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Characterization of a novel carbonic anhydrase from freshwater pearl mussel *Hyriopsis cumingii* and the expression profile of its transcript in response to environmental conditions

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ABSTRACT

Gene encoding for α-carbonic anhydrases (α-CAs) and their functions in fundamental metabolism and biomineralization are widely identified in mollusks. However, the transcriptional regulation of α-CA genes in response to various environmental conditions remains unknown. In the present study, we characterized a cDNA encoding for an α-CA (*HcCA*) from the freshwater pearl mussel *Hyriopsis cumingii*. The spatial and temporal expression patterns of *HcCA* indicate that this gene is mainly expressed in the mantle of juvenile mussels. The expression profile of *HcCA* under various environmental conditions reveals that the transcription of *HcCA* is significantly regulated by Ca^{2+} concentration, water temperature, pH and air exposure. Our results suggest that *HcCA* is a crucial target gene by which the external environmental conditions affecting shell growth and pH homeostasis of *H. cumingii*.
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1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) form a family of enzymes that catalyze the process of reversible hydration of CO_2 to yield HCO_3^- and H^+ in the carbonic acid equilibrium ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$) (Badger and Price, 1994). CAs can participate in various physiological processes, such as respiration, pH homeostasis, ion transport, photosynthesis, synthesis of fatty acid and amino acid and biomineralization (Henry, 1996; Medakovic, 2000). To date, five CA (α, β, γ, δ, and ζ-CAs) families have been identified, and the α-CA is the predominant group in metazoans (Bertucci et al., 2013).

Numerous CA genes and proteins have been identified from mollusks, exhibiting versatile functions related to biomineralization (Le Roy et al., 2012; Marie et al., 2008; Miyamoto et al., 1996; Norizuki and Samata, 2008) since Freeman and Wilbur (1948) first reported the carbonic anhydrase activity in the mantle of mollusks. Basically, CAs provide the substrate of HCO_3^- by hydrating CO_2 for $CaCO_3$ crystallization in molluscan biomineralization. Some studies revealed that CAs can regulate Ca^{2+} transport and maintain Ca^{2+} homeostasis in the mantle of mollusks (Ebanks et al., 2010; Istin and Girard, 1970; Lopes-Lima et al., 2008). Nacrein, a type of CAs, is composed of a carbonic anhydrase domain and a Gly-X-Asn repeat domain, and is one of the organic matrix proteins accumulated in shells (Miyamoto et al., 2005). Nacreins in oysters *Pinctada fucata* and *Crassostrea nippona* can regulate the structure of $CaCO_3$ crystal in shells by the calcium-bonding NG repeat domain (Miyamoto et al., 1996; Norizuki and Samata, 2008). Recent studies reported that CAs participate in pH homeostasis of mollusks (Connor and Gracey, 2011; Dickinson et al., 2012).

Hyriopsis cumingii (Bivalvia: Unionidae) is a commercially important freshwater mussel in pearl farming, and provides more than 95% pearl production in the world (Wang et al., 2009). This mussel is widely cultured in ponds, lakes and rivers of middle and lower reaches of Yangtze River, China, and exhibits growth variation under different environmental conditions such as Ca^{2+} concentration, water temperature and pH. The optimal water temperature (24–28 °C) and neutral pH allow fast growth of *H. cumingii* and fast secretion of nacre in the mantle (Qiu and Shi, 1999; Xu et al., 1988). In commercial farming, *H. cumingii* is occasionally exposed to air for an extended period of time (e.g., over 1 day). The molecular mechanisms of the mussel in adaption to environmental change are unknown. In the present study, we identified an α-CA cDNA, designated as *HcCA* from *H. cumingii*, and analyzed its spatial and temporal expression patterns by real-time quantitative PCR. We also analyzed the expression profiles of *HcCA* under different Ca^{2+} concentrations, temperatures, pHs and air exposure. The objective of the

Abbreviations: CA, carbonic anhydrase; RACE, rapid amplification of cDNA ends; NCBI, National Center for Biotechnology Information; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; NLP, nacrein-like protein; ANOVA, analysis of variance; UTR, untranslated region.

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present study is to explore the function and transcriptional regulation mechanism of *HcCA* in metabolism and biomineralization of *H. cumingii*.

2. Materials and methods

2.1. Mussel

Hyriopsis cumingii at various developmental stages (embryos, larvae, mussels of 1, 2 or 4 years old) was collected from a commercial pearl mussel farm near Fengqiao Town, Zhuji City, Zhejiang Province, China (29°48 E, 120°23 N). 2-Year old mussels were used for cloning of *HcCA* cDNA fragment and expression analysis of *HcCA* mRNA under different Ca^{2+} concentrations, temperatures, pHs and air exposure. The spatial expression profile of *HcCA* in different tissues was tested with grafted mussels of 4 years old, and the temporal expression profile of *HcCA* was tested with embryos, larvae and mussels of 1, 2 or 4 years old.

2.2. Identification of characterization of HcCA

2.2.1. cDNA fragment amplification

Total RNA was extracted from the mantle pallial in non-grafted mussels of 2 years old using RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's protocol, and quantified by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). 2.0 μg of total RNA was reversely transcribed with a PrimeScript® reverse transcriptase kit (TaKaRa) and primed with Oligo$(dT)_{18}$. The partial *HcCA* cDNA fragment was amplified with the gene specific primers HcCAF1 and HcCAR1 which were designed on the conserved region of *P. fucata nacrein* cDNA sequence (GenBank accession no. D83523). The PCR product was electrophoresed on an agarose gel, purified with a DNA fragment purification kit (TaKaRa), and cloned into a pMD19-T vector with a TA cloning kit (TaKaRa). The recombinant plasmids were transformed into trans 5α chemically competent cell of *Escherichia coli* (TransGen Biotech, Beijing, China). The positive clones were screened by PCR with primers the HcCAF1 and HcCAR1 primers, and then subsequently sequenced.

