snap-frozen in liquid nitrogen and stored at -80 °C.

*Bacterial challenge experiment*

*Vibrio splendidus* D450 was cultured and harvested as previously described by Yang *et al.* (2015), and the cell pellet was resuspended in

**Table 2** Primer sequences used for the analysis of the binding of $Ca^{2+}$ to protein gene

| Primers | Sequences (5'-3') | Application | Melting temperatures |
|---|---|---|---|
| $C_a^{2+}$-F | TCCTCCACTCTGACAACACCTACG | qPCR | 60 °C |
| $C_a^{2+}$-R | AGTCCTCTGGTTTCTTGGCTTCGG | qPCR | 60 °C |

phosphate buffered saline (PBS, 0.1 mM, PH 7.4) to a final concentration of $10^7$ CFU/mL. Sea cucumbers were divided into two groups: control group and a bacterial-challenged group. The control group (a total of 25 individuals) was immersed in a tank containing PBS only whereas the bacterial-challenged group (also 25 individuals) was immersed in a tank containing PBS plus *V. splendidus* D4501 at a concentration of $10^7$ CFU/mL. The celomic fluids were collected from the sea cucumbers at 0, 4, 8, 12, 24, 48 and 72 h post-immersion, and three individuals were randomly selected at each time point.

*Total RNA extraction and cDNA synthesis*

Total RNA was isolated from each of the six different tissues using Trizol (Ambion) according to the manufacturer's instructions. Then, the quantity and quality of extracted RNA were assessed by UV spectrophotometry (Nanophotometer, Munich, Germany) and agarose gel electrophoresis. The cDNA was synthesized using a PrimerScript™ RT reagent Kit (TaKaRa, Japan) for real-time PCR (RT-PCR), diluted to 1:10 and stored at -20 °C until used.

*Cloning and sequencing of AjCRT*

A partial cDNA sequence of *AjCRT* was obtained from our transcriptome assembly data (unpublished data). Based on the partial sequence, 5' and 3'-rapid amplification of cDNA ends (RACE) was conducted using the SMARTer®RACE 5'/3' Kit (TaKaRa, Japan). All primers used are listed in Table 1. PCR product from RACE was detected in 1.2 % agarose gel and purified using EasyPure Quick Gel Extraction Kit (Transgen Biotech, China). The purified product was then ligated to the PEASY®-1 Cloning Vector (Transgen Biotech, China). Trans1-$T_1$ Phage Resistant Chemically Competent Cells (Transgen Biotech, China) were immediately transformed with the resulting construct. Positive transformants were verified by colony PCR using M13 primers (Tran, China), and three independent clones were subjected to DNA sequencing to confirm the sequence of the insert.

*Bioinformatics analysis of AjCRT*

The full-length of the *AjCRT* gene was analyzed using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast) program. Open reading frame (ORF) was identified by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi). The deduced amino acid sequence was analyzed online using the Expert Protein Analysis System (http://www.expasy.org/), and the molecular weight of the predicted polypeptide was calculated using the Expasy compute PI/MW tool (http://www.expasy.org/). SignaIP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide sequence of *AjCRT* whereas Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de/) was used to analyze its domain structure. Phylogenetic tree was conducted by the Neighbor-joining (NJ) method using MEGA 5.2 program. Multiple sequence alignment was performed by the DNAman.

*Quantification analysis of AjCRT expression*

Spatial and temporal expression levels of *AjCRT* were analyzed by *q*RT-PCR using the Applied Biosystem 7500 Real-time system (Applied Biosystem, USA). The sample contained 2 μL of 1:10 diluted original cDNA, 10 μL of 2 × SYBR Green Master mix (SYBR PrimeScript™ RT-PCR kit Ⅱ, TaKaRa, Japan), 0.4 μL of ROX Reference Dye Ⅱ, 0.8 μL (10μM) of each primer and 6 μL $ddH_2O$ in a total volume of 20 μL. The condition of the amplification consisted of the followings: a holding step at 95 ℃ for 30 s, followed by 40 cycles of 95 ℃ for 5s, and 60 ℃ for 32 s. The specific amplification of *AjCRT* was confirmed by melting curve. The gene for cytochrome b was selected as the reference gene (Yang *et al*., 2010). The sequences of all the primers used are shown in Table 1. The relative mRNA levels of *AjCRT* were quantified using the $2^{-\Delta\Delta Ct}$ method and the results were given as relative expression patterns (means ± S.D., n = 3), and the differences were obtained by one-way Analysis of Variance (ANOVA).

