Scientific Research Publishing

American Journal of Molecular Biology, 2021, 11, 38-50
https://www.scirp.org/journal/ajmb
ISSN Online: 2161-6663
ISSN Print: 2161-6620

Characteristics of a Critical Calcium Transportation Regulator: Plasma Membrane Ca^{2+}-ATPase Involved in Calcium Homeostasis from *Hyriopsis cumingii* (Lea)

Aiju Zhang, Zhiming Zhou

Agriculture Ministry Key Laboratory of Healthy Freshwater Aquaculture, Key Laboratory of Freshwater Aquaculture Genetic and Breeding of Zhejiang Province, Zhejiang Research Center of East China Sea Fishery Research Institute, Zhejiang Institute of Freshwater Fisheries, Huzhou, China

Email: zjhz-zzm@163.com

How to cite this paper: Zhang, A.J. and Zhou, Z.M. (2021) Characteristics of a Critical Calcium Transportation Regulator: Plasma Membrane Ca^{2+}-ATPase Involved in Calcium Homeostasis from *Hyriopsis cumingii* (Lea). *American Journal of Molecular Biology*, **11**, 38-50.
https://doi.org/10.4236/ajmb.2021.112004

Received: February 2, 2021
Accepted: March 23, 2021
Published: March 26, 2021

Abstract

Plasma Membrane Calcium ATPase (PMCA) plays a critical role in transporting Ca^{2+} out of the cytosol across the plasma membrane. Here, a full-length cDNA sequence of plasma membrane Ca^{2+}-ATPase gene was isolated from the gill of *Hyriopsis cumingii* (HcPMCA) by using SMART RACE technique. The entire cDNA was 5230 bp, including a 417-bp 5'-UTR, a 3588-bp ORF and a 1225-bp 3'-UTR, encoding a 1195-amino acid protein, and no putative signal peptide was predicted. Compared with PMCA homologs from seawater mollusks, HcPMCA had high similarity with them in both sequence and structure. Tissue-specific expression analysis revealed that HcPMCA mRNA was detected in all the sampled tissues, but was prominently expressed in the gill and mantle. When exposed to a serie of increasing Ca^{2+} that lasted for 7 days, the mRNA expression of HcPMCA in the mantle was slightly downregulated, but peaked at 60 mg/L. Moreover, the temporal expression of HcPMCA transcripts in the mantle after 60 mg/L Ca^{2+} exposure was shown to be bell-shaped, which was slightly downregulated at 24 h, but upregulated from 24 h to 48 h post-treatment, peaking at 48 h. The result of present study provides useful information for further studies on function and regulation mechanism of HcPMCA gene.

Keywords

Hyriopsis cumingii (Lea), HcPMCA, Cloning, Gene Expression, Calcium Stimulation

1. Introduction

As an essential iron in living cells, Calcium (Ca^{2+}) is not only a crucial regulatory element in cell signaling, but also a primary cation that is used for biomineralization of shell/nacre in mollusk [1]. The concentration of extracellular and cytoplasmic Ca^{2+} in resting cells are both relatively stable, which is called Ca^{2+} homeostasis, and the former is nearly 10,000-fold more than the latter in mollusk [2]. Environmental Ca^{2+} concentration is one of the important factors affecting calcium metabolism of mollusk that is of great significance to elucidate the formation mechanism of shell/nacre and improve the production of high-quality pearl. Absorption calcium from the environment directly is an important way for Ca^{2+} to enter in freshwater mollusk. As a result, the efflux mechanism of solute Ca^{2+} is very important for the return of cytoplasmic Ca^{2+} to a static state and the maintenance of intracellular Ca^{2+} homeostasis. Meanwhile, Ca^{2+} pumps on the plasma membrane and sarco/endoplasmic reticulum excretes the cytosol Ca^{2+} out of the cell or into the intracellular calcium pool, which are also important bases for maintaining cellular regulation.

As one of the major Ca^{2+} transporters, plasma membrane Ca^{2+}-ATPase (PMCA) has been researched nearly 50 years [3]. It has high Ca^{2+} affinity and plays an important role in keeping the higher extracellular free calcium concentration and maintaining Ca^{2+} homeostasis by using energy to pump Ca^{2+} out of the cytosol into the extracellular milieu, usually against a strong chemical gradient [4] [5]. Previous studies suggested that PMCA may be involved in calcified layer formation in pearl oyster, and several PMCA or putative PMCA genes have been cloned from the seawater mollusk, such as *Tridacna squamosa*, *Mizuhopecten yessoensis* and *Pinctada fucata* [6]. While, the researches about this gene on freshwater mollusk were nearly few. The triangle sail mollusk, *Hyriopsis cumingii* (Lea) is the most important mollusk in commercial freshwater pearl production of China. In order to maintain its pearl industry development sustainably, it is very important to clarify the Ca^{2+} transporters, or even its regulatory mechanisms of calcium metabolism. Hence, in this study, a putative PMCA from *H. cumingii* (HcPMCA) was cloned by using SMART RACE technique. The gene structure and phylogenesis were analyzed, and also its expression profile in different calcium stress was explored using quantitative real-time polymerase chain reaction (qRT-PCR). These results will help us to further understand the function of PMCA during mollusk biomineralization.

