Molecular cloning and characterization of calmodulin-like protein CaLP from the Scleractinian coral *Galaxea astreata*

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Abstract

Optimal temperature and light are both necessary conditions for coral survival. Light enhances calcification, and thermal stress disrupts Ca^{2+} homeostasis. As calcium is involved in many important metabolic activities, in this study, we cloned the calmodulin-like protein (CaLP) gene of one of the scleractinian corals, *Galaxea astreata*. We also detected the relative mRNA expression levels of *gaCaLP* using the calcium channel blocker verapamil and CaCl_2 treatment under conditions of light and dark, and compared expression levels under controlled temperature conditions. Full-length *gaCaLP* cDNA comprised 1290 nucleotides and contained 498 bp open reading frame that encoded a protein with 165 amino acids. With CaCl_2 , expression levels of *gaCaLP* only increased in the presence of light, suggesting that light may be a restrictive factor in CaLP expression when sufficient calcium is available in the environment. In addition, after verapami treatment, we noted that a down regulation of *gaCaLP*, suggesting that the expression of CaLP is closely related to extracellular Ca^{2+} influx. Under temperature stress at both high (30 °C) and low (20 °C) temperatures, expression levels of *gaCaLP* showed an initial increase, followed by a decreasing trend as treatment progressed. Expression levels reached their maximum value at 24 h. This result showed that CaLP participated in a temperature stress response, and Ca^{2+} homeostasis was disrupted during stress. The findings of the present study will help determine the function and regulatory mechanisms of *gaCaLP*.

Keywords *Galaxea astreata* · Calmodulin-like protein · Light · Calcium · Temperature stress

Introduction

Calcium not only plays a crucial role in various physiological processes but also functions as the primary cation used in biomineralization systems, such as in coral, shellfish, and coralline algae. The formation of biomineralized structures, which are products of Ca^{2+} metabolism, is controlled by biochemical and physiological activities and many Ca^{2+} -binding proteins are involved, such as calmodulin (CaM) and calmodulin-like protein (CaLP), which have EF-hand domains for calcium binding. The EF-hands are characterized by a helix-loop-helix structure, and are closed in the absence

of Ca^{2+} ; the Ca^{2+} -free form of the EF-hand protein is not active (Chen et al. 2015). In calcium signal transduction, calcium triggers CaM and CaLP conformational changes, which result in activity changes and allow interaction with downstream proteins (Maki et al. 2002).

Calmodulin-like protein (CaLP), a multi-functional calcium-sensing element that belongs to the CaM superfamily, has already been studied in nematodes (Karabinos 2016), fruit fly (Fyrberg et al. 1994), rats (Tomizawa et al. 1993), and humans (Hashimoto et al. 2017). CaLP is involved in human epithelial cell differentiation (Me'hul et al. 2000), and the regulation of Ca^{2+} -induced release of Ca^{2+} in human and rat cell lines (Kasri et al. 2003). In addition, it has been shown to be modulated by elevated pCO_2 exposure in bivalves (Jeffrey et al. 2017). Compared with CaM, CaLP had a greater role and more research significance in calcified organisms. For example, it has the maximal expressed levels in scallop *Chlamys farreri* mantle, a key organ involved in shell formation, whereas CaM expression levels are basically similar in each organization (Lin et al. 2014). Moreover, its expression increased more in the shell notching experiment in *C. farreri* and shell

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regeneration in *Pinctad fucata* than that of CaM, thereby suggesting that CaLP participated in biomineralization (Lin et al. 2014; Fang et al. 2008). Among highly calcified organisms, coral calcification results in the formation of approximately 284,300 km² of coral reefs (Spalding et al. 2001), which are formed through enriched bonding of Ca²⁺, C, and O atoms in the ectoderm (Geng et al. 2017). Corals are temperature-sensitive organisms; in fact, 20 °C is the minimum seawater temperature for scleractinian coral growth and 30 °C is the maximum temperature for mass propagation (Zhu 1987). Regrettably, high sea water temperature has already caused major coral bleaching events (De'ath et al. 2012). Huang et al. (1998) showed that intracellular Ca²⁺ sustained increases in isolated coral cells over 24 h of thermal stress. Given that calcium signaling is closely connected to exocytosis, some studies have pointed out that one of the relationships between calcium signaling and bleaching can be explained by zooxanthellae exocytosis triggered by increasing intracellular Ca²⁺ concentration (Fang et al. 1997). Since CaLP has an important role in the calcium signaling pathway, temperature stress also affects CaLP expression. In a previous study, our transcriptome data (unpublished) revealed that *Galaxea astreata* CaLP was up-regulated after 12 h in 35 °C. However, this temperature was too high to conform with real conditions, and a moderate temperature treatment was needed to obtain more meaningful data.