*Primary coelomocytes culture and AjCRT silencing in vitro*

Primary celomocytes were cultured as described by Yang *et al.* (2016) and Lu *et al.* (2015). In brief, the washed cells were re-suspended in Leibovitz's L-15 cell culture medium (Invitrogen, USA) containing penicillin (100 U/mL), streptomycin sulfate (100 μg/mL) and Gentamycin sulfate (100 μg/mL). The final concentration of the cells was $10^6$ cells/mL. The osmotic pressure of the cell suspension was adjusted with 0.39 M NaCl, and 500 μL aliquots were then dispensed into a 24-well plate and incubated at 17 ℃ for 12 h before the *AjCRT* knockdown experiment. The *AjCRT* targeting small interfering RNA (*AjCRT* siRNA) and a negative control siRNA (NC siRNA) were designed and

synthesized by GenePharma (shanghai, China). The sequences of these siRNAs are shown in Table 1. For *AjCRT* knockdown, 3 μL of *AjCRT* siRNA (20 μM) or NC siRNA was mixed with an equal volume of Lipotap Liposomal Transfection Reagent (Beyotime Biotechnology, China) and 500 μL of the cultured coelomcytes prepared earlier was transfected with this siRNA sample. Twenty-four hours after the transfection, the cells were harvested and used in subsequent experiments.

*Analysis of the binding $Ca^{2+}$ to protein gene after AjCRT knockdown*

Total RNA harvested from the celomocytes following the *AjCRT* knockdown was used to synthesize the cDNA. Then, the expression levels of *AjCRT* and the binding $Ca^{2+}$ to protein gene were conducted as described above. The primers of the binding $Ca^{2+}$ to protein are listed in Table 2.

*Measurement of intracellular $Ca^{2+}$ level*

Intracellular $Ca^{2+}$ concentration ($[C_a{}^{2+}]_i$) was measured according to Ding *et al.* (2008) and Girard *et al.* (2002) with little modification. Briefly, 500 μL coelomocytes at the final concentration of $10^6$ cells/mL was mixed with 1.3 μL of 2 μM Fura-2/AM and 0.8 μL 0.05 % (w/v) of pluronic-F127, and then incubated in 37 ℃ for 30 min. Following incubation, the coelomocytes were collected by centrifugation at 1000*g* and 4 °C for 5 min, and then washed thrice with Hanks' Balanced Salt Solution (HBSS) without calcium chloride (Sangon, China). The washed cells were re-suspended in 250 μL HBSS. $[C_a{}^{2+}]_i$ was automatically recorded with a Hitachi F-4500 fluorimeter at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm.

*Statistic analysis*

Data were represented as mean ± standard error (SE). Difference in relative expression of *AjCRT* and the binding of $Ca^{2+}$ protein was analyzed by one way ANOVA or independent-T-test on SPSS 13.0. The asterisk (*) and double asterisks (**) above the bars represent difference at $p < 0.05$ and significant difference at $p < 0.01$, respectively.

## Results

*Sequence analysis of AjCRT*

The full-length cDNA of *AjCRT* (GenBank accession No. MF960913) was obtained by 5'and 3'-RACE according to an EST obtained from an *A. japonicus* cDNA library. The gene was 3316 bp long, containing 1242-bp of open reading frame, 204-bp of 5'-untranslated region (UTR) of and 1870-bp of 3'-UTR, which included a putative polyadenylation consensus signal (AATAAA) and a poly A tail (Fig. 1) The open reading frame was found to encode a polypeptide of 480 amino acids with a predicted molecular mass and a theoretical pI of 48 kDa and 4.19, respectively. Domains and motifs analysis revealed two conserved domains reminiscent of the CRT family proteins: $Lys^{94}$-$Phe^{109}$ and $Lle^{126}$-$Gly^{134}$ NG finger domain (residues 119 - 160). Three CRT family repeat motifs (DWD), one putative ER targeting motif HDEL as well as a coiled coil (residues 346 - 383) were also found in *AjCRT*. CRT was also found to contain a putative signal peptide of 16 amino acids (MKFLVALAILCYTASA). Blastp analysis revealed 69 - 75 % sequence identity between *AjCRT* and CRTs from other species, including *Strongylocentrotus purpuratus* (XP 006824896), *Haliotis discus discus* (ALY 11013), *Danio rerio* (NP 956007) and *Sus scrofa* (NP 001167604).

*Multiple alignment and phylogenetic analysis*

The deduced amino acid sequence of *AjCRT* and the corresponding CRT sequences of invertebrates and vertebrates were aligned using the DNAman program (Fig. 2). *AjCRT* was found to have significant percentages of sequence identity (> 50 %) with the CRT sequences from other species, *e.g.*, 59 % with *Homo sapiens* CRT and 65 % with *S. purpuratus* CRT (Fig. 3). Moreover, phylogenetic analysis showed that *AjCRT* was clustered into the invertebrate subgroup with closer evolution relationship with *S. purpuratus*. Figure 3 depicts the phylogenetic analysis of CRTs, which was constructed by the Neighbor-joining method. The relationships among the 10 CRTs shown by the phylogenetic tree were consistent with traditional taxonomy.

