Acta Oceanol. Sin., 2020, Vol. 39, No. 6, P. 58–64
https://doi.org/10.1007/s13131-020-1590-4
http://www.hyxb.org.cn
E-mail: hyxbe@263.net

Cloning and functional analysis of the calreticulin gene from the scleractinian coral *Galaxea astreata*

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Received 9 May 2019; accepted 17 June 2019

Abstract

Calreticulin (CRT) is a highly conserved Ca^{2+}-binding protein and chaperone in the endoplasmic reticulum, which mainly participates in adjusting calcium level and directing proper conformation of proteins. Here, we cloned the *crt* gene of the scleractinian coral *Galaxea astreata*, named *Gacrt*, and analysed its ability to drive bacterial agglutination. The full-length *Gacrt* cDNA consisted of 1 792 nucleotides and contained a 77 bp 5′ untranslated region (UTR), a 380 bp 3′ UTR and a 1 335 bp open reading frame (ORF) that encoded a 444 amino acid protein. The deduced peptide possessed a signal peptide domain, an endoplasmic reticulum retrieval signal sequence (KDEL), two potential calreticulin family signature motifs and a set of triplicate repeats. We also found that the recombinant GaCRT protein could promote agglutination of both the Gram-positive bacterium *Micrococcus luteus* and the Gram-negative bacterium *Escherichia coli*. These results show that the GaCRT protein can enhance bacterial agglutination, hinting that GaCRT is an immune-relevant molecule involved in host defense against bacterium.

Key words: *Galaxea astreata*, calreticulin, bacterial agglutination

Citation: Huang Yuanjia, Zhang Yanping, Peng Huipai, Yuan Jigui, Liu Li. 2020. Cloning and functional analysis of the calreticulin gene from the scleractinian coral *Galaxea astreata*. Acta Oceanologica Sinica, 39(6): 58–64, doi: 10.1007/s13131-020-1590-4

1 Introduction

Calreticulin (CRT) was first isolated from the endoplasmic reticulum (ER) of rabbit skeletal muscle (Ostwald and MacLennan, 1974), and first cloned in 1989 from mouse liver (Smith and Koch, 1989). Typical CRT has three functional domains: the N-terminal domain, the P-terminal domain and C-terminal domain (Michalak et al., 1999; Baksh and Michalak, 1991). The N-terminal domain has a signal peptide and two conserved CRT-family label sequences (KHEQNIDCGGGY and IMFGPDICG). The P-terminal domain is proline rich and has a high affinity and low capacity Ca^{2+} binding site and two groups of three repeat sequences. The C-terminal domain has a high capacity and low affinity Ca^{2+} binding site and a typical endoplasmic reticulum retention signal peptide (K/H)DEL, which enables CRT to enter and reside in the ER.

As an ER retention protein, CRT is responsible for maintaining Ca^{2+} homeostasis and functions as a molecular chaperone to help proteins fold correctly (Michalak et al., 1999). In addition, CRT is also found in other membrane structures and is involved in many physiological processes, such as transcriptional regulation and gene expression, cell adhesion and recognition, and removal of apoptotic cells (Qiu and Michalak, 2009; Gu et al., 2008; Gold et al., 2010). As an important Ca^{2+} binding protein, CRT participates in a variety of biological calcification processes, such as in the production of teeth (Kobylewski et al., 2017) and bone (Fischer et al., 2017). Studies of highly calcified organisms, including the marine shellfish *Pinctada martensi* and the freshwater shellfish *Hyriopsis cumingii*, have shown that CRT is highly expressed in the mantle, suggesting that it is important for calcification (Xia et al., 2013). Moreover, the role of CRT in immunity has attracted more and more attention. In plants, over-expression of CRT confers resistance to drought or cold weather (Jia et al., 2008). In mammals, CRT is considered to be a potential tumour-associated molecular marker (Wang et al., 2013). In fish, CRT expression is significantly upregulated after infection with *Edwardsiella*, a pathogen associated with septicaemia (Peatman et al., 2007). The amphioxus CRT protein can specifically bind the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Escherichia coli* (Liu et al., 2013). In invertebrates, CRT regulates insect immune responses by participating in early encapsulation (Zhang et al., 2006), and the transcription of CRT is upregulated in the white prawn *Exopalaemon carinicauda* (Duan et al., 2014) and the shrimp *Penaeus monodon* (Wang and Chen. 2006) after infection with white spot syndrome virus (WSSV). In one study, it was found that the expression of CRT in the reef coral *Acropora tenuis* increases in response to exposure to the antifouling agent tributyltin chloride (TBT-Cl); the same authors found that the protein works alongside the heat shock proteins in the high-temperature stress recovery process (Yuyama et al., 2012). In fact, Dunlap et al. (2013) have classified

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Foundation item: The National Marine Welfare Industry Research Project under contract No. 201105012; the Guangdong Provincial Natural Science Foundation under contract No. S2011010000269; the Guangdong Marine Fishery Science and Technology Extension Project under contract No. A201308E02; the Guangdong Ocean University Scientific Research Start-up Fund for the Doctoral Program under contract No. R17066.

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CRT as a stress response protein after analysing the proteome of *Acropora digitifera*. Here, to enrich our knowledge of this protein, we identified the *crt* gene of one of the scleractinian corals, *Galaxea astreata* (designated as *Gacrt*) and investigated its bioactivity in bacterial agglutination.