2. Materials and Methods

2.1. Animal Material and Tissue Collection

One year old individuals of *H. cumingii*, with an average shell length of 65 mm were collected from Weiwang Pearl Farm of Jinhua, Zhejiang Province, China, which were transported to the laboratory and kept in a PVC tank with aerated pond water at 25˚C for 48 h. After that, the mollusks were sampled, and five tissue samples were isolated for RNA extraction, including gonad, hepatopancreas,

foot, mantle and gill. All tissue samples were immediately washed with sterile PBS, stored in liquid nitrogen and stored at −80˚C.

2.2. RNA Preparation and cDNA Synthesis

Total RNA from the gill was extracted using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol. The quality and quantity of total RNA were then checked using gel electrophoresis system (Bio-Rad, USA) and Spectrophotometer (Thermo, USA). First-strand cDNA was synthesized with 5 μg DNA-free total RNA as the PCR template using a RevertAid First Strand cDNA Synthesis Kit (Thermo, USA), according to the manufacturer's instructions.

A partial cDNA sequence of PMCA from *H. cumingii* had been harvested via high-throughput transcriptome sequencing using the mRNA extracted from pooled six tissues before this study [7]. Here, a rapid amplification of cDNA ends (RACE) cDNA library was constructed with a Clontech Smart cDNA Amplification kit using RNA from the gill, according to the manufacturer's instructions. To obtain the 3' terminal sequence of the PMCA end, two rounds of the forward primers, including RC146-R1 and RC146-R2 was designed based on the EST sequence obtained from our previous transcriptomic analysis of *H. cumingii* [1] (**Table 1**). The 25-μL reaction mixture consisted of 12.5 μL of 2 × GC buffer I, 4.0 μL of dNTP Mix (2.5 Mm), 1.0 μL of template, 0.5 μL of universal primer mix, 0.5 μL of RC146-R1 or RC146-R2, 0.2 μL of Polymerase Mix (5 U/μL) and 6.3 μL ddH_2O was programmed as follows: 3 min at 95˚C; 40 cycles of 30 s at 94˚C, 30 s (and 58 s in the 2nd round) at 58˚C and 60 s at 72˚C; 7min at 72˚C. The amplified fragment was then sub-cloned into a pMD18-T vector prior to sequencing by a commercial company (Sangon, China). Based on the 3'-cDNA end sequence of HcPMCA, gene-specific primers were designed for two rounds of 5'-RACE PCR and listed in **Table 1**. PCR of 5'-cDNA ends and cloning of the products were carried out as described above for 3'-cDNA ends. The complete HcPMCA cDNA sequence was obtained by overlapping these three fragments.

Table 1. Primers designed for cloning and expression analysis of HcPMCA gene.

Primer use	Primer name	Primer sequence
1st round 3'RACE	RC146-R1	5'-TCCCTCTGCATCAAGACACTTACCCATT-3'
2nd round 3'RACE	RC146-R2	5'-TTGAGCCCAGGAGTTTGAGACCAGC-3'
1st round 5'RACE	PMCAF1	5'-CGCCTGACCATCCTCCCTGCTCT-3'
2nd round 5'RACE	PMCAF2	5'-GGGGAGGCGAGTTCTCTGCCTTAA-3'
Realtime PCR forward	*β*-actin-F	5'-CGGATAACACAAGGAAAGGAAAC-3'
Realtime PCR reverse	*β*-actin-R	5'-ATGGATGGAAACACGGCTCT-3'
Realtime PCR forward	RT-PMCAF	5'-GGTGTATGAAGACGGACCAAAAC-3'
Realtime PCR reverse	RT-PMCAR	5'-GCCATGAAGTCAACGTAGAGGA-3'

2.3. Sequencing and Analysis

All recombinant DNA was sequenced, and determination of the gene, ORF, and protein sequence was performed using the Expert Protein Analysis System (ExPASy, http://au.expasy.org). Multiple sequence alignments and the phylogenetic tree were analyzed using the DNAMAN 8. Domain prediction was undertaken using the simple modular architecture research tool (SMART, http://smart.embl-heidelberg.de/). Analysis of the C-terminal sequence of the protein with the Calmodulation Database and Meta-Analysis Predictor (CDMAP) server (http://cam.umassmed.edu), was performed to search the putative CaM-binding motifs within HcPMCA. HMMTOP [8] and TMHMM (http://www.cbs.dtu.dk/services/TMHMM), were used to predict transmembrane helices and to perform a topology analysis of HcPMCA.