*Tissue distribution and mRNA expression pattern of AjCRT in coelomocytes after V. splendidus challenge*

*AjCRT* transcript was detected in all the different tissues examined, suggesting that it was ubiquitously expressed in *A. japonicus* (Fig. 4), but the expression varied, depending on the tissue. For comparison purpose, the transcript level of *AjCRT* in the intestine was taken as 1 and the transcript levels in the other tissues were compared against that of the intestine. Such comparison yielded the highest *AjCRT* transcript level in the cleomocytes (3.2 fold the level found in the intestine), followed by tube feet (1.24-fold), respiratory tree (1.05-fold), body wall (0.92-fold), and lastly, the longitudinal muscle (0.3-fold). The different levels of *AjCRT* expression in the different tissues suggested that *AjCRT* may be involved in important physiological functions.

A significant ($p < 0.01$) increase in the expression level of *AjCRT* was found in the coelomocytes of *A. japonicas* following *V. splendidus* challenge. The expression of *AjCRT was* 4.97-fold and 2.56-fold the expression of non-infected *A. japonicas* 4 h and 72 h, respectively, after the bacterial challenge (Fig. 5).

*AjCRT knockdown affects the expression of the binding of $Ca^{2+}$ to protein gene $[C_a{}^{2+}]_i$*

Knockdown of *AjCRT* by siRNA in the primary celomocytes was found to affect the expression of the binding to $Ca^{2+}$ protein gene in the cells. The level of *AjCRT* transcript in the cells transfected with the *AjCRT*-specific siRNA was 0.46 fold and 0.44 fold the levels *AjCRT* in the cells transfected with a negative control siRNA or in the non-transfected cells (Fig. 6A). At the same time, the transcript level of a binding of $Ca^{2+}$ to protein gene in the *AjCRT*-knocked down celomocytes was also reduced to about 0.52-fold the level found in the negative control cells (Fig. 6B).