2 Materials and methods

2.1 *Identification and characterization of Gacrt cDNA*

Galaxea astreata colonies collected from the Xuwen Coral Reef National Nature Reserve (20°10′36″–20°27′00″N, 109°50′12″–109°56′24″E), Zhanjiang, Guangdong Province, China, were transferred to a flow-through aquaria system and maintained in (27±0.5)°C seawater at a salinity of 31, pH 8.1, and with a light:dark cycle of 12 h:12 h. Total RNA was extracted by the TRI Reagent (MRC Inc., USA), and the concentrations and quality were determined using the Thermo NanoDrop 2000 and 1% agarose gel. Reverse transcription of 1 μg total RNA was carried out using a PrimeScript$^{\text{TM}}$ RT reagent Kit with gDNA Eraser (Takara, Japan) according to the manufacturer’s instructions. Gene-specific primers were designed based on the *G. astreata* transcriptome data (unpublished) (Table 1). To amplify the *Gacrt* cDNA fragment, polymerase chain reaction (PCR) was performed at 94°C for 3 min, followed by 35 cycles at 95°C for 30 s, 55°C for 45 s, 72°C for 30 s, and a final extension step at 72°C for 10 min.

To clone the full-length *Gacrt* gene, the cDNA template was synthesized with the SMARTer$^{\text{TM}}$ RACE cDNA Amplification Kit (Takara, Japan), followed by nest- and touchdown-PCR for amplification of the 5' and 3' ends. The first and nested primers were designed based on the obtained partial nucleotide sequences of *Gacrt*. The universal primer mix (UPM) was a mixture of long and short primers (supplied with the SMARTer$^{\text{TM}}$ RACE cDNA Amplification Kit). The *Gacrt*-5′-outer or *Gacrt*-3′-outer primers and UPM were used in the first round of PCR, and the second round of PCR was performed using the *Gacrt*-5′-inner or *Gacrt* -3′-inner primers and the NUP (nested universal primer) for the 5′ and 3′ cDNA ends, respectively. The touchdown PCR procedure involved 3 min of initial denaturation at 94°C, 10 cycles of denaturation at 94°C for 30 s, annealing at 65°C (with 1°C decreases from 65°C to 55°C in every cycle) for 45 s, and extension at 72°C for 90 s followed by 94°C for 30 s, 55°C for 45 s, 72°C for 90 s for 30 cycles, and 72°C for 10 min.

The PCR products were purified and subcloned into the pEASY-T5 Zero cloning vector (TransGen). After transformation into the *Trans*1-T1 Phage Resistant Chemically Competent Cells (TransGen), positive clones were sequenced by Sangon Biotech (Shanghai). Full length *Gacrt* cDNA was synthesized by aligning the three overlapping fragments.

2.2 *Sequence analysis*

Coding regions were predicted using ORF Finder (https://www.ncbi. nlm.nih.gov/orffinder/). ProtParam was used to predict the molecular weight and isoelectric point (http://web. Expasy.org/protparam/). Functional domain prediction was performed at http://smart.embl- heidelberg.de/. The SignalP 4.1 server was used to predict the signal peptide. Putative phosphorylation sites and N-glycosylation sites were predicted at http://www.cbs.dtu.dk/services /NetPhos/ and http://www.cbs.dtu.dk/services/NetNGlyc/, respectively. MEGA 6.0 was used to construct a phylogenetic tree by the neighbour-joining method, with the bootstrap setting as 1 000. The protein sequences used for the alignment and to construct the phylogenetic tree were as follows: *Orbicella faveolata* (XP_020627206.1), *Exaiptasia pallida* (XP_020900968.1), *Lottia gigantea* (XP_009056939.1), *Pinctada fucata* (ABR68546.1), *Ascaris suum* (ADY44096.1), *Danio rerio* (AAH68336.1), *Mus musculus* (CAA33053.1) and *Homo sapiens* (AAA36582.1).

2.3 *Construction and induction of the Gacrt expression vector*

The *Gacrt* ORF was amplified with primers containing BamHI and HindIII restriction enzyme cutting sites (Table 1). The PCR program is the same as the touchdown-PCR mentioned above. This PCR product was digested with BamHI and HindIII and sub-cloned into the pET32a vector, which was previously cut with the same enzymes. *Escherichia coli* BL21 competent cells were transformed with the recombinant plasmid and cultured in LB liquid medium containing ampicillin. When the culture reached an OD_{600} of 0.6–0.9, IPTG was added to a final concentration of 1 mmol/L to induce expression of the recombinant protein at 37°C for 4 h. The recombinant protein was concentrated and purified by a Ni-NTA resin column and Amicon® Ultra-0.5 centrifugal filter (Merck Millipore). The protein concentration was determined by the Easy Protein Quantitative Kit (Bradford) (TransGen).

2.4 *Effect of recombinant protein on bacterial agglutination*

The Gram-positive bacterium *Micrococcus luteus* and the Gram-negative bacterium *Escherichia coli* were grown to an OD_{600} of 0.07. Moreover, 30 μL of the recombinant protein (100 μg/mL) was mixed with an equal volume of *E. coli* or *M. luteus*, the mixture was incubated at room temperature for 30 min, and then 30 μL aliquots of the microbial suspensions were sampled and applied to microscope slides, the bacterial agglutination was observed under a microscope. BSA was substituted in the control groups.

3 Results

3.1 *Sequence analysis of Gacrt*

The full-length *Gacrt* cDNA was 1 792 bp long, with a 77 bp 5′-untranslated region (UTR), 380 bp of 3' UTR, and a 1 335 bp open reading frame (ORF), which encodes 444 amino acid residues.

