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Cloning, differential tissue expression of a novel *hcApo* gene, and its correlation with total carotenoid content in purple and white inner-shell color pearl mussel *Hyriopsis cumingii*

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ABSTRACT

As a molecular carrier and storage protein, apolipoprotein (Apo) mediates the intracellular uptake of lipids, proteins, vitamins and carotenoids. In this study, we identified a novel *Apo* gene, designated *hcApo*, from the freshwater pearl mussel *Hyriopsis cumingii*. The complete *hcApo* cDNA consists of 4104 nucleotides with an open reading frame encoding 1155 amino acid residues. The hcApo protein contains a conserved lipoprotein N-terminal domain (LPD-N) that is a characteristic of the large lipid transfer protein (LLTP) superfamily. The *hcApo* mRNA is constitutively expressed in a wide range of tissues with the highest expression level in the liver. Moreover, differential expression analysis revealed that the *hcApo* gene is more highly expressed in the liver, kidney, mantle and gill of purple line mussels compared to white line mussels. *In situ* hybridization investigations of the precise expression site of *hcApo* mRNA in the mantle showed that *hcApo* mRNA is specifically expressed in the outer epithelial cells of the middle fold and the inner epithelial cells of the outer fold of the mantle, as well as throughout the outer epithelium of the outer fold and ventral mantle. Another very important finding is that significantly positive correlation existed between the *hcApo* gene expression level and the total carotenoid content in purple line mussels. These findings may provide a better understanding of the roles of *hcApo* in the molecular mechanisms of shell formation and coloring of *H. cumingii*.

1. Introduction

The freshwater pearl mussel *Hyriopsis cumingii*, also known as triangle mussel, is a freshwater bivalve widely distributed in China and is the most commercially important mussel species exploited for freshwater pearl production in the country (Hua and Gu, 2002). China is the largest commercial producer of freshwater pearls, producing 3108 t in 2010 (FAO, 2012). The quality and value of freshwater non-nucleated pearl are correlated with five variations, including size, shape, color, luster and surface complexion, of which color is one of the most important criteria (Li and Liu, 2011). *H. cumingii* displays polymorphism in inner-shell colors including purple, faint yellow, pink and white. Purple pearls hold the greatest economic value among freshwater pearls with otherwise similar quality characteristics. Variations in shell and pearl colors have often been related to environmental effects, but they have also been demonstrated to be genetically controlled (Jerry et al., 2012). Recently, increasing attention has been paid to research into the factors affecting pearl coloring, including body coloring and structural coloring (Ji et al., 2013; Karampelas et al., 2009).

It is well known that the presence of carotenoids is related to body colors appearing in the tissues of many aquatic animals (Li et al., 2010; Ytrestoyl et al., 2004; Zheng et al., 2010). Previous studies have shown that carotenoids are abundant in some mollusks including Polyplacophora, Gastropoda, Bivalvia, and Cephalopoda (Matsuno, 2001). The principal carotenoids in shellfish are β-carotene, lutein A, zeaxanthin, diatoxanthin and pectenolone (Maoka, 2011; Schubert et al., 2006). However, mollusks are unable to synthesize carotenoids; therefore, carotenoids are absorbed from food and accumulated, with some carotenoids modified to other forms during the process of accumulation (Kantha, 1989). In our laboratory, purple line mussels (mussels with purple nacre inside the shell) and white line mussels (mussels with white nacre inside the shell) have been under mass selection since 2001, and the fourth selected generation was reached in 2009 (Bai et al., 2013). Previous studies on this species showed that the total carotenoid content in purple line mussels is higher than that in white line mussels, and shell colors have been confirmed to be partially inherited (Zhu, 2011). However, until now, no studies on the molecular

Abbreviations: Apo, apolipoprotein; LLTP, large lipid transfer protein; MTP, microsomal triglyceride transfer protein; TCC, total carotenoid content; qRT-PCR, quantitative real-time PCR; RACE, Rapid Amplification of cDNA Ends; ISH, *in situ* hybridization; ORF, open reading frame; DIG, digoxigenin.

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mechanisms of carotenoid absorption and shell coloring in *H. cumingii* have been reported.