2.4. Tissue Specific Expression of HcPMCA Gene

The tissue-specific expression analysis was performed by qRT-PCR in a real-time thermal cycler (ABI, USA) using synthesized cDNA as the template, based on a previous protocol [9]. Here, the sampled tissues were gonad, hepatopancreas, foot, mantle and gill. RNA preparation and cDNA synthesis were conducted using the methods mentioned above. Gene-specific primers for qRT-PCR were designed (**Table 1**). The 25-μL reaction mixture consisted of 12.5 μL of 2× qPCR Mix, 2.0 μL of 7.5 μM primers, 2.5 μL cDNA and 8.0 μL of dd H_2O was programmed as follows: 10 min at 95˚C; 40 cycles at 95˚C for 15 s, 60˚C for 60 s and 72˚C for 30 s; and a melt from 75˚C to 95˚C.

2.5. Calcium Stimulation Experiment

A series of increasing Ca^{2+} concentrations, 0 (control group), 40, 60, 80 and 100 mg/L, were established by adding $CaCl_2$ into 20L-tanks. 15 mollusks were collected and averagely divided into these five groups. After a 7-days' culture with aerated pond water at 25˚C, mantle tissue were sampled.

According to the former result, a calcium stimulation test with Ca^{2+} concentration of 60 mg/L was designed for a 7-days' culture. Mantle samples of three individual mollusks were isolated at 0 h, 24 h, 48 h, 96 h and 168 h, respectively. All samples were immediately washed with sterile PBS, frozen in liquid nitrogen and stored at −80˚C. The expression profiles of HcPMCA mRNA were detected with the qRT-PCR method mentioned above.

2.6. Statistical Analysis

When using qRT-PCR method, the β-actin was used as an internal control. All qRT-PCR tests were conducted three times using individual templates. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of HcPMCA [10]. Significant differences were determined by one-way analysis of variance and the Tukey's HSD test for multiple-range comparison using SPSS 16.0 software, with significant levels accepted at $p < 0.05$.

3. Results

3.1. Cloning of the HcPMCA cDNA

The HcPMCA cDNA had a full-length of 5230 bp (GenBank accession No. KR 080192.1), which comprised of a 417-bp 5'-UTR, a 3588-bp ORF, and a 1225-bp 3'-UTR. Also, it had a stop codon TAA and a poly (A) tail, but it had no poly (A) addition signal.

The deduced HcPMCA protein contained 1195 amino acids, within which Ile had the highest content (8.5%). The inferred HcPMCA protein had a theoretical molecular mass of 131.64 kDa and an isoelectric point (pI) of 6.05, but no putative signal peptide was predicted.

SMART analysis showed that the deduced amino acid sequence of HcPMCA presented typical characteristics of Ca^{2+}-ATPase, which was constitute by five domains, including Cation transporter/ATPase, N-terminus (Cation ATPase N) domain (residues 40 - 115), E1-E2 ATPase domain (residues 136 - 288 and residues 345 - 460), haloacid dehalogenase-like hydrolase (HAD) domain (residues 468 - 811), Cation transporting ATPase, C-terminus (Cation ATPase C) domain (residues 884 - 1066) and Plasma membrane calcium transporter ATPase C terminal (ATP Ca trans C) domain (residues 1106 - 1166). These domains contained common structures for PMCAs, such as Ca^{2+} translocation site, phosphorylation site, ATP binding site (**Figure 1**). In addition, the analysis of the C-terminal region from HcPMCA (1106-1166 residues) predicts a potential CaM-BD based on the density of canonical binding motifs and a number of patterns, including 1 - 10, 1 - 12, 1 - 14, 1 - 16, 1 - 5 - 8 - 14, 1 - 8 - 14 and IQ-LIKE Ca^{2+}/CaM-binding motifs were predicted (**Table 2**). Generally, PMCA consists of 10 transmembrane α-helices [11], which is also consistent with our result of HcPMCA.