Table 1. Primers for *Gacrt* cloning and prokaryotic expression

Primer	Sequence (5′-3′)	Function
Gacrt-F	ATAGCAATGCGTCAGTTGT	middle fragment PCR
Gacrt-R	TAGCAGAAGACCCGAAAA	
Gacrt-5′-outer	GGTGAAGAGAAATCAGAGGAAGGGGGA	5′-RACE
Gacrt-5′-inner	TCTGCGCTTCACATAATAGATCCTGTG	
Gacrt-3′-outer	CGTAACCACCTCCACAATCAATGCTCTG	3′-RACE
Gacrt-3′-inner	CGTCGAACTTGGCCGAAAGTCCGTA	
Gacrt-ORF F	CGCGGATCCATGGAACTTTGCCGTTTAAT	double enzyme digestion primer
Gacrt-ORF R	CCCAAGCTTTTAAAGTTCATCTTTGGGTGC	

The 3' UTR had a polyadenylation signal AATAAA at nucleotide positions 1 744–1 749 and a 29 bp polyadenylation tail. A Kozak consensus sequence (PuNNATGPu) was found surrounding the initiation codon (ATG). This cDNA featuring 99% nucleotide was compared with the corresponding sequence of the published *G. astreata* transcriptome (Kenkel and Bay, 2017). We therefore concluded that *Gacrt* encoded the *G. astreata crt* gene. The gene has been uploaded to GenBank with the accession number MH128130.

According to the deduced amino acid sequence, GaCRT has the molecular formula $C_{2295}H_{3474}N_{570}O_{778}S_{11}$, a molecular weight of 51.85 kDa, and a theoretical isoelectric point of 4.43. This deduced protein can be considered hydrophilic, since the grand average of hydropathicity (GRAVY) is –1.301. Signal P 4.1 server analysis revealed that it had a signal sequence of 17 amino acid residues. Putative phosphorylation sites were predicted, showing there were 13 serine (Ser),16 threonine (Thr), and 9 tyrosine (Tyr) phosphorylation sites. However, no N-glycosylation site existed. Two conserved CRT-family sequences were found (K^{100}HEQSIDCGGGYVKIF^{115} and I^{132}MFGPDICG^{140}), and a set of triplicate repeats was also present (I^{210}KDPEAKKPEDWD^{222}, I^{227}DDPEDKKPEDWD^{239}, and I^{244}PDPDAKKPDDWD^{256}). In addition, an ER signal sequence (KDEL) is located at the C-terminal (Fig. 1).

3.2 *Multiple sequences and phylogenetic analysis*

When compared to other animal species, Clustal Omega alignment result indicated that the peptide sequence is highly conserved in all of the selected animals (Fig. 2). However, the C-terminal end of CRT from coelenterates is more than 20 amino acid residues longer than in the other animals. In addition, the ER signal sequence of the sea anemone *E. pallida* is RDEL, which is inconsistent with the typical motif KDEL or HDEL. According to the neighbour-joining method (MEGA 6.0), all of the CRT amino acid sequences clustered into 2 branches: one is invertebrate, and the other is vertebrate. *Galaxea astreata* CRT clustered with *O. faveolata* and *E. pallida* in the coelenterate clade (Fig. 3).

3.3 *Prokaryotic expression of rGaCRT*

The recombinant protein was analyzed by SDS-PAGE, and one major protein with an apparent molecular weight between 70–100 kDa was detected, consistent with the predicted molecular mass of the GaCRT protein (51.85 kDa), plus the fusion protein added during pET-32a expression (approximately 21 kDa) (Fig. 4). The concentration of the purified recombination protein was 103.02 μg/mL, as determined by the Bradford method.

3.4 *The promoting effect of rGaCRT on bacterial agglutination*

Compared with the blank control group and the BSA control group, the obvious agglutination phenomenon was observed in the Gram-positive bacterium *M. luteus* and the Gram-negative bacterium *E. coli* after incubation with the recombinant protein rGaCRT. These results indicate that rGaCRT can promote the agglutination of these two bacteria (Fig. 5).