Light provides a primary source of energy for coral reefs and accelerates CaCO₃ skeleton precipitation in scleractinian corals, which is called light-enhanced calcification. This positive correlation between light and coral calcification has been observed in both laboratory and field studies. For example, Moya et al. (2006) reported that coral calcification rates in *Acropora* spp. showed a 2.6-fold enhancement at 12 AM compared with that observed at 12 PM. In *Acropora horrida* and *Porites cylindrical*, light caused significant changes in calcification rates by measuring related indexes after nubbins were incubated for approximately 4 h (Suggett et al. 2013). Higher coral calcification rates also mean higher calcium uptake rates. In a study of calcium incorporation, calcium uptake was through calcium channels, thereby suggesting the involvement of verapamil-sensitive Ca²⁺ channels in some marine invertebrates, such as crustaceans, echinoids and corals, based on pharmacological evidence (Zoccola et al. 1999). Tambutté et al. (1996) studied the transcellular passage of Ca²⁺ in *Stylophora pistillata* by using verapamil. In addition, CaCl₂ was also studied in coral Ca²⁺ incorporation (Al-Horani et al. 2005). Using verapamil and CaCl₂ to study calcium-sensing elements in both light and dark conditions can better elucidate the molecular mechanism of coral calcium uptake.

Unlike *Acropora* spp., which is one of the most sensitive coral species, *Galaxea astreata* (Oculinidae, *Galaxea*) is more resistant to environmental changes. This was one of

the most abundant coral species in Xuwen Coral Reef National Nature Reserve (Shen et al. 2016). However, coral coverage rate in Xuwen rapidly declined because of human activities and other environmental factors, thereby resulting in a corresponding decline of *G. astreata*. It is therefore necessary to understand the physiological and stress tolerance molecular mechanism in *G. astreata*. To this end, we cloned and characterized the full-length CaLP cDNA of *G. astreata* and conducted a transcript expression analysis in CaCl₂ and verapamil culture under light and dark conditions respectively, and at varying times after hot (30 °C) and cold (20 °C) treatments.

Materials and methods

Animals and maintenance

G. astreata, was collected from the Xuwen Coral Reef National Nature Reserve, Zhanjiang, Guangdong Province, China, at the specific geolocations of 109° 50'12"–109°56' 24E", 20°10'36"–20°27'00"N. Specimens were acclimatized in 27 ± 0.5 °C seawater at a salinity of 31 ppt, pH 8.1, and a light:dark cycle of 12 h:12 h in the flow-through aquaria system of the aquarium. All tanks were cleaned in order to prevent algal growth. After division into approximately 4 × 4 cm nubbins by a hammer and chisel, the colonies were maintained for a week under the same condition (as observed in another study (Desalvo et al. 2012), 1 week was long enough for acclimation). During this acclimation period, nubbins were visually inspected three times a day to check for bleaching, tissue degradation, mortality, gross morphological aspect, and mucus production to confirm that no mortality occurred and that tissues grew completely and were in good condition.

Molecular cloning

Total RNA was extracted from *G. astreata* by the TRI Reagent (MRC Inc., USA). Quantity and quality of extracts were assessed by NanoDrop 2000 (Thermo Scientific, USA) and 1% agarose gel. Reverse transcription of 1 µg total RNA was carried out using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan) according to the manufacturer's instructions. All primers were synthesized by Sangon Biotech (Shanghai) (Table 1). Target gene fragments were amplified under the following PCR procedure: 1 cycle at 94 °C for 3 min; then 35 cycles at 95 °C for 30 s, 62.6 °C for 45 s and 72 °C for 30 s; and 72 °C for 10 min.