3.2. Phylogenetic Analyses

The BLASTp search analyses demonstrated that HcPMCA shared the highest identity (74%) with *T. squamosa* PMCA (AML22897.1), followed by *M. yessoensis* PMCA 2-like (XP_021343203.1), *Crassostrea virginica* PMCA 2-like (XP_022299339.1), *P. fucata* PMCA (ABL63470.1) and *Crassostrea gigas* PMCA 2 (XP_019926614.1) successively with 71%, 69%, 68% and 66% identity respectively. In contrast, although the deduced amino acid sequence of HcPMCA has 60% similarity with human PMCA4b (P23634-6), which was a little lower than that between HcPMCA and other mollusk PMCA sequences, their functional domains are highly conserved (**Figure 1**).

Based on the BLASTp results and the reported PMCA homologs in mollusks and arthropods, a phylogenetic tree was constructed to show their evolutionary relationships (**Figure 2**). In this tree, PMCA homologs were grouped into two clusters. HcPMCA together with TsPMCA, PfPMCA, CgPMCA, CvPMCA and MyPMCA formed a cluster, and the other PMCA homologs from insects belonged to another cluster, indicating that HcPMCA had a closer evolutionary relationship with other mollusks' PMCAs.

Figure 1. Putative Ca^{2+}-ATPase amino acid sequence from *H.cumingii*. Clustal alignment of *H.cumingii* PMCA and *Homo sapiens* PMCA4b (P23634-6). Identical residues are shown with an asterisk (*), and semi-conservative substitutions are labeled with a period (·). Transmembrane segments M1–M10 are shaded in grey. The ATP binding, phosphorylation and Ca^{2+} translocation site are highlighted in bold, indicating D and K residues in red bold.

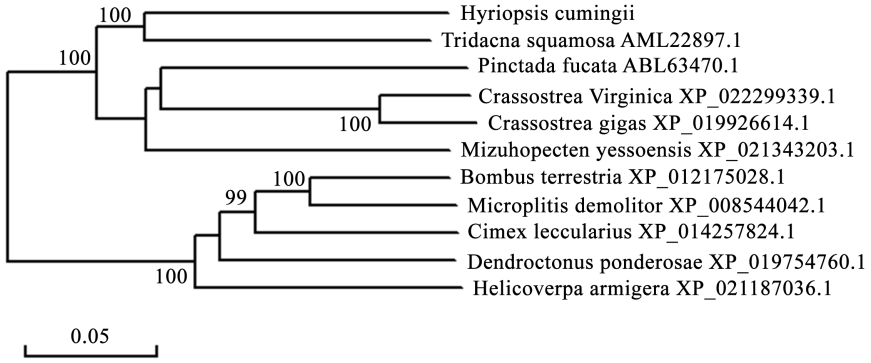

Figure 2. Phylogenetic analysis of PMCAs from shellfish and insects based on the BLASTp results. A neighbor-joining tree was constructed using the DNAMAN 8 software, and was adjusted by 1000 boot strap replicates. The corresponding GenBank accession No. are shown. The mollusk group is indicated with a brace.

Table 2. Predicted Calmodulin binding motifs in C-terminal region of HcPMCA gene.

Motif	Sequence	Residues
1 - 10	ILWVRGLTRL	1108 - 1117
1 - 10	LTRLQQQIRV	1114 - 1123
1 - 10	LQSLQAAQAF	1151 - 1160
1 - 10	LQAAQAFRGI	1154 - 1163
1 - 12	WVRGLTRLQQQI	1110 - 1121
1 - 12	VVNAFQMDIEAL	1123 - 1134
1 - 12	ISLQSLQAAQAF	1149 - 1160
1 - 14	ILWVRGLTRLQQQI	1108 - 1121
1 - 14	WVRGLTRLQQQIRV	1110 - 1123
1 - 14	VRGLTRLQQQIRVV	1111 - 1124
1 - 14	LTRLQQQIRVVNAF	1114 - 1127
1 - 14	IRVVNAFQMDIEAL	1121 - 1134
1 - 14	VNAFQMDIEALGGF	1124 - 1137
1 - 16	ILWVRGLTRLQQQIRV	1108 - 1123
1 - 16	LWVRGLTRLQQQIRVV	1109 - 1124
1 - 16	LGGFDSYDKRSRPSMI	1134 - 1149
1 - 5 - 8 - 14	WVRGLTRLQQQIRV	1110 - 1123
1 - 8 - 14	WVRGLTRLQQQIRV	1110 - 1123
1 - 8 - 14	LTRLQQQIRVVNAF	1114 - 1127
1 - 8 - 14	VNAFQMDIEALGGF	1124 - 1137
IQ-LIKE	LQQQIRVVNAFQMD	1117 - 1130

3.3. Tissue Expression of HcPMCA mRNA

The tissue-specific expression of HcPMCA in the gonad, hepatopancreas, foot, mantle and gill was analyzed by qRT-PCR using β-actin as an internal control.