In animals, the assembly, transportation, and metabolism of lipoproteins require members of the large lipid transfer protein (LLTP) superfamily, including the cytosolic large subunit of the microsomal triglyceride transfer protein (MTP), vertebrate apolipoprotein B (apoB), vitellogenin (Vtg), and the insect apolipophorin II/I precursor (apoLp-II/I) (Smolenaars et al., 2007). Apolipoprotein (Apo) plays a crucial role in the assembly and transportation of lipids, proteins, vitamin and carotenoids (Weers and Ryan, 2006). To date, cloning and gene expression levels of Apo have been mainly focused on mammals, insects, and fishes (Choudhury et al., 2011; Ramsoondar et al., 1998; Smolenaars et al., 2005). However, few studies regarding this gene have been conducted in mollusks, and none in *H. cumingii*. The purpose of this study was to clone and characterize the *Apo* gene from *H. cumingii*, and to investigate tissue-specific and differential expression levels of *hcApo* in purple and white line mussels. Furthermore, total carotenoid content was determined simultaneously, in order to investigate whether there is correlation between hcApo and carotenoids. *In situ* hybridization was performed to detect the precise position of *hcApo* in the mussel mantle — the main tissue responsible for shell and pearl formation. The results of the present study may provide the basis for further studies on Apo and its function in the molecular mechanisms of shell formation and coloring in *H. cumingii*.

2. Materials and methods

2.1. Animals and tissue sample

Live freshwater pearl mussels (200 ± 30 g body weight and 10 ± 2.0 cm shell length) with purple (P-line) and white (W-line) innershell colors were collected from a freshwater mussel farm in Jinhua (Zhejiang, China) in April 2013, and all mussels were 2-year old, with 30 P-line individuals and 30 W-line individuals (Fig. 1). The mussels were kept in a 120 L aerated circulating freshwater aquaria at 23 ± 2.0 °C and fed twice daily (in the morning and late in the afternoon) with *Chlorella vulgaris* for one week before experimental analyses. For expression analysis, various tissues including the blood, hepatopancreas, kidney, gill, axe foot, intestine, mantle, and adductor muscle were separately sampled from six P- and W-line individuals. All samples were immediately frozen in liquid nitrogen and then stored at −80 °C for the following experiments.

2.2. Full-length cDNA cloning of the hcApo gene

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and stored at −80 °C after incubation with RNase-free gDNA Eraser (TaKaRa, Kyoto, Japan). Based on expressed sequence tags (ESTs) from a *de novo* transcriptomic library of *H. cumingii*, primers were designed to target the *hcApo* gene (Bai et al., 2013). The synthesized cDNA was used as the template to amplify the central fragment with the gene-specific primers F1 and R1 (Table 1), which corresponded to the *hcApo* fragment sequence obtained from the transcriptomic library. The PCR program was as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min; a final elongation step of 72 °C for 10 min. The PCR products were isolated using a Gel Extraction Kit (Tiangen Biotech Ltd., Beijing, China) and cloned into a PMD19-T vector (TaKaRa). The recombinant vector was used to transform competent *Escherichia coli* DH5a cells which were plated on LB-agar and incubated overnight at 37 °C. Colony PCR was used to screen positive clones. Three positive clones were picked from each plate and sequenced.

The full-length of the *hcApo* gene was obtained using 5′- and 3′-RACE-PCR respectively. The gene-specific primers (A5race and A3race) were designed according to the conserved region sequences. RACE-PCR was conducted with the SMART™ RACE cDNA Amplification Kit and Advantage 2 PCR Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. The PCR conditions were five cycles of 94 °C for 30 s; 70 °C for 1 min; 72 °C for 3 min, followed by 32 cycles of 94 °C for 30 s; 68 °C for 1 min; 72 °C for 3 min. A final elongation step of 10 min was carried out at 72 °C. PCR products were cloned and sequenced as previously described. All primers used in this study are listed in Table 1.

2.3. Sequence and phylogenetic analysis

The cDNA and deduced amino acid sequences of *hcApo* were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/). The signal peptide cleavage site was predicted by the SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011). The sequences of Apo from different species were compared in BLASTx search of the GenBank sequences. The phylogenetic tree was constructed with CLUSTAL X2.0 (Larkin et al., 2007) and MEGA 5.0 (Tamura et al., 2011) using the neighbor-joining (NJ) algorithm and the reliability of the branching was evaluated by the bootstrap method with 1000 pseudoreplicates.

2.4. Tissue-specific gene expression of hcApo

In order to examine the expression of the *hcApo* mRNA, tissues were collected separately from six P-line mussels. Total RNA treated with DNase I (TaKaRa, Japan) from the tissues was submitted to reverse transcription (RT) using RNAiso plus reagent (TaKaRa, Japan). The sequences of *hcApo* cDNA specific primers used in semi-quantitative RT-PCR were showed in Table 1. The reaction conditions were: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and finally, 72 °C for 5 min. Meanwhile, *H. cumingii* β-actin was used as an internal control. The volume of each cDNA pool was adjusted to give the same amount of the β-actin product in the exponential phase. The analyses were representative of six independent RT reactions followed by PCR assays from each set of total RNA. The reaction conditions were the same as those stated above. The RT-PCR products were separated by electrophoresis on a 1.5% agarose gel and verified by sequencing.

Fig. 1. Inner-shell color of *H. cumingii*. The purple color variant is shown on the left.