For cloning of the full-length target gene, the cDNA template was synthesized with a SMARTer™ RACE cDNA Amplification Kit (Takara, Japan), followed by nest- and touchdown-PCR for amplification of 5' and 3' end. The first

Table 1 Primers used for various experimental procedures

Primer	Sequence (5'-3')	Function
CaLP-F	AGCGCCAACTCACTGAGG	Middle fragment PCR
CaLP-R	TTGCATGGAAGCTGGGATA	
CaLP-5'-outer	GTAGCCAGAAGGTATTGAGCGGGG	5'-RACE
CaLP-5'-inner	TCGGTCAAAAACCTCTGAATGCCTCTCG	
CaLP-3'-outer	GATCCCAGTTGTTATTTTAATCAGCGCG	3'-RACE
CaLP-3'-inner	AAAATGGCAAAGGTTGAAACGCTCTTCGT	
CaLP-qPCR-F	ACAGATAAGCCCGATGGAAC	qRT-PCR ($E \approx 106.4769\%$)
CaLP-qPCR-R	TGGTAGCCAGAAGGTATTGAGG	
Actin-F	AATGTATGTTGCCATTACAG	Reference gene ($E \approx 103.7466\%$)
Actin-R	TCACGCACAATCTCACGT	

round of PCR was performed using a UPM (Universal Primer Mix) and CaLP-5'-outer or CaLP-3'-outer. The second round of PCR was performed using a NUP (Nested Universal Primer) and CaLP-5'-inner or CaLP-3'-inner for 5' and 3' cDNA ends, respectively. 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 1 min followed by 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min for 25 cycles, and 72 °C for 10 min.

PCR products were visualized upon agarose gel electrophoresis, subcloned into pEASY-T5 Zero Cloning vector (TransGen), and sequenced by Sangon Biotech (Shanghai).

Sequence analyses

Nucleotide sequences were assembled by MEGA 6.0. Coding regions were predicted using ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). Functional domain prediction was performed at <http://smart.embl-heidelberg.de/>. Another online tool, the ProtParam, was used to predict the molecular weight and isoelectric point ([http:// web. Expasy.org/ protparam/](http://web.expasy.org/protparam/)). The secondary structure was predicted by Motif scan (http://hits.isb-sib.ch/cgi-bin/motif_scan).

Pharmacological experiment

For pharmacological studies, calcium channel blockers Verapamil (Vp, Shanghai Yuanye Bio-Technology) and CaCl_2 (Guangdong Guanghua Sci-Tech Co.) were dissolved in seawater from the coral aquarium at final concentrations of 0.6 and 15 mM respectively. Each nubbin was cultivated in seawater with Vp or CaCl_2 in a 1000 ml beaker, and the control group contained no extra reagent. The pharmacological and control groups were kept for 4 h in light with an intensity of $90 \mu\text{moles photons m}^{-2} \text{s}^{-1}$ (as observed in another study, $90 \mu\text{moles photons m}^{-2} \text{s}^{-1}$ was suitable for coral normal life) (Schutter et al. 2008) or in dark incubators at a constant temperature of 27 ± 0.5 °C before harvesting of

mRNA for every three polyps. Including the treated and control groups, there were six experimental groups.

Temperature stress experiment

For thermal stress experiments, nubbins were randomly transferred into three experimental aquarium tanks at 20 ± 0.5 , 27 ± 0.5 , and 30 ± 0.5 °C respectively. In each sampling, every three polyps were cut from each incubator at the same time with scissors at 12, 18, 24, 36, and 48 h intervals after treatment. Every nubbin was subjected to sampling once before being abandoned to minimize the possibility of undesirable effects.

Quantitative real-time PCR

mRNA expression levels of *gaCaLP* gene were measured using quantitative real-time PCR (qRT-PCR). RNA was extracted and reverse transcription were carried out as mentioned above. Volume reactions (10 μL) contained 5 μL Top Green qPCR SuperMix (Transgen), 3.5 μL water, 0.5 μL of 5 μM of each primer, and 0.5 μL cDNA. PCR procedure was as follows: 3 min at 94 °C, followed by 40 cycles of 10 s at 95 °C, 15 s at 55 °C, and 15 s at 72 °C. Melting curve analysis was recorded every 0.5 °C at a temperature gradient of 65 °C–95 °C. All reactions were performed in triplicate, and the fold expression level of *gaCaLP* was calculated by the $2^{-\Delta\Delta\text{Ct}}$ method, using β -actin as a reference gene (Rocker et al. 2015; Hayakawa et al. 2005).