HcPMCA mRNA was detected in all the tested tissues, and expressed the highest level in the gill, whereas the lowest level in the foot (Figure 3).

3.4. Expression of HcPMCA mRNA under Calcium Stimulation

The HcPMCA transcripts in the mantle were significantly decreased under environmental calcium stimulation, with the highest expression level at the Ca^{2+} concentration of 60 mg/L, whereas the lowest level at the 40 mg/L (Figure 4(a)). Also, The temporal expression profile of HcPMCA at Ca^{2+} concentration of 60 mg/L was examined, which showed that mRNA expression of HcPMCA gene in mantle was bell-shaped with the exposure time increased. Detailly, the HcPMCA transcripts in mantle were slightly decreased at 24 h after treatment, but were upregulated from 24 to 48 h post-treatment, peaking at 48 h post-treatment (Figure 4(b)). Moreover, the HcPMCA transcripts were downregulated at the following period, with the expression amounts at 96h and 168 h post-treatment were nearly in line with that at 24 h.

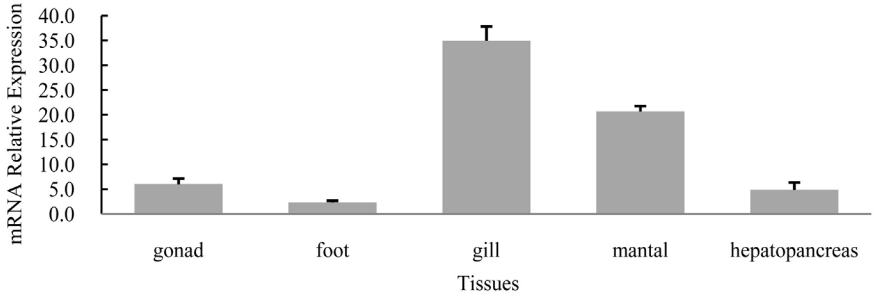

Figure 3. The relative expressions of HcPMCA gene in different tissues from *H. cumingii*.

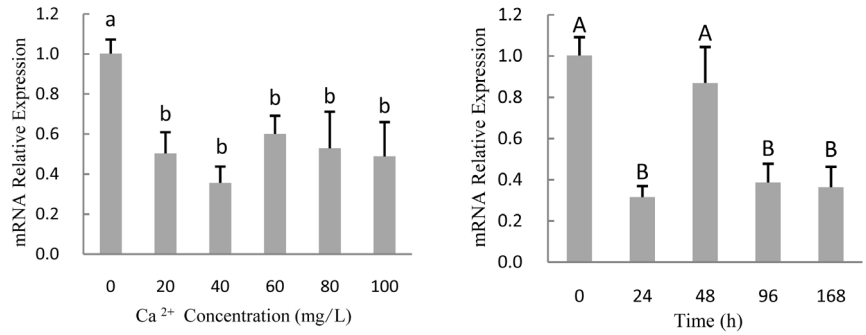

Figure 4. Relative expression level of HcPMCA detected by real-time PCR from *H. cumingii*. *β*-actin was amplified as an internal control. The transcripts expression profile of HcPMCA (a) in the mantle under different Ca^{2+} concentration treatment, and (b) the temporal expression profile under Ca^{2+} concentration of 60 mg/L. The columns represent mean value and the error bars represent SD. The lowercase letters represent the results of Tukey's HSD test among Ca^{2+} concentration of 0, 20, 40, 60, 80 and 100 mg/L. The capital letters represent the temporal results of Tukey's HSD test under Ca^{2+} concentration of 60 mg/L. The different lowercase letters and capital letters mean significant difference at $p < 0.05$ and $p < 0.01$ respectively.

4. Discussion

4.1. The Structural Characteristics of HcPMCA

Compared with PMCA homologs from seawater mollusks, HcPMCA had high similarity with them in both sequence and structure. Despite some differences in amino acid sequence, our results suggest that HcPMCA folds in a similar manner to "P-type" Ca^{2+}-ATPase proteins from higher eukaryotes, and contains all the conserved protein domain found in other P-type ATPases [12] [13], suggesting that HcPMCA might possess the same physiological function as other PMCA homologs. Meanwhile, it's found that a series of predicted patterns, such as 1 - 10, 1 - 12, 1 - 14, 1 - 16 and IQ-LIKE motifs at the C-terminal region from HcPMCA were the canonical classical Ca^{2+}/CaM-binding ones that have been described in other CaM-modulated proteins such as CaM-dependent protein-kinase II, MLCK and KCNQ channels [14] [15] [16], indicating HcPMCA may be one CaM-modulated protein.