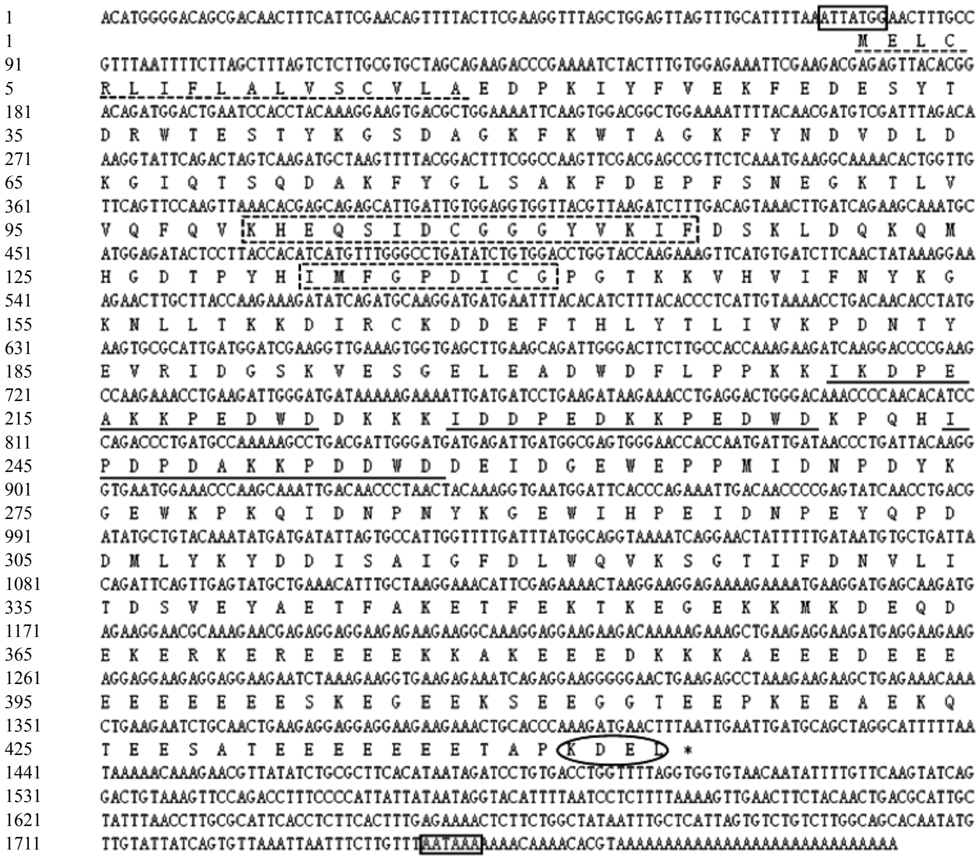

Fig. 1. Nucleotide and putative amino acid sequence of *Gacrt*. The predicted signal peptide is indicated by the dotted line. The two calreticulin-family signature sequences are marked with dotted boxes. Triplicate repeats are highlighted with lines. The ER retrieval signal sequence (KDEL) is enclosed in an oval. The Kozak consensus sequence is in a transparent box, and the polyadenylation signal is in the grey box.

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A. suum         -MRSALVVLAIVIAAVA--AEVYFREEFLD-DSWESRWVQSKHK-DDYGAFKLSHGKFYN
E. pallida      MALYFLVLACLASSVFATD---HFVERFDD-SKWEDRWIYSTYKGSQMGKFKWTAGKFYG
G. astreata     MELCRLIFLALVSCVLAEDPKIYFVEKFED-ESYTDRWTESTYKGSDAGKFKWTAGKFYN
O. faveolata    MALCRLVFLALVSCTLAQDPTIYFVEKFEE-QSYQDRWIESTFKGSDSGKFVWTAGKFYG
L. gigantea     -MKAGLLFLALVGTVLC-DPKVYFNEEFKD-ESYTDNWVESTKKGAEQGKFVWSAGKFYG
P. fucata       ---MKAVFLLVLVGVLYSDATIHFEEKFDS-DGWKDKWVDSTNKGAEQGKFEWTAGKFYG
D. rerio        -MRITAAVCFISALAFIAHADVYFKEQFLDGDGWKSRWVESKHK-SDYGQWKLTSGKFYG
M. musculus     MLLSVPLLLGLLGLAAA-DPAIYFKEQFLDGDAWTNRWVESKHK-SDFGKFVLSSGKFYG
H. sapiens      MLLSVPLLLGLLGLAVA-EPAVYFKEQFLDGDGWTSRWIESKHK-SDFGKFVLSSGKFYG
                       .  :   .       :* * * . . : ..*  *. *  : * :  : ****.

A. suum         DAVKDKGIQTSQDAKFYSRAASFP-KFSNKGKTVVIQFTVKHEQNIDCGGGYLKVMSSNV
E. pallida      DAEKDKGIQTSEDAKFYGVSAKFEKPFSNEGKDLVVQFSVKHEQNIDCGGGYAKIFGKDV
G. astreata     DVDLDKGIQTSQDAKFYGLSAKFDEPFSNEGKTLVVQFQVKHEQSIDCGGGYVKIFDSKL
O. faveolata    DANLDKGIQTSQDAKFYGISAKFEEPFSNEDKTLVIQFQVKHEQNIDCGGGYVKVYDSKV
L. gigantea     DADKDKGIQTSQDARFYGLSAKFD-KFSNEGKSLVVQFTVKHEQNIDCGGGYVKVFSSDL
P. fucata       DADKDKGIQTSQDAKFYGISAKLDKPFSNEGKKLVVQFTVKHEQNIDCGGGYLKVFSSDL
D. rerio        DAELDKGLQTSQDARFYALSSRFD-SFSNEGKTLVIQFTVKHEQKIDCGGGYVKVFPAEM
M. musculus     DLEKDKGLQTSQDARFYALSAKFE-PFSNKGQTLVVQFTVKHEQNIDCGGGYVKLFPSGL
H. sapiens      DEEKDKGLQTSQDARFYALSASFE-PFSNKGQTLVVQFTVKHEQNIDCGGGYVKLFPNSL
                *   ***:***:**.**. :: :   ***:.: :*:** *****.******* *:    :

A. suum         NLEDFHGETPYNVMFGPDICGPGTKKVHVIFSYKGKNHLIKKEIRCKDDEMTHMYTLILS
E. pallida      DQKNLHGDSPYMIMFGPDICGPGTKKVHVIFNYKGKNLLTKKDIRCKDDEMSHLYTLIVK
G. astreata     DQKQMHGDTPYHIMFGPDICGPGTKKVHVIFNYKGKNLLTKKDIRCKDDEFTHLYTLIVK
O. faveolata    DQKNIHGDTPYHIMFGPDICGPGTKKVHVIFNYKGKNLLTKKDIRCKDDEFTHLYTLIVK
L. gigantea     DQENMHGDSPYLIMFGPDICGPGTKKVHVIFNYKGKNLLIKKDIRCKDDVYTHLYTLVVN
P. fucata       DQAGMHGDSPYLIMFGPDICGPGTKKVHVIFNYKGKNLLVKKEIRCKDDVFTHLYTLIVN
D. rerio        DQTEMHGESQYYIMFGPDICGYSTKKVHVIFNYKGQNHLIKKDIKCKDDELTHLYTLILR
M. musculus     DQKDMHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKNVLINKDIRCKDDEFTHLYTLIVR
H. sapiens      DQTDMHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKNVLINKDIRCKDDEFTHLYTLIVR
                :   :**:: * :******** .********.***:* * :*:*.****  :*:***::