Statistical analysis

All data were presented as mean $\pm$ standard deviation. After confirmed that the data conformed to normal distribution, the significance of the results was tested using a t test (SPSS 17.0 software, Chicago, IL, USA) to compare light and dark for each of the three pharmacological treatments and each time point under temperatures stress.

Results

Cloning of CaLP gene from *G. astreata*

Based on our previous study of the transcriptome of *G. astreata* (unpublished data), we designed gene-specific primers and isolated cDNA clones encoding the CaLP gene from the total RNA extract of *G. astreata* using the RACE method. Full-length *gaCaLP* cDNA comprised 1290 nucleotides and possessed a 109-bp 5'-untranslated region (UTR), 683-bp 3'-UTR, and 498-bp open reading frame (ORF), which corresponded to a protein consisting of 165 amino acids (a.a.). The Kozak consensus sequence (PuNNATGPu) surrounding the initiation codon (ATG) of cDNA was found. Two polyadenylation signals (AATAAA) were also identified at nucleotides 1232–1237 and 1260–1265 (Fig. 1).

Upon BlastN search, the isolated cDNA featured 97% nucleotide was compared with the CaM-like gene of the published *G. astreata* transcriptome (isogroup 67,746) (Kenkel and Bay 2017), which was only 16 bp longer than this transcriptome sequence. We therefore concluded that *gaCaLP* (MH006890) encoded the CaLP gene of *G. astreata*.

Theoretical molecular weight and isoelectric point of *gaCaLP* protein totaled 18.89 and 4.22 kDa, respectively. The deduced *gaCaLP* protein contained four EF-hand calcium-binding domains at 13–41, 49–77, 86–114, and 122–150 after functional domain prediction. According to the Motif online tool, 16 EF-hand motifs were located in the *gaCaLP* a.a. sequence. *gaCaLP* contained a PPxY motif at the C-terminus. The mature peptide was classified as a stable protein owing to its instability index of 38.39.

Effects of calcium concentration on *gaCaLP* gene expression in light and dark conditions

To determine how calcium regulated the expression levels of *gaCaLP* gene under dark conditions compared with light conditions, we regulated the calcium concentration pharmacologically and measured mRNA levels of *gaCaLP* using qRT-PCR. After adding Vp to the culture, mRNA levels of *gaCaLP* were down-regulated both under light and dark conditions. In contrast, *gaCaLP* expression levels significantly increased after CaCl_2 addition only under light conditions (Fig. 2).

Fig. 1 Nucleotide and deduced a.a. sequence of *gaCaLP* cDNA. Polyadenylation signals are designated with black boxes; the transparent oval box indicates PPxY motif, and Kozak consensus sequence is underlined

Fig. 2 Expression patterns of *gaCaLP* gene under different pharmacologic treatments. Asterisks (*) indicate statistical differences at $P < 0.05$, as determined by t test

Expression levels of *gaCaLP* under different temperatures

Using qRT-PCR, we measured the time course of mRNA levels of *gaCaLP* gene under hot and cold stresses. Similar trends of expression level changes were observed under either stress, that is, *gaCaLP* expression increased in the first 24 h, and then decreased with prolonged incubation under continued thermal stress. Expression levels peaked at 24 h and were approximately three and nine times higher than those of the control group at 30 and 20 °C, respectively. However, mRNA levels of *gaCaLP* were barely detectable after 48 h at 20 °C (Fig. 3).

Fig. 3 Expression patterns of *gaCaLP* gene in 30 ± 0.5 °C (a) and 20 ± 0.5 °C (b) temperature stresses and different time points. Asterisks (*) indicate statistical differences at $P < 0.05$, as determined by t test

Discussion

In this study, the gene sequence of *gaCaLP* was determined, and its structure was characterized, thereby showing that *gaCaLP* contained four EF-hand domains to bind calcium. In addition, *gaCaLP* had a PPxY motif. Proline-rich motifs such as PPxY were exposed to combine and break away from proteins rapidly. Hence, proline sequences are normally present in some proteins with rapid interaction with others, such as during initial transcription, signal cascade, and cytoskeletal rearrangement (Wang et al. 2008).