As we all know, Mammalian PMCA exists in four isoforms, with each isoform possessing multiple splice variants [17]. PMCA1 and 4 are generally universally expressed and serve housekeeping functions. Whereas, PMCA2 and 3 are differentiated by cell type [18]. Here, aignment between HcPMCA and Eukaryota PMCAs using SMART revealed that HcPMCA had the same domain with many PMCAs, such as PMCA1 from *Homo sapiens,* PMCA1 from *Sus scrofa,* PMCA1 from *Rattus norvegicus*, PMCA2 from *Homo sapiens*, PMCA2 from *Mus musculus,* PMCA3 from *Homo sapiens and* PMCA4 from *Homo sapiens*. Obviously, it is necessary to carry out further exploration that involves the separate isoforms of this gene and concrete functions in the calcium homeostasis of *H. cumingii*.

4.2. Tissue Specific Expression Profile of HcPMCA Gene

Both freshwater and seawater mollusks absorb calcium actively from environment, but their calcium metabolism mechanisms are significant differences. Some of the Ca^{2+} absorbed by freshwater mollusks can be stored as calcium spheres for later usage [19], while seawater mollusks are opposite. In freshwater mollusks, foot is the main locomotion organ, gonads are an important part of reproductive system, hepatopanpancreas are closely related to digestive and immune functions, mantle is an important tissue secreting calcium to the mineralization site [20], and gill is a pivotal tissue for calcium uptake from water [21]. Tissue-specific expression analysis revealed that HcPMCA mRNA was detected in all these five tissues, indicating HcPMCA may be a housekeeping PMCA isoform. Among these sampled tissues, mantle is the site of pearl formation, and is highly permeable to Ca^{2+} [22] [23]. Both of its inner and outer epidermis have the functions of absorbing and storing Ca^{2+} actively with a high utilization rate of Ca^{2+} [24]. Gill, as respiratory and filter feeding organ of mussels, is another important tissue for calcium metabolism with a strong affinity and a high metabolic rate of Ca^{2+}. Therefore, calcium metabolism in the mantle and gill is vigorous, and the main-

tenance of calcium homeostasis is especially vital. The highest expression level of HcPMCA in the gill and mantle indicates that the gene plays an important role in Ca^{2+} transportation and extruding, engaging in calcium homeostasis and biomineralization processes, such as pearl formation. In addition, the lower mRNA expression of this gene in hepatopancreas, gonad and foot, may indicate that the abilities of extruding calcium from the cytosol into the extracellular space of these there tissues were weak. Further studies on the mechanisms of calcium absorption, storage and transport are still needed.

4.3. Expression Profile of HcPMCA Gene under Calcium Stimulation

Environmental calcium concentration is one of the important factors affecting calcium metabolism of mollusk. when Ca^{2+} was added, the flow direction of extracellular Ca^{2+} gradually changed from efflux to internal flow, and the flow velocity of Ca^{2+} increased with the increase of Ca^{2+} concentration, which resulted in the enhancement of intracellular fluorescence signal [19]. A similar view was drawn in this study. Here, the HcPMCA transcripts in the mantle were significantly decreased after calcium was added into the aquatic water, suggesting the existence of free enviromemtal Ca^{2+} may inhibit the expression of this gene, accumulating more cytosolic calcium concentration in cells in order to guarantee future use.

The regulatory factors related to calcium metabolism may participate in or regulate the formation of shell/pearl through calcium absorption, transport, storage and deposition. The absorption and transport of Ca^{2+} play an important role in improving the yield of pearls in the freshwater pearl cultivation process. Accelerating the deposition of Ca^{2+} in the pearl sac is an important way to promote the growth of pearls rapidly. However, the calcium content is very low in natural water. Thus, maintaining appropriate Ca^{2+} concentration in water is a prerequisite for the growth of pearls. A appropriate concentration of Ca^{2+} could promote the absorption, transport and storage of Ca^{2+} in the mantle of mussels, promoting the calcium metabolism process and accelerating the formation of pearls, while excessive content of Ca^{2+} would inhibit the calcium metabolism of mantle [25] [26].