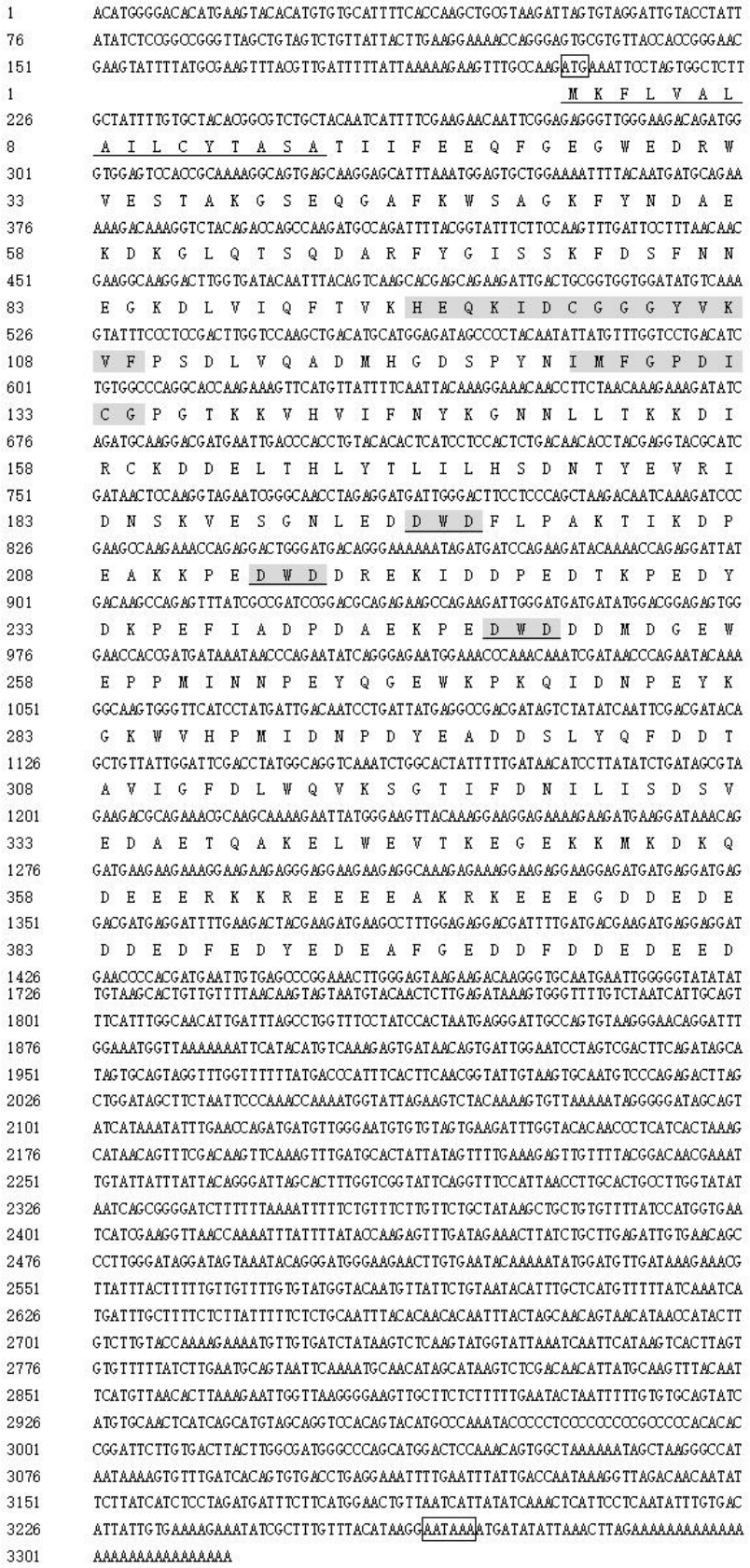

**Fig. 1** Nucleotide and deduced amino acid sequences of the *CRT* gene cloned from *A. japonicus*. The start codon (ATG) and the putative polyadenylation consensus signal (AATAAA) are boxed. The asterisk represents the stop codon. The signal peptide is underlined. The two CRT family signature motifs (KHEQKIDCGGGYVKVF and IMFGPDICG) are shaded. The three CRT family repeat motifs (DWD) and the putative ER targeting motif HDEL are underlined and shaded.

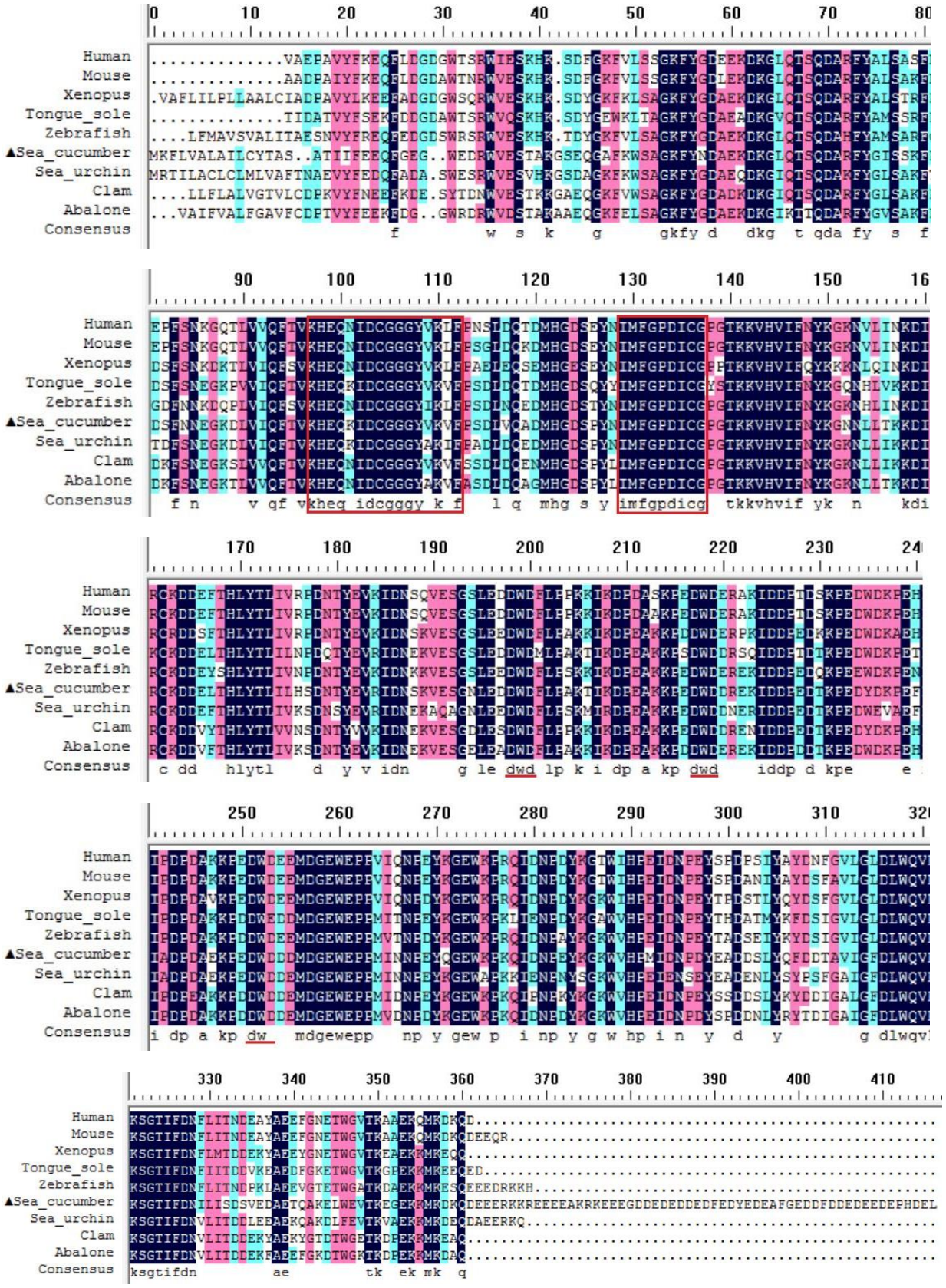


**Fig. 2** Multiple alignment of the deduced amino acid sequence of *AjCRT* with CRTs from other organisms. *H. discus* (ALY11013), *L. gigantea* (XP_009056939), *S. purpuratus* (NP_999643), *D. rerio* (AJG06014), *C. semilaevis* (XP_008336669), *X. tropicalis* (NP_001001253), *M. musculus* (NP_031617) and *H. sapiens* (NP_004334). The two CRT family signature motifs are indicated by two boxes. The three CRT family repeat motifs (DWD/E) are underlined.

