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The characterization of *RHEB* gene and its responses to hypoxia and thermal stresses in the small abalone *Haliotis diversicolor*

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ABSTRACT

RHEB (**R**as **H**omolog **E**nriched in **B**rain) is a GTP-binding protein that is ubiquitously expressed in humans and other mammals. The protein is largely involved in the mechanistic target of rapamycin (mTOR) pathway, and regulates the cell cycle progression and growth. The goal of this study was to characterize the *RHEB* gene in the small abalone *Haliotis diversicolor*, and identify the responses of *RHEB* gene to stresses of hypoxia or/and thermal. The objectives were to: 1) clone the full-length cDNA *RHEB* gene in the *H. diversicolor* (*HdRHEB*); 2) quantify the expression of *HdRHEB* gene in tissues of haemocytes, mantle, kidney, gill, digestive tract, colleterial gland, and hepatopancreas by using RT-PCR, and 3) evaluate the responses of *HdRHEB* in gill and haemocyte to stresses of hypoxia (0.2 mg/l00 ml), thermal (31 °C), and combination of hypoxia (0.4 mg/l00 ml) and thermal (30 °C) at exposure time of 0, 4, 24, 96, and 192 h. The full length cDNA of *HdRHEB* was 1044 bp encoding a peptide of 182 amino acid residues. Expression of *HdRHEB* gene was detected in all of the 7 tissues and showed the highest in mantle ($P < 0.05$). Under hypoxia, expression of *HdRHEB* in gill increased significantly at 4 h, 24 h and 96 h ($P < 0.05$), and that in haemocyte increased significantly at 24 h, 96 h and 192 h ($P < 0.05$). Under thermal stress, expression of *HdRHEB* gene in gill decreased significantly at 4 h and 24 h, while expression in haemocyte decreased significantly all the time. Under thermal and hypoxia stresses, expression of *HdRHEB* gene in gill and haemocyte was up-regulated significantly at 24 h and 96 h ($P < 0.05$). The results in this study demonstrated for the first time that *RHEB* gene in abalones is able to response to stress stimuli of hypoxia or/and thermal.

1. Introduction

The small abalone *Haliotis diversicolor* is an important aquaculture species in the southern coast of China (Wang et al., 2004). With the rapid expansion of abalone farming, mass mortality occurred often in hot summer, and has become the major threat to the industry (Cai et al., 2014). Therefore, understanding of the molecular mechanism about responses to environmental stresses, such as high temperature and hypoxia, is essential for disease control and treatment, and will make contribution to sustain this large-scale industry.

The Ras Homolog Enriched in Brain (RHEB), a Ras-related GTP-binding proteins, was first cloned and identified in hippocampal granule cells of *Rattus norvegicus* (Yamagata et al., 1994). RHEB belongs to the Ras/Rap/Ral subfamily, and has a similar activity regulating mode to other small G proteins of the Ras family. It has been found in different tissues in mice (Goodman et al., 2010; Tamai et al., 2013), and is an important regulatory factor in a series of biochemical reactions (Goodman et al., 2010; Tamai et al., 2013). Different types of RHEB was observed among different species, for example, one type of RHEB (either *sRHEB* or *dRHEB*) occurs in fission yeast (Urano et al., 2005) and *Drosophila* (Stocker et al., 2003), but two types of RHEB (*hRHEB1* and *hRHEB2*) co-exist in human cells. In mollusks, research about RHEB has only been reported in *Admete californica* (Weatherill et al., 2010).

Recent research about RHEB focused majorly on the regulation of signaling pathway of mTOR (mammalian target of Rapamycin) and the occurrence of disease (Martin et al., 2014; Marshall et al., 2014; Xu et al., 2015). To inhibit mTOR - mediated downstream signaling, RHEB plays a role and is regulated by tuberous sclerosis complex (TSC) genes, which is an autosomal-dominant genetic disorder yielding the formation of benign tumors known as hamartomas. Two TSC genes, which encode protein products of hamartin (TSC1) and tuberin (TSC2) (Tee et al., 2002) with activity of GTPase (Inoki et al., 2003a, 2003b), function together with RHEB for this signal pathway. Recently, RHEB was also found to play an essential role in NF-κB pathway which is

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related to stresses in cells (Lee et al., 2007; Dan and Baldwin, 2008; Weichhart et al., 2008). Furthermore, overexpression of RHEB can be induced when TSC2 inhibits NF-κB signaling pathway (Ghosh et al., 2006). So far, most studies about RHEB were reported in mammals. Since RHEB is expressed in a variety of animal organs, and is involved in several important pathways for environmental stresses, we hypothesize that it plays roles in responses to hypoxia and thermal in shellfish too. In this study, the goal was to characterize the *RHEB* gene in the small abalone *Haliotis diversicolor*, and identify the responses of *RHEB* gene to stresses of hypoxia or/and thermal. The objectives were to: 1) clone the full-length cDNA *RHEB* gene in the *H. diversicolor* (*HdRHEB*); 2) quantify the expression of *HdRHEB* gene in tissues of haemocytes, mantle, kidney, gills, digestive tract, colleterial gland and hepatopancreas by using RT-PCR, and 3) evaluate the responses of *HdRHEB* in gill and haemocyte to stresses of hypoxia (0.2 mg/l00 ml), thermal (31 °C), and combination of hypoxia (0.4 mg/l00 ml) and thermal (30 °C) at exposure time of 0, 4, 24, 96, and 192 h.