In this study, the expression level of HcPMCA gene in the mantle reached the highest expression levels at the Ca^{2+} concentration of 60 mg/L, and the lowest level at the 40 mg/L. It was speculated that the mantle secreted calcium actively to the biomineralization site to participate in pearl growth at the the Ca^{2+} concentration of 60 mg/L, resulting in enhancement of extruding function. While, the result was opposite when the Ca^{2+} concentration was 60 mg/L. In addition, the present study also found that the mRNA expression of HcPMCA gene in mantle was bell-shaped with the exposure time increased at Ca^{2+} concentration of 60 mg/L, and peaked at 48 h post-treatment, indicating that the mantle of molusck had a strong ability of transporting Ca^{2+} at 48 h, and then decreased.

5. Conclusion

In this study, a putative PMCA from *H. cumingii* (HcPMCA) was cloned by using SMART RACE technique. The entire HcPMCA cDNA was 5230 bp, encoding a 1195-amino acid protein, and no putative signal peptide was predicted. HcPMCA had high similarity with PMCA homologs from seawater mollusks in both sequence and structure, and the mRNA was detected in all the sampled tissues. Meanwhile, its expression profile in different calcium stress was explored using quantitative real-time polymerase chain reaction (qRT-PCR). It's confirmed that the putative HcPMCA is a critical calcium transportation regulator that involved in calcium homeostasis for *H. cumingii*. However, further studies are needed to clarify whether the expression profiles of the mantle in different growth stages or in different positions are consistent, and further explorations that involve the separate isoforms of this gene and concrete functions in the calcium homeostasis of *H. cumingii* are also necessary.

Acknowledgements

This research was provided by Zhejiang Science and Technology Project (No. 2019C02047) to Zhiming Zhou.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Docampo, R. and Huang, G. (2015) Calcium Signaling in Trypanosomatid Parasites. *Cell Calcium*, **57**, 194-202. https://doi.org/10.1016/j.ceca.2014.10.015

[2] Peterson, J.A., Oblad, R.V., Mecham, J.C. and Kenealey, J.D. (2016) Resveratrol Inhibits Plasma Membrane Ca^{2+}-ATPase Inducing an Increase in Cytoplasmic Calcium. *Biochemistry & Biophysics Reports*, **7**, 253-258. https://doi.org/10.1016/j.bbrep.2016.06.019

[3] Schatzmann, H.J. (1966) ATP-Dependent Ca^{++}-Extrusion from Human Red Cells. *Experientia*, **22**, 364. https://doi.org/10.1007/BF01901136

[4] Carafoli, E. and Stauffer, T. (2010) The Plasma Membrane Calcium Pump: Functional Domains, Regulation of the Activity, and Tissue Specificity of Isoform Expression. *Developmental Neurobiology*, **25**, 312-324. https://doi.org/10.1002/neu.480250311

[5] Rasmussen, H. and Barrett, P.Q. (1984) Calcium Messenger System: An Integrated View. *Physiological Reviews*, **64**, 938-984. https://doi.org/10.1152/physrev.1984.64.3.938

[6] Wang, X., Fan, W.M., Xie, L.P. and Zhang, R.Q. (2008) Molecular Cloning and Distribution of a Plasma Membrane Calcium ATPase Homolog from the Pearl Oyster *Pinctada fucata*. *Journal of Tsinghua University* (*Science and Technology*), **13**, 439-446. https://doi.org/10.1016/S1007-0214(08)70071-3

[7] Zhang, A.J., Liu, S.L., Zhu, J.Y., Gu, Z.M. and Lu K.H. (2016) Transcriptome Analysis of the Freshwater Pearl Mussel, *Hyriopsis cumingii* (Lea) Using Illumina Paired-

End Sequencing to Identify Genes and Markers. *Iranian Journal of Fisheries Sciences*, **15**, 1425-1440.

[8] Tusnády, G.E. and Simon, I. (2001) The HMMTOP Transmembrane Topology Prediction Server. *Bioinformatics*, **17**, 849-850. https://doi.org/10.1093/bioinformatics/17.9.849

[9] Li, X.C., Zhu, L., Li, L.G., Ren, Q. and Huang, Y.Q. (2013) A Novel Myeloid Differentiation Factor 88 Homolog, SpMyD88, Exhibiting Sp Toll-Binding Activity in the Mud Crab *Scylla paramamosain*. *Developmental and Comparative Immunology*, **39**, 313-322. https://doi.org/10.1016/j.dci.2012.11.011

[10] Livak, K.J. and Schmittgen, T.D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta}$CT Method. *Methods*, **25**, 402-408. https://doi.org/10.1006/meth.2001.1262

[11] Lopreiato, R., Giacomello, M. and Carafoli, E. (2014) The Plasma Membrane Calcium Pump: New Ways to Look at an Old Enzyme. *Journal of Biological Chemistry*, **289**, 10261-10268. https://doi.org/10.1074/jbc.O114.555565