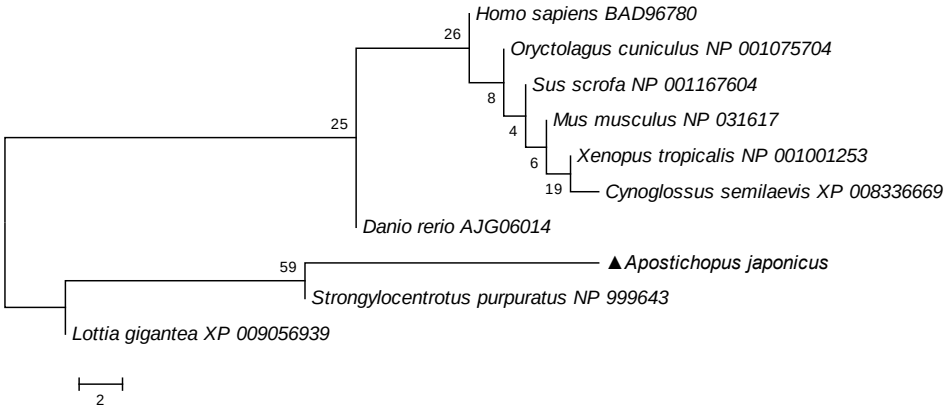


**Fig. 3** Consensus neighbor-joining tree constructed from the amino acid sequences of CRTs from other species. The phylogenetic tree was constructed according to the Neighbor-joining method using MEGA 5.2 software. The numbers at the forks indicate the bootstrap.

Intracellular $Ca^{2+}$ concentration ($[C_a^{2+}]_i$) in the primary celomocytes increased following the knockdown of *AjCRT*. A $[C_a^{2+}]_i$ of 61.83 ± 2.79 nM was obtained for cells transfected with the control siRNA, 59.51 ± 2.65 nM for non-transfected cells, and 115.2 ± 2.25 nM for cells transfected with *AjCRT*-specific siRNA. Thus knockdown of *AjCRT* in primary coelomocytes increased the level of intracellular $Ca^{2+}$ by almost 2 fold compared to cells without *AjCRT* knockdown (Fig. 6C).

## Discussion

Sea cucumbers lack an adaptive immune system and therefore they rely completely on an innate system for protection against potential pathogens. The vital roles involved in immune responses from the calcium-related proteins were identified in *A. japonicus*, such as calumenin, annexin, calreticulin and phospholipase C-gamma (Andrew *et al*., 2007; Zhang *et al*., 2014). $Ca^{2+}$ acts