2.2.2. Rapid amplification of cDNA ends (RACE)

The 3′- and 5′-ends of cDNA were amplified by nested PCR using the FirstChoice® RLM-RACE kit (Ambion, Austin, TX, USA) and the SMART® RACE kit (Clontech, Palo Alto, CA, USA) respectively according to the manufacturer's protocols. The gene specific primers for RACE were designed according to the partial *HcCA* cDNA sequence obtained above (Table 1). 3′- and 5′-RACE products were isolated, purified, cloned, and sequenced as described in the cDNA fragment amplification.

2.2.3. Bioinformatics analysis

The full-length cDNA sequence was identified by BLASTx analysis in NCBI database (http://www.ncbi.nlm.nih.gov/). The predicted amino acid sequence was determined using the BLAST Network Service on the expert protein analysis system (http://web.expasy.org/blast/). Prediction of CA domain was performed using the simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de/). The potential N-glycosylated site was predicted by NetNGlyc 1.0 tool (http://www.cbs.dtu.dk/services/NetNGlyc/). In silico analysis tools including SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/), PredictProtein (http://www.predictprotein.org), TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM), and TMpred (http://www.ch.embnet.org/software/TMPRED) were integrated to predict the subcellular localization of HcCA protein. Multiple alignments of amino acid sequences of molluscan CA domains were performed using the ClustalX 1.81 software (http://www.clustal.org/), and edited by the GeneDoc software (http://www.psc.edu/biomed/genedoc). The Gly–Aln repeats in nacrein and nacrein-like protein sequences were removed before the alignment. The parameters of all bioinformatics tools mentioned above are set by default.

2.3. Temporal and spatial expression of HcCA

2.3.1. Sample collection

Spatial expression of *HcCA* in tissues was tested with grafted mussels of 4 years old (shell length 170 ± 8 mm, mean ± SD, n = 6). Thirteen samples were collected from gill (GL), labial palp (LP), hepatopancreas (HP), intestine (IN), adductor muscle (AM), pearl sac (PS), mantle center (MC), outer epithelium of the posterior mantle pallial (OpMP), inner epithelium and connective tissue of the posterior mantle pallial (IpMP), mantle edge (ME), foot (FT), hemolymph (HM) and gonad (GN), respectively. The samples used to test of temporal expression profile of *HcCA* at different development stages including whole embryos, whole larvae and the mantle center collected from mussels of 1, 2 or 4 years old (shell length of the mussels were 30 ± 2 mm, 80 ± 7 mm, and 170 ± 8 mm respectively, mean ± SD, n = 3). The embryos and larvae were isolated from the gill of mother mussels in May 2013 (n = 3). The samples were frozen in liquid nitrogen immediately and stored at −80 °C until RNA isolation.

2.3.2. Real-time PCR assay

Total RNAs from the embryos, larvae, and tissues of 1, 2 or 4 year old mussels were extracted using RNAiso Plus (TaKaRa). The cDNA from total RNA was synthesized by a PrimeScript® Reverse Transcriptase kit (TaKaRa), using oligo $(dT)_{18}$ as a primer. The gene-specific primers (RT-HcCAF and RT-HcCAR) for real-time quantitative PCR were designed according to the complete *HcCA* cDNA sequence (Table 1) with a correlation coefficient of $R^2 = 0.998$ and an amplification efficiency of 93.2%. *β-actin* (GenBank accession no. HM045420) was used as an endogenous reference gene for calibration with a correlation coefficient of $R^2 = 0.999$ and an amplification efficiency of 94.7% for the primers RT-actinF and RT-actinR. qRT-PCR was performed on an iQ™5 Real Time PCR detection system (Bio-Rad Laboratories, Hercules, USA) with the SYBR® Premix Ex Taq™ PCR kit (TaKaRa) according to the manufacturer's

Table 1
Primers used in the present study.

Primer name	Sequence 5′ → 3′	Sequence information
HcCAF1	CAATCTCCAATCAACATCGT	PCR amplifying of cDNA fragment
HcCAR1	TGGTCAGGGATCCCTCGTATG	PCR amplifying of cDNA fragment
3′ RACE adapter	GCGAGCACAGAATTAATACGACTCACTATAGGT12VN	3′ RACE reverse translation
HcCAF2	GCCGTACAGCGAGGGACAAG	1st round 3′ RACE
3′ RACE outer primer	GCGAGCACAGAATTAATACGACT	1st round 3′ RACE
HcCAF3	CTGGCGGACATTATCGGAAGC	2nd round 3′ RACE
3′ RACE inner primer	CGCGGATCCGAATTAATACGACTCACTATAGG	2nd round 3′ RACE
HcCAR2	GGGAGCGGCGTAACGTCCTTTTG	1st round 5′ RACE
HcCAR3	TTGACCACCTGGGCACAGCTTCC	2nd round 5′ RACE
RT-HcCAF	GTCTACAGTCAAAATGGTGGAAACG	qRT-PCR of *HcCA*
RT-HcCAR	CAGACCCAATGAGTTCACCCG	qRT-PCR of *HcCA*
RT-actinF	CCCTGGAATCGCTGACCGTAT	qRT-PCR of *β-actin*
RT-actinR	GCTGGAAGGTGGAGAGAGAAG	qRT-PCR of *β-actin*

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