[12] Brini, M. and Carafoli, E. (2011) The Plasma Membrane Ca^{2+} ATPase and the Plasma Membrane Sodium Calcium Exchanger Cooperate in the Regulation of Cell Calcium. *Cold Spring Harbor Perspectives in Biology*, **3**, 487-496. https://doi.org/10.1101/cshperspect.a004168

[13] Brini, M. and Carafoli, E. (2014) Calcium Pumps in Health and Disease. *Cardiac Electrophysiology from Cell to Bedside*, **89**, 43-53. https://doi.org/10.1016/B978-1-4557-2856-5.00005-4

[14] Barbato, G., Ikura, M., Kay, L.E., Pastor, R.W. and Bax, A. (1992) Backbone Dynamics of Calmodulin Studied by 15N Relaxation Using Inverse Detected Two-Dimensional NMR Spectroscopy: The Central Helix Is Flexible. *Biochemistry*, **31**, 5269-5278. https://doi.org/10.1021/bi00138a005

[15] Moore, C.M., Hoey, E.M., Trudgett, A. and Timson, D.J. (2012) A Plasma Membrane Ca^{2+}-ATPase (PMCA) from the Liver Fluke, *Fasciola hepatica*. *International Journal for Parasitology*, **42**, 851. https://doi.org/10.1016/j.ijpara.2012.06.003

[16] Mruk, K., Farley, B.M., Ritacco, A.W. and Kobertz, W.R. (2014) Calmodulation Meta-Analysis: Predicting Calmodulin Binding via Canonical Motif Clustering. *Journal of General Physiology*, **144**, 105-114. https://doi.org/10.1085/jgp.201311140

[17] Carafoli, E. and Guerini, D. (1993) Molecular and Cellular Biology of Plasma Membrane Calcium ATPase. *Trends in Cardiovascular Medicine*, **3**, 177-184. https://doi.org/10.1016/1050-1738(93)90003-O

[18] Carafoli, E. and Lim, D. (2012) Plasma Membrane Calcium ATPase. *Acta Biophysica Sinica*, **28**, 581-596. https://doi.org/10.1007/978-0-387-30370-3_32

[19] Hao, Y.Y. (2011) Effect of Three Different Factors on the Calcium Ion Metabolism and Expression of Alkaline Phosphatase Gene in *Hyriopsis cumingii*. Shanghai Ocean University, Shanghai.

[20] Cho, J.H., Bandyopadhyay, J., Lee, J., Park, C.S. and Ahnn, J. (2000) Two Isoforms of Sarco/Endoplasmic Reticulum Calcium ATPase (SERCA) Are Essential in *Caenorhabditis elegans*. *Gene*, **261**, 211-219. https://doi.org/10.1016/S0378-1119(00)00536-9

[21] Beirão, P.S., Hamilton, J. and Nascimento, M. (1989) Sodium- and Calcium-Dependent Mechanisms in the Action Potential of the Secretory Epithelium of a Clam Mantle. *Journal of Experimental Biology*, **145**, 395-402.

[22] Kirschner, L.B., Sorenson, A.L. and Kriebel, M. (1960) Calcium and Electric Poten-

tial across the Clam Mantle. *Science*, **131**, 735.
https://doi.org/10.1126/science.131.3402.735-a

[23] Coimbra, J., Machado, J., Fernandes, P.L., Ferreira, H.G. and Ferreira, K.G. (1988) Electrophysiology of the Mantle of *Anodonta cygnea*. *Journal of Experimental Biology*, **140**, 65-88.

[24] Tang, M. and Shi, A.J. (2000) Studies of Environmental Calcium Concentration Effect on the Calcium Metabolism of the Mantle and Pearl Sac of the Freshwater Pearl Mussel. *Journal of Sichuan University*, **37**, 741-747.

[25] Li, W.J., Shi, Z.Y., Hao, Y.Y., Han, J., Qi, L.L. and Ye, X.F. (2011) Research of Calcium Flux by Ion-Selective Microelectrodes in Mantle Tissue of *Hyriopsis cumingii*. *Acta Hydrobiologica Sinica*, **35**, 545-549.

[26] Shu, M.A., Hu, H.J., Lu, J.Y., Xu, B.P., Wang, Y., Liu, G.X. and Guo, X.L. (2013) Full-Length cDNA Cloning and Expression Analysis of *calreticulin* Gene from *Hyriopsis cumingii*. *Acta Hydrobiologica Sinica*, **37**, 999-1006.