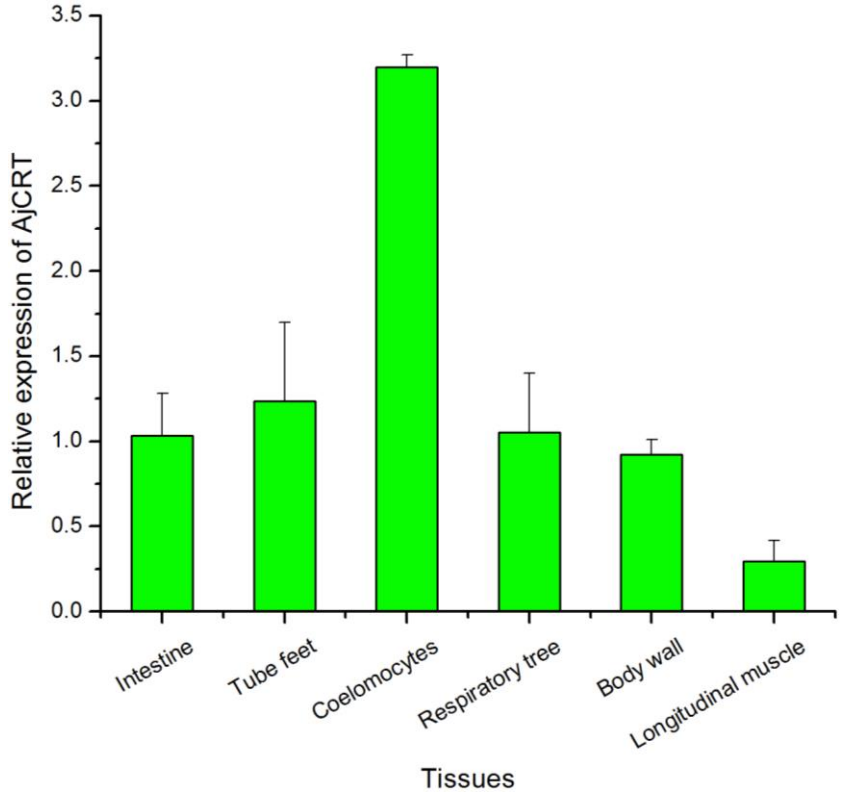


**Fig. 4** Relative expression of *AjCRT* in different tissues. Each vertical bar represents the mean ± SD (n = 3).

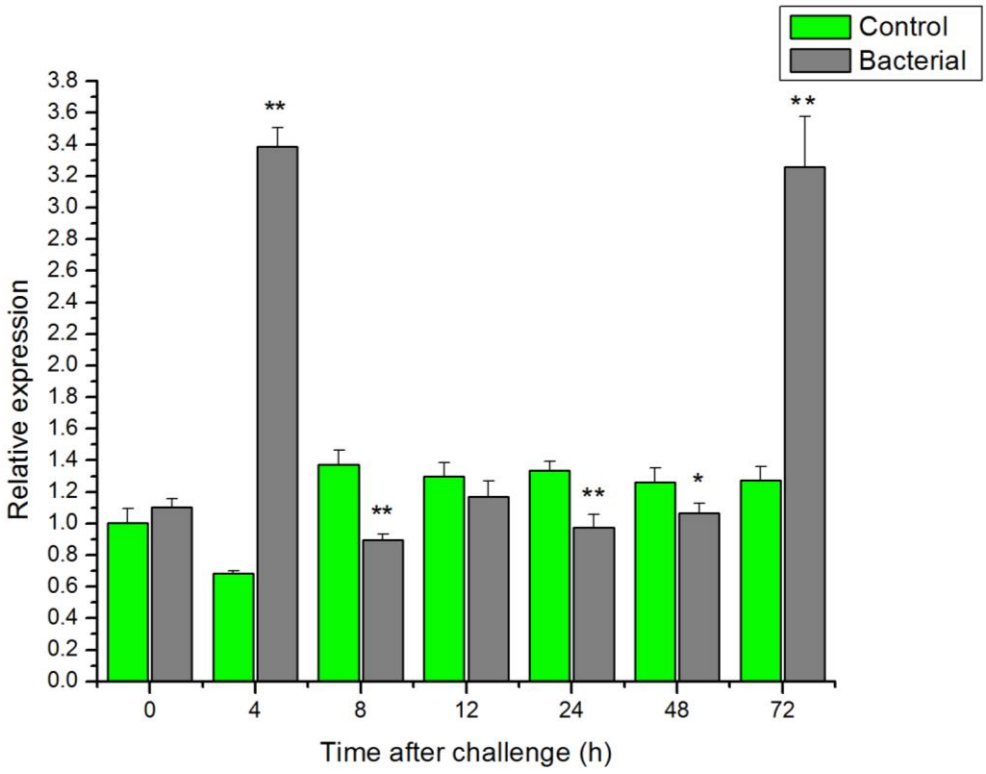


**Fig. 5** Temporal expression of *AjCRT* in the coelomocytes after infection of the *A. japonicus* by *V. splendidus*. Each vertical bar represents the mean ± SD (n = 3). Significant difference between the challenge group and the control group are indicated by asterisk (* represented $p < 0.05$, ** represented $p < 0.01$).

as a diffusible second messenger and plays a crucial role in the metabolism and physiology of eukaryotes. In addition, the maintenance of intracellular $Ca^{2+}$ homeostasis is essential in the metabolic processes of and physiology of eukaryote. Intracellular $Ca^{2+}$ concentration can be affected by various external signals, such as drugs, stress, light and pathogens (Carafoli *et al*., 1999; Chamilani *et al*., 2010). Therefore, the modulation of calcium-related proteins is critical for intracellular $Ca^{2+}$ homeostasis. Calreticulin (CRT) is a highly conserved protein that modulates calcium binding to proteins and storage (Wang *et al*., 2012; Zhang *et al*., 2014). In order to better understand the role of CRT in the processes of immune responses and the maintenance of intracellular $Ca^{2+}$ homeostasis, we identified and characterized a CRT gene from *A. japonicus.* As a result, a total of 3316 bp nucleotide sequences represented the complete cDNA sequence of *AjCRT*. Typical domains of CRT proteins were found in the deduced *AjCRT* protein sequence, suggesting that *AjCRT* might perform similar functions as other CRTs from other animals, including invertebrates and vertebrates. Similar results of CRT domains have also been reported in plenty of species, such as *Trypanosoma carassii*, *Pieris rapae*, *Exopalaemon carinicauda*, *et al*., which further supporting the notion that *AjCRT* being an ancient and highly conserved protein (Johnson *et al*., 2001; Gelebart *et al*., 2005; Ayoola and Miodrag, 2010; Wang *et al*., 2012).