A. suum         PDNTYEVQIDGEKVESGELESDWDFLPPKKIKDPDAKKPDDWDEREYIDDPEDKKPDDWE
E. pallida      PDNTYEVRIDNEKVESGKLEEDWDFLPPKKIKDPEAKKPEDWDDKKQIDDPEDKKPEDWD
G. astreata     PDNTYEVRIDGSKVESGELEADWDFLPPKKIKDPEAKKPEDWDDKKKIDDPEDKKPEDWD
O. faveolata    PDNTYEVRIDGSKVESGDLESDWDFLPPKKIKDPEAKKPEDWDDKPKIDDPEDSKPEDWD
L. gigantea     SDNTYVVKIDNEKVESGDLESDWDFLPPKKIKDPEAKKPEDWDDRENIDDPEDTKPEDYD
P. fucata       SDNTYEVRIDNEKAESGNLEEDWDFLPPKKIKDPEAKKPEDWDDREKIDDPDDTKPEDWD
D. rerio        PDQTYEVKIDNEKVESGSLEEDWDFLPPKKIKDPEAKKPDDWDDRAKIDDPEDTKPEDWD
M. musculus     PDNTYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDAAKPEDWDERAKIDDPTDSKPEDWD
H. sapiens      PDNTYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDASKPEDWDERAKIDDPTDSKPEDWD
                .*:** *.**..:.***.** *************:* **:***:.  **** *.**:*::

A. suum         KPEHIPDPEAKKPEDWDDEMDGEWEPPMIDNPEYKGEWKPKQIKNPAFKGKWIHPEIDNP
E. pallida      KPQHIPDPDAKKPEDWDDEIDGEWEAPMIDNPDYKGEWKPKQIDNPNYKGEWVHPEIDNP
G. astreata     KPQHIPDPDAKKPDDWDDEIDGEWEPPMIDNPDYKGEWKPKQIDNPNYKGEWIHPEIDNP
O. faveolata    KPQNIPDPDAKKPDDWDDEIDGEWEPPMIDNPDYKGEWKPKQIDNPNYKGEWVHPEIDNP
L. gigantea     KPEHIPDPEAKKPDDWDDEMDGEWEPPMIDNPEYKGEWKPKQIPNPKYKGKWVHPEIDNP
P. fucata       KPEHIPDPDAKKPDDWDDEMDGEWEPPQIDNPEYKGEWKPKQIDNPAYKGKWIHPEIDNP
D. rerio        KPENIPDPDAKKPDDWDEDMDGEWEPAMIPNPEYKGEWKPKQIDNPSYKGTWVHPEIDNP
M. musculus     KPEHIPDPDAKKPEDWDEEMDGEWEPPVIQNPEYKGEWKPRQIDNPDYKGTWIHPEIDNP
H. sapiens      KPEHIPDPDAKKPEDWDEEMDGEWEPPVIQNPEYKGEWKPRQIDNPDYKGTWIHPEIDNP
                **::****:****:***:::*****.. * **:*******.** ** :** *:*******

A. suum         EYTPDDELYLYEDWGAVGFDLWQVKSGTIFDNILITDSIEEAKSFAAETFEKTKEGEKKM
E. pallida      DYTADDKLYKHDDIGAIGFDLWQVKSGTIFDNVLITDSVESAEEMAKE-FEKTKEGEKAM
G. astreata     EYQPDDMLYKYDDISAIGFDLWQVKSGTIFDNVLITDSVEYAETFAKETFEKTKEGEKKM
O. faveolata    EYQLDDKLYKYDDIGAIGFDLWQVKSGTIFDNILVTDSVEYAETFAKETFEKTKEGEKKM
L. gigantea     EYSSDDSLYKYDDIGALGFDLWQVKSGTIFDNVLITDDEKYAEKYGTDTWGETKDPEKKM
P. fucata       EYVADDKLYKYDDIGAIGFDLWQVKSGTIFDNIIITDDIKEAEEDAKL-WSATKDAEKKM
D. rerio        EYSADDAIYKFDSIGVLGLDLWQVKSGTIFDNFLITDDVEEAEKFGTDTWGATKGPEKKM
M. musculus     EYSPDANIYAYDSFAVLGLDLWQVKSGTIFDNFLITNDEAYAEEFGNETWGVTKAAEKQM
H. sapiens      EYSPDPSIYAYDNFGVLGLDLWQVKSGTIFDNFLITNDEAYAEEFGNETWGVTKAAEKQM
                :*  *  :* .:. ..:*:*************.::*:.   *:  .   :  **  ** *

A. suum         KDKHDEEERKKAEEEEKKRKEEE-DKKKKEEKEDDEEDEK---EKEDDKKDKDEDS----
E. pallida      KDKQDEEERKKQEEEDKKRKEEE-DKKKAEEEDEDDEEEDADKKDEKKEEPKEEKEEAKE
G. astreata     KDEQDEKERKEREEEEKKAKEEE-DKKKAEEEDEEEEEEE---EEESKEGEEKSEEGGTE
O. faveolata    KDEQDEKERKEREEEEKKAKEEE-DKKKAEEEDEEEEEEE---EEEEESKETEEEGKETE
L. gigantea     KEAQDEEESKKREEEEKKRKEEE-DAKK--DDDDEEEDKE---EEEDEKEPD--------
P. fucata       KDQQDEEERKQREEEEKKRKEEEKDSKKDDDDDDDDADDD---EEKDDDKKEDSDDHD--
D. rerio        KDQQEEEERKKREEEEKSKKDDNEEDEEDEDEDEPEEDDH---TEEPPEEEEEGEDDA--
M. musculus     KDKQDEEQRLKEEEEDKKRKEEE-EAEDKEDDDDRDEDED----EEDEKEEDEEESPG--
H. sapiens      KDKQDEEQRLKEEEEDKKRKEEE-EAEDKEDDEDKDEDEE---DEEDKEEDEEEDVPG--
                *: ::*::  : ***:*. *::: : :.  :.:: : :.     .:  .