2. Materials and methods

2.1. Animals and tissue sampling

Adult abalones with body length of 6.30 ± 0.50 cm and body weight of 16.70 ± 2.50 g (n = 300) were collected from Hongyun abalone farm (Zhangpu, Zhangzhou, Fujian Province) in July 2014. The abalones were maintained in a seawater recirculating system with sand-filter at controlled temperature of 26 °C, and fed with sea tangles once a day.

All sampled abalones were dissected immediately on ice. Tissues (gill, mantle, kidney, digestive tract, colleterial gland, and hepatopancreas) were removed and immediately stored in liquid nitrogen or RNAlater (Ambion, USA) until use for RNA isolation. Haemocytes were isolated by centrifugation 4000 × g at 4 °C for 10 min from haemolymph collected from foot cutoff, and were frozen immediately in liquid nitrogen after discarding the supernatant.

2.2. RNA isolation

Total RNA was extracted from all tissue samples using Trizol Reagent (Invitrogen, USA) per manufacture's protocol (Wang and Zhang, 2003). Total RNA quality was assessed by agarose gel electrophoresis and quantified by spectrophotometry (Nano Drop ND-2000, Thermo, USA).

2.3. Reverse transcription and clone of the full-length cDNA of HdRHEB

Two microgram total RNA and 2 μl of 10 μM Random primer were used to synthesize cDNA by M-MLV reverse transcriptase (Promega, USA). The synthesized cDNA was diluted by 10-fold and 100-fold and stored at − 20 °C until use. Primers used in reverse transcription included UPM, NUP, 5′CDS primer, 3′CDS primer and SMARTII, as listed in Table 1.

EST of *HdRHEB* was screened from the abalone transcriptome database in our laboratory. Based on the partial *HdRHEB* sequences, the other parts of the cDNA were obtained by 5′ and 3′ rapid amplification of cDNA ends (5′ and 3′ RACE) with annealing temperatures from 59 °C to 63 °C (Primers reference Table 1).

Sequence characterization and phylogenetic analysis were performed by using the following free software from different resources. The open reading frame (ORF) of the gene was found by ORF Finder of NCBI (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi). Theoretical isoelectric point and molecular weight prediction were computed at EXPASY database (http://web.expasy.org/compute_pi/). Potential N-glycosylation, phosphorylation and isoelectric point sites were predicted with CBS Prediction Servers (http://www.cbs.dtu.dk/services/). The protein domain was predicted with the simple modular architecture research tool (SMART) program (http://smart.embl-heidelberg.de/). Protein multiple alignments were performed with the EMBL-EBI server (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenies of protein sequences were estimated in MEGA 5.05 using the Neighbor-Joining (NJ) method. Bootstrap values were replicated 1000 times to obtain the required confidence value for the analysis.

2.4. Gene expression of HdRHEB in different tissues

Abalones (n = 6) cultured as normal condition (at 25 °C and 0.62 mg/100 ml) were sampled, tissues of haemocytes, mantle, kidney, gills, digestive tract, colleterial gland and hepatopancreas were samples for RNA isolation to evaluate the gene expression of *HdRHEB*.

2.5. Gene expression of HdRHEB to stresses of hypoxia, thermal, and hypoxia plus thermal

After one week of acclimation, abalones were exposed the stresses of: 1) *hypoxia* (0.2 mg/l00 ml): dissolved oxygen was adjusted to 0.2 mg/l00 ml at temperature of 25 °C (Zhang et al., 2014), and the control was set as dissolved oxygen of 0.62 mg/l00 ml at the same temperature (25 °C); 2) *thermal* (31 °C): thermal treatment was achieved by increasing the temperature from 25 °C at 1 °C per hour, and

Table 1
Oligo nucleotide primers used in this study.

Primer name	Nucleotide sequence (5′ → 3′)	Purpose
HdRHEB-out	GCCAGCAGTGTCCACAAGC	5′ race
HdRHEB-inner	GCCCATAATGGCTATTCGTCTC	
HdRHEB-out1	AATGGAGCGTTCAGAAAACATAGGG	3′ race
HdRHEB-inner1	CAGAAAACATAGGGCAACAGAAGAG	
HdRHEB-out2	AACATAGGGCAACAGAAGAGTGGAT	3′ race
HdRHEB-inner2	ACAAGGACGGTCAGAACCAAGG	
SMART II	AAGCAGTGGTATCAACGCAGAGTACGCGGG	5′ race
UPM (Universal Primer Mix) Long:	CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT	race
UPM (Universal Primer Mix) Short:	CTAATACGACTCACTATAGGGC	
NUP (Nested Universal primer)	AAGCAGTGGTAACAACGCAGAGT	
5′CDS primer[a]	(T)25VN	5′ race
3′CDS primer[a]	AAGCAGTGGTATCAACGCAGAGTAC(T)30VN	3′ race
HdRHEB-F	GAAGGCGCCATTTCTGGAATC	Realtime PCR
HdRHEB-R	TCCACTCTTCTGTTGCCCTA	Realtime PCR
β-actin-F	CCGTGACCTTACAGACTACCT	Realtime PCR
β-actin-R	TACCAGCGGATTCCATAC	Realtime PCR
elfa-F	ATGCCTTGTTGGCTTACACC	Realtime PCR
elfa-R	CAACAGCCTTGGGGTTGTAT	Realtime PCR

[a] N = A, C, G or T; V = A, G or C. Note: HdRHEB: Ras Homolog Enriched in Brain from *H. diversicolor*; SMART: Simple Modular Architecture Research Tool; SMARTII: Switching Mechanism At 5′ end of RNA Transcript4.