In the white prawn *E. carinicauda*, CRT could be detected in all the tissues examined, including hemocytes, gill, hepatopancreas, muscle, ovary, intestine, stomach and eyestalk, with the highest expression level in hepatopancreas, whereas the lowest was found in eyestalk (Duan *et al*., 2014). In this study, *AjCRT* was distributed in all the tested tissues, including celomocytes, tube feet, respiratory trees, intestine, body wall and longitudinal muscle. The highest expression level of *AjCRT* mRNA was detected in the celomocytes, cells that are regarded as the main cellular component of the immune system and the metabolic center for reactive oxygen species (ROS) production in sea cucumber. Similar to the hemocytes of vertebrates, celomocytes are freely circulating cells and they play an irreplaceable role in the immune responses of sea cucumber (Wang *et al*., 2007; Wang *et al*., 2009; Cheng *et al*., 2016). Thus *AjCRT* may act as an immune-related gene and its function may be to defend the animal against bacterial, fungal and viral pathogens (Duan *et al*., 2014; Zhang *et al*., 2014). In addition, the ubiquitous distributing pattern of *AjCRT* suggested that it may have a multifunction in many cellular processes, both intracellular and extracellular processes, such as calcium-binding in ER and folding of newly synthesized protein (Thomas and David, 1974; Michalak *et al*., 1999). Infection by pathogen can induce the generation of excessive