A. suum         ---------------------------HDEL
E. pallida      ETKEDTAEETKEPEEEKKEDETTEETKRDEL
G. astreata     EPKEEA--EKQTEESATEEEEEEEETAPKDEL
O. faveolata    EKPEEGSEEPDTPETATEEEEEEETTTAKDEL
L. gigantea     ---------------------------HDEL
P. fucata       ---------------------------HDEL
D. rerio        -------------------------LPKDEL
M. musculus     -------------------------QAKDEL
H. sapiens      -------------------------QAKDEL
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Fig. 2. Multiple sequence alignments of CRT amino acid sequences.

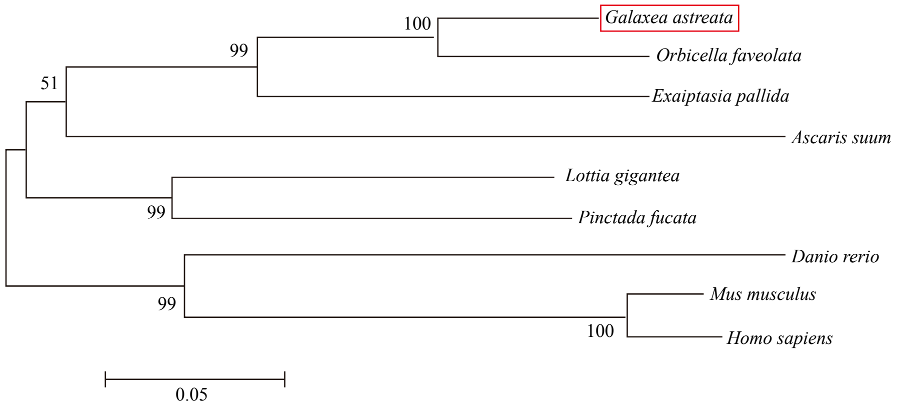

Fig. 3. Cluster analysis of CRT amino acid sequences. The sequence isolated in this study is marked with box. Numbers of each node indicated the percentage of bootstrapping of a 1 000 replications.

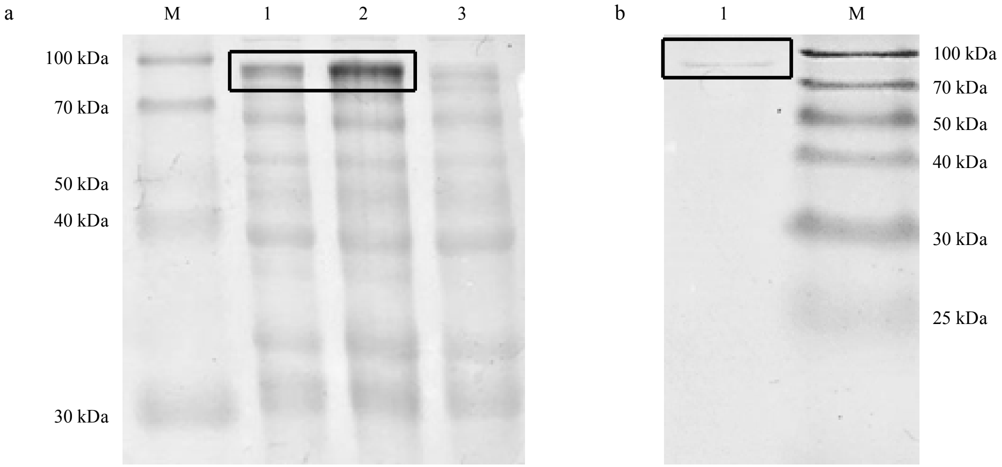

Fig. 4. pET32a-GaCRT prokaryotic expression. a. M: protein molecular weight marker; 1: rGaCRT without induction; 2: rGaCRT induced with IPTG; 3: pET-32a. b. 1: purified protein, M: protein molecular weight marker. The rGaCRT protein is highlighted with a box.

4 Discussion

This study identified the *crt* gene sequence of *G. astreata* (*Gacrt*) and analysed its structure, expression and biological effects. GaCRT possesses typical characteristics of calreticulin, including conserved CRT-family sequences (KHEQSIDCGGGYVKIF and IMFGPDICG), a set of triplicate repeats and an ER retrieval signal sequence (KDEL). Since the signature motif MELCRLIFLALVSCVLA was found in the N-terminal domain, *G. astreata* CRT can be predicted to be a secretory protein. Considering CRT is expressed at higher level at the post-settlement stage of *A. millepora* than at the pre-settlement stage (Hayward et al., 2011) and it plays an important role in cell adhesion, we speculated that CRT may participate in the attachment process of planula larvae. Through multiple sequence alignment, we found that CRT has strong similarity in both vertebrates and in invertebrates, indicating that CRT has been highly conserved during animal evolution. It is noteworthy that the C-terminal of the CRT amino acid sequence from coelenterates is obviously longer than in other animals by more than 20 amino acid residues. The C-terminal plays important roles in protein function and location. For example, the last 9 amino acids at the C-terminal end of myostatin (a member of the β-transforming growth factor family) are identical in vertebrates and invertebrates. When the C-terminal end was changed, myostatin had an opposite function (Zhuo et al., 2017). A study of the oyster (*Pinctada fucata*) calmodulin-like protein (CaLP) also indicated that the C-terminal tail plays important roles in the interactions between CaLP and its targets (Li et al., 2006). Based on the observation that the extra C-terminal fragment is located before the ER retrieval signal sequence, and the C-terminal domain is responsible for regulating the interaction with ER proteins (Corbett et al., 1999), the extra fragment may be related to a function in the ER.