In accordance with light-enhanced calcification, the expression of some calcification-associated genes, such as carbonic anhydrase and galaxin (Desalvo et al. 2012), were also down-regulated in the dark. However, as a calcium transport protein, CaLP showed no significant difference in expression in light and dark at the transcriptional level in this study. Somewhat similarly, Ca²⁺-ATPase and Ca²⁺ channel genes did not show differential expression between light and dark conditions (Moya et al. 2008). On the contrary, a significant up-regulation of *gaCaLP* was observed during incubation with CaCl₂ under light conditions. In addition, Al-Horani et al. (2005) also showed that light-incubated colonies had a higher Ca²⁺ incorporation rate in its skeletons compared with the dark-incubated ones after the addition of CaCl₂. This result hinted that light may be a restrictive factor for coral uptake calcium when calcium in the environment was abundant.

The calcium channel blocker verapamil can inhibit cell uptake Ca²⁺, and has been used in a study of Ca²⁺ transcellular transport in *S. pistillata* (Tambutté et al. 1996). Our study found that *gaCaLP* expression level was reduced in the treatment of verapamil under both light and dark conditions, suggesting that CaLP expression was affected by extracellular calcium influx. In *C. farreri* (Lin et al. 2014) and *Hyriopsis cumingii* (Ren et al. 2013), CaLP expression increased with the increase of the concentration of Ca²⁺ in the environment within a certain range. This result was consistent with the role of CaLP as a key calcium sensor, storage, and trafficking protein.

Under prolonged temperature stress, the expression pattern of *gaCaLP* first increased and then decreased. The stress protein was highly expressed at the beginning of the stress conditions, and protein synthesis decreased due to heat tolerance or to the gradual decrease in energy supply. Heat shock proteins (HSPs), observed under heat stress, also have a similar expression pattern (Carpenter et al. 2010). Li et al. (2004) reported that the expression of HSP70 in heat stress is regulated by CaM. Since the structure and function of CaLP have high similarity with CaM, the reaction mechanism of CaLP after heat stress may also be consistent with CaM. In addition, CaLP expression changes provided evidence of disruption of Ca²⁺ homeostasis during temperature stress. Ca²⁺ homeostasis disruption was observed during thermal stress experiments on Caribbean coral *Montastraea faveolata* for 10 days and 17 h,

which was demonstrated by down regulation of CaM and an EF-hand protein (Desalvo et al. 2008). A previous study also showed intracellular calcium concentrations sustained increases under heat treatment over 24 h (Huang et al. 1998). This same trend was observed in our experiments. Compared with thermal stress, cold stress also generated remarkable fluctuation in *gaCaLP* expression. CaLP belonged to a member of the CaM superfamily. Earlier studies have shown that cold can lead to CaM expression increase in fish, and over expression experiments on tobacco showed that CaM enhances plant cold resistance (Yang et al. 2013). Under cold stimuli, membrane depolarization was generated by an increase in the Ca^{2+} conductance and a decrease in the K^{+} conductance (Kuriu et al. 1997). Previous studies also showed that some calcium channels were activated in cold temperatures and cold-induced Ca^{2+} increase in cytosol (Yang et al. 2013). However, how CaLP participates in temperature stress response requires further study.

Conclusions

In this study, a Calmodulin-like protein gene was isolated from the scleractinian coral *G. astreata* using RACE-PCR, and its expression levels were analyzed at different temperatures and with calcium-related reagents. Pharmacological studies on diurnals and nocturnals confirmed that light may be a restrictive factor in Ca^{2+} intake under sufficient calcium conditions, and expression of CaLP was influenced by extracellular calcium influx. Regulation of CaLP first increased and then decreased during the time course of under both hot and cold treatments, suggesting that *gaCaLP* participated in the thermal stress response and that Ca^{2+} homeostasis was disrupted during temperature stress.

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