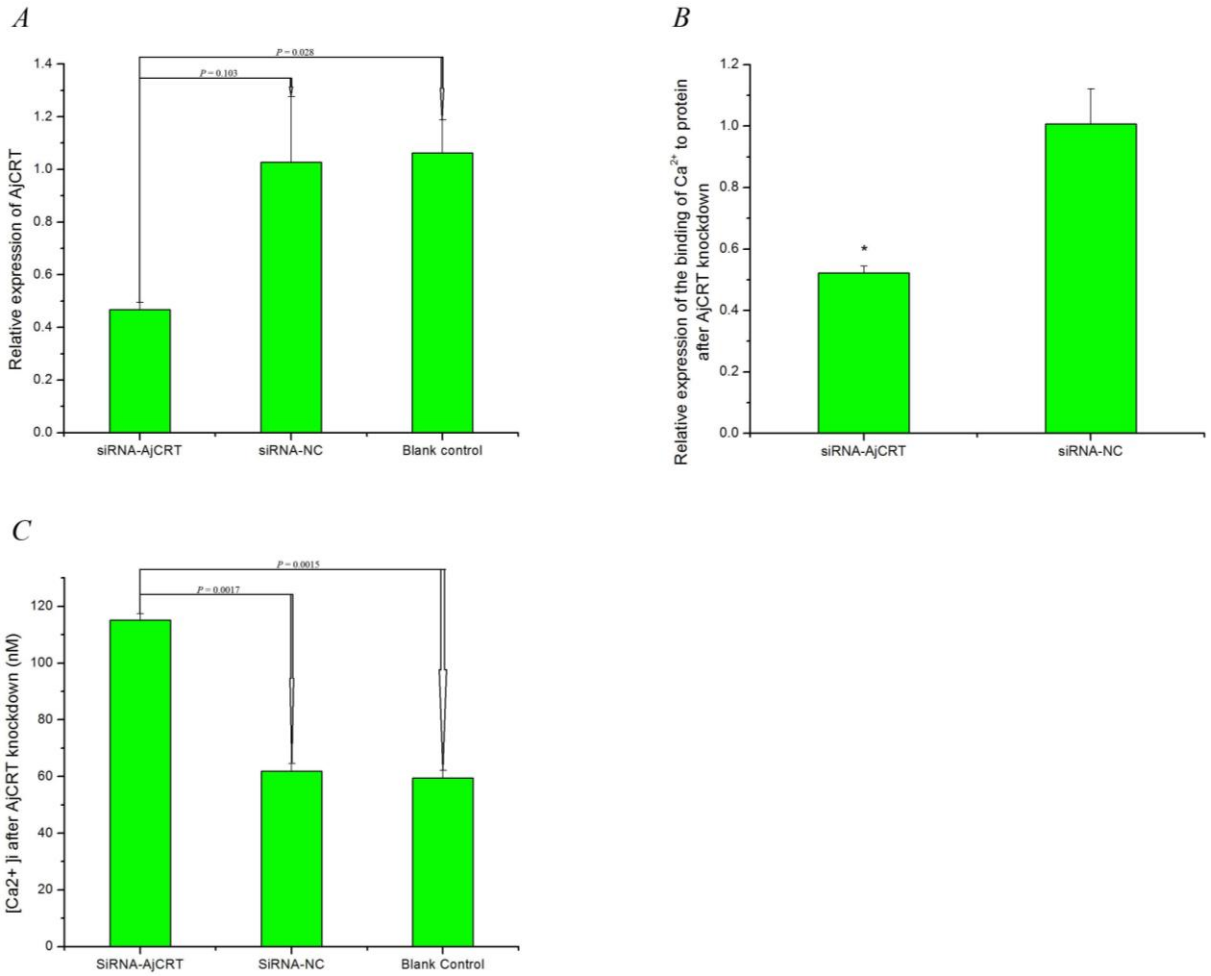


**Fig. 6** *AjCRT* knockdown in *A. japonicas* coelomocytes and its effect on intracellar $Ca^{2+}$ and $Ca^{2+}$-binding protein expression. (A) *AjCRT* knockdown. Effect of *AjCRT* knockdown on intracellular $Ca^{2+}$ concentration (B) and $Ca^{2+}$-binding protein expression (C). Data are the means ± SDs (n = 3). Significant difference between the *AjCRT* siRNA group and the negative control group is indicated by asterisk (*represented $p < 0.05$, **represented $p < 0.01$).

ROS, which could cause mass mortality in marine organisms (Siripong *et al*., 2014). *Vibrio splendidus* was regarded as the major pathogen of sea cucumbers (Zhao *et al*., 2011). To better understand the role of *AjCRT* in the immune response, the animals were challenged with *V. splendidus* D450 to simulate a state of infection. Pathogens infection can elicit variation in the concentration of intracellular $Ca^{2+}$ in eukaryotes. Maintenance of intracellular $Ca^{2+}$ homeostasis is essential to the processes of metabolism and physiological function (Chamilani *et al*., 2010). As the result, *AjCRT* expression was significantly up-regulated at 4 h after *V. splendidus* challenge, which indicated that *AjCRT* expression was quickly induced upon bacterial infection. At the same time, our data also showed that the concentration of intracellular $Ca^{2+}$ increased when *AjCRT* was knocked down, further suggesting that *AjCRT* was involved in the homeostasis of intracellular $Ca^{2+}$ directly. However, the second peak of *AjCRT* expression was observed at 72 h post bacterial challenge, and a possible explanation for the emergence of this peak could be due to the momentarily disruption of the innate immune system of *A. japonicus*, caused by the growing number of bacteria inside the animal. Similar results have been observed in *Exopalaemon carinicauda* after WSSV challenge and in the hepatopancreas *Fenneropenaeus chinensis* following WSSV infection (Duan *et al*., 2014; Luana *et al*., 2014). This further demonstrated that a role for *AjCRT* in the immune response against bacterial infection.

CRT is initially thought to be responsible for the high calcium-binding capacity in ER and the folding of newly synthesized proteins (Michalak *et al*., 1999). A growing number of studies have focused on unravelling the function of CRT since its discovery in mammals (Ostwald and MacLennan, 1974). It is now known that CRT has many functions, including lectin-like chaperone activity, $C_a^{2+}$ storage and signaling, and regulation of gene expression, cell adhesion, wound healing, cancer and autoimmunity

(Corbett *et al*., 2000; Ellgaard *et al*., 2001; Johnson *et al*., 2001; Wang *et al*., 2012). The role of CRT in immune reaction has been reported in different invertebrates (Luana *et al*., 2007; Duan *et al*., 2014). In this study, the connection between CRT and the binding of $Ca^{2+}$ to protein gene was investigated by looking at the effect of *AjCRT* knockdown on the level of intracellular $Ca^{2+}$ and the expression of a binding of $Ca^{2+}$ to protein gene. The binding of $Ca^{2+}$ to protein gene known as EF hand domain-containing calcium regulation protein plays important roles in signal transduction and calcium binding to proteins (Ikura *et al*., 1996). The modulation of the binding of $Ca^{2+}$ to protein gene is considered to be vital during thermal stress (Zhang *et al*., 2013). The level of the binding of $Ca^{2+}$ to protein gene in the celomocytes of *A. japonicus* decreased when *AjCRT* in these cells was knocked down. This basically consistent decreasing of *AjCRT* and the binding of $Ca^{2+}$ to protein gene after *AjCRT* knockdown maybe suggested that *AjCRT* and the binding of $Ca^{2+}$ to protein gene are involved in the general physiological processes of *A. japonicus*. Intracellular $Ca^{2+}$ was regarded as critical for various biological events. Particularly, increase in intracellular $Ca^{2+}$ is associated with many defense responses elicited by the host cell during an infection (Michalak *et al*., 2002; Zhang *et al*., 2013). Significant increase in intracellular $Ca^{2+}$ concentration resulting from the knockdown of *AjCRT* in *A. japonicus* celomocytes indicated that *AjCRT* was directly affected intracellular $Ca^{2+}$ homeostasis. A possible perspective of *AjCRT* involved in different biological events like intracellular $Ca^{2+}$ homeostasis.

In conclusion, we have cloned and characterized a full-length *CRT* gene from *A. japonicus* and analysis of its spatial and temporal expression pattern suggested that this gene might be involved in the immune response, perhaps by mediating the regulation of intracellular $Ca^{2+}$ homeostasis in sea cucumber.

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