CRT has been shown to be an immune-relevant molecule associated with immune responses in both vertebrates and invertebrates, including plants (Jia et al., 2008), mammals (Wang et al., 2013), fish (Peatman et al., 2007), cephalochordates (Liu et al., 2013), shrimp (Duan et al., 2014; Wang and Chen, 2006) and insects (Zhang et al., 2006). In coral, it was shown that CRT is involved in a stress response caused by pollutants and high temperature stress (Yuyama et al., 2012), and is considered to be a stress response protein (Dunlap et al., 2013). In this study, rGaCRT could promote the agglutination of the Gram-positive bacterium *M. luteus* and the Gram-negative bacterium *E. coli*, suggesting that rGaCRT can participate in immune-related reactions, directly or indirectly play an immune-related role, to avoid the interference of bacteria. Amphioxus CRT also has a similar func-

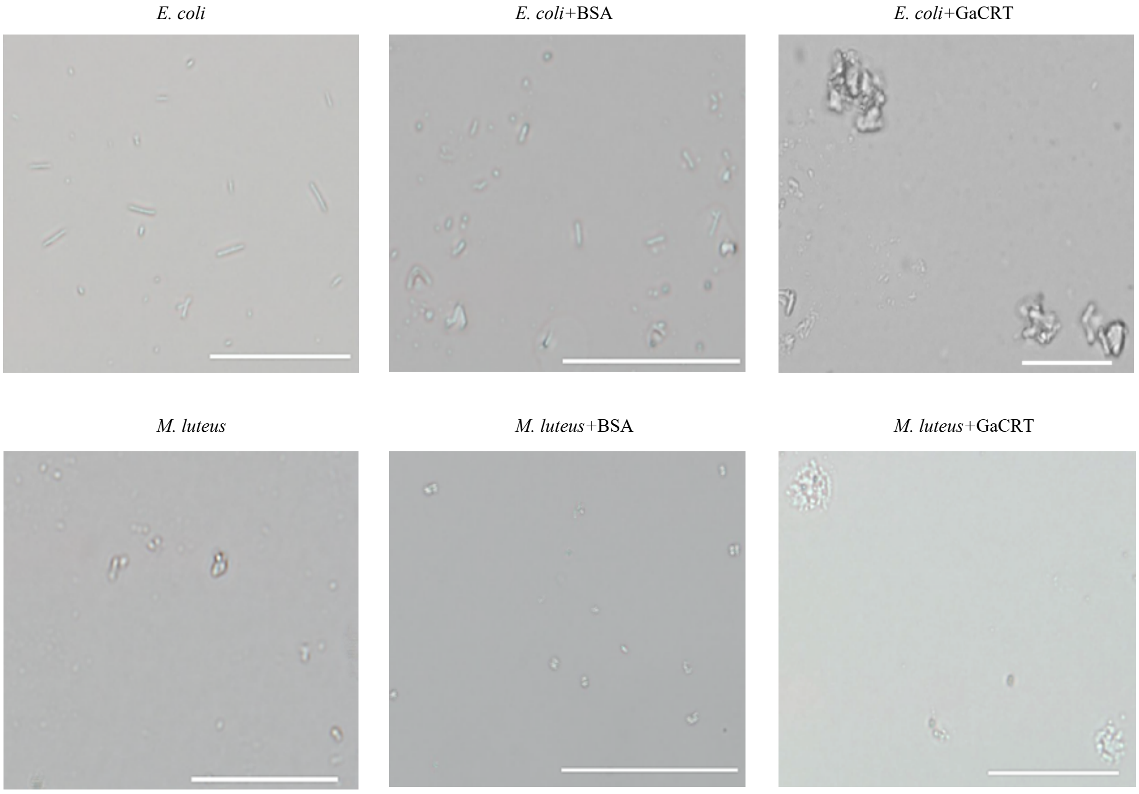

Fig. 5. Bacterial agglutination induced by rGaCRT (bars = 20 μm).

tion, as demonstrated by work showing that *Branchiostoma japonicum* recombinant CRT was able to bind *E. coli* and *S. aureus* (Liu et al., 2013). It is likely that the ability of CRT to act as an immune-relevant molecule is highly conserved throughout animal evolution. In addition, since GaCRT is secreted, it may exist in the mucus of coral to support the mucus' immune function.

In conclusion, in this study, the *crt* gene from *G. astreata* was cloned and characterized. The results show that rGaCRT can promote the agglutination of Gram-positive and Gram-negative bacteria, such as *M. luteus* and *E. coli*, supporting the hypothesis that CRT is an immune-relevant molecule that participates in host defense against bacterium.

References

Baksh S, Michalak M. 1991. Expression of calreticulin in *Escherichia coli* and identification of its Ca^{2+} binding domains. The Journal of Biological Chemistry, 266(32): 21458–21465, doi: 10.1007/BF00006813

Corbett E F, Oikawa K, Francois P, et al. 1999. Ca^{2+} regulation of interactions between endoplasmic reticulum chaperones. The Journal of Biological Chemistry, 274(10): 6203–6211, doi: 10.1074/jbc.274.10.6203

Duan Yafei, Liu Ping, Li Jitao, et al. 2014. Molecular responses of calreticulin gene to *Vibrio anguillarum* and WSSV challenge in the ridgetail white prawn *Exopalaemon carinicauda*. Fish & Shellfish Immunology, 36(1): 164–171, doi: 10.1016/j.fsi.2013.10.024

Dunlap W C, Starcevic A, Baranasic D, et al. 2013. KEGG orthology-based annotation of the predicted proteome of *Acropora digitifera*: zoophyteBase-an open access and searchable database of a coral genome. BMC Genomics, 14: 509, doi: 10.1186/1471-2164-14-509

Fischer C R, Mikami M, Minematsu H, et al. 2017. Calreticulin inhibits inflammation-induced osteoclastogenesis and bone resorption. Journal of Orthopaedic Research, 35(12): 2658–2666, doi: 10.1002/jor.23587

Gold L I, Eggleton P, Sweetwyne M T, et al. 2010. Calreticulin: non-endoplasmic reticulum functions in physiology and disease. The FASEB Journal, 24(3): 665–683, doi: 10.1096/fj.09-145482

Gu V Y, Wong M H, Stevenson J L, et al. 2008. Calreticulin in human pregnancy and pre-eclampsia. Molecular Human Reproduction, 14(5): 309–315, doi: 10.1093/molehr/gan017

Hayward D C, Hetherington S, Behm C A, et al. 2011. Differential gene expression at coral settlement and metamorphosis—a subtractive hybridization study. PLoS One, 6(10): e26411, doi: 10.1371/journal.pone.0026411

Jia Xiaoyun, Xu Chongyi, Jing Ruilian, et al. 2008. Molecular cloning and characterization of wheat calreticulin (CRT) gene involved in drought-stressed responses. Journal of Experimental Botany, 59(4): 739–751, doi: 10.1093/jxb/erm369

Kenkel C D, Bay L K. 2017. Novel transcriptome resources for three scleractinian coral species from the Indo-Pacific. Gigascience, 6(9): 1–4, doi: 10.1093/gigascience/gix074

Kobylewski S E, Henderson K A, Yamada K E, et al. 2017. Activation of the EIF2α/ATF4 and ATF6 pathways in du-145 cells by boric acid at the concentration reported in men at the US mean boron intake. Biological Trace Element Research, 176(2): 278–293, doi: 10.1007/s12011-016-0824-y

Li Shuo, Xie Liping, Meng Qingxiong, et al. 2006. Significance of the extra C-terminal tail of CaLP, a novel calmodulin-like protein involved in oyster calcium metabolism. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 144(4): 463–471, doi: 10.1016/j.cbpb.2006.04.010

Liu Xuemei, Xu Na, Zhang Shicui. 2013. Calreticulin is a microbial-binding molecule with phagocytosis-enhancing capacity. Fish & Shellfish Immunology, 35(3): 776–784, doi: 10.1016/j.fsi.2013.06.013

Michalak M, Corbett E F, Mesaeli N, et al. 1999. Calreticulin: one protein, one gene, many functions. Biochemical Journal, 344(2): 281–292, doi: 10.1042/bj3440281

Ostwald T J, MacLennan D H. 1974. Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum. The Journal of Biological Chemistry, 249(3): 974–979, doi: 10.1007/BF01520862

Peatman E, Baoprasertkul P, Terhune J, et al. 2007. Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium. Developmental & Comparative Immunology, 31(11): 1183–1196, doi: 10.1016/j.dci.2007.03.003

Qiu Yuanyuan, Michalak M. 2009. Transcriptional control of the calreticulin gene in health and disease. The International Journal of Biochemistry & Cell Biology, 41(3): 531–538, doi: 10.1016/j.biocel.2008.06.020

Smith M J, Koch G L. 1989. Multiple zones in the sequence of calreticulin (CRP55, calregulin, HACBP): a major calcium binding ER/SR protein. The EMBO Journal, 8(12): 3581–3586, doi: 10.1002/j.1460-2075.1989.tb08530.x

Wang F I, Chen J C. 2006. Effect of salinity on the immune response of tiger shrimp *Penaeus monodon* and its susceptibility to *Photobacterium damselae* subsp. *damselae*. Fish & Shellfish Immunology, 20(5): 671–681, doi: 10.1016/j.fsi.2005.08.003

Wang Yan, Zhou Jianjiang, Xie Yuan, et al. 2013. Expressions of calmodulin, calreticulin and cyclin-dependent kinase 1 genes in gastric cancer tissues. Cancer Research on Prevention and Treatment (in Chinese), 40(2): 151–154

Xia Xiulin, Wang Guiling, Bai Zhiyi, et al. 2013. Molecular characterization and expression analysis of calreticulin cDNA from *Hyriopsis cumingii*. Journal of Fisheries of China (in Chinese), 37(5): 679–687, doi: 10.3724/SP.J.1231.2013.38454

Yuyama I, Ito Y, Watanabe T, et al. 2012. Differential gene expression in juvenile polyps of the coral *Acropora tenuis* exposed to thermal and chemical stresses. Journal of Experimental Marine Biology and Ecology, 430–431: 17–24, doi: 10.1016/j.jembe.2012.06.020

Zhang Guangming, Schmidt O, Asgari S. 2006. A calreticulin-like protein from endoparasitoid venom fluid is involved in host hemocyte inactivation. Developmental & Comparative Immunology, 30(9): 756–764, doi: 10.1016/j.dci.2005.11.001

Zhuo Ruiqun, Zhou Tingting, Yang Shiping, et al. 2017. Characterization of a molt-related myostatin gene (*FmMstn*) from the banana shrimp *Fenneropenaeus merguiensis*. General and Comparative Endocrinology, 248: 55–68, doi: 10.1016/j.ygcen.2017.03.010