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Research paper

# Identification of a tyrosinase gene and its functional analysis in melanin synthesis of *Pteria penguin*

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ABSTRACT

Tyrosinase is a key rate-limiting enzyme in melanin synthesis. In this study, a new tyrosinase gene (*Tyr*) was identified from *Pteria penguin* and its effect on melanin synthesis was deliberated by RNA interference (RNAi). The cDNA of *PpTyr* was 1728 bp long, containing a 5′untranslated region (UTR) of 11 bp, a 3′UTR of 295 bp, and an open reading fragment of 1422 bp encoding 473 amino acids. Amino acid alignment showed *PpTyr* had the highest (50%) identity to tyrosinase-like protein 1 from *Pinctada fucata*. Phylogenetic tree analysis classified *PpTyr* into α-subclass of type-3 copper protein. Tissue expression analysis indicated that *PpTyr* was highly expressed in mantle, a nacre formation related tissue. After *PpTyr* RNA interference, *PpTyr* mRNA was significantly inhibited by 71.0% ($P < 0.05$). For other melanin-related genes, *PpCreb2* and *PpPax3* expression showed no significant change, but *PpBcl2* was obviously increased. By liquid chromatograph-tandem mass spectrometer (LC-MS/MS) analysis, the total content of PDCA (pyrrole-2, 3-dicarboxylic acid) and PTCA (pyrrole-2,3,5-tricarboxylic acid), as main markers for eumelanin, was sharply decreased by 66.6% after *PpTyr* RNAi ($P < 0.05$). The percentage of PDCA was also obviously decreased from 20.1% to 13.9%. This indicated that tyrosinase played a key role in melanin synthesis and color formation of *P. penguin*.

## 1. Introduction

*Pteria penguin* (*P. penguin*) is an important marine bivalve to produce high quality seawater pearls in aquaculture of China. The color of pearl is the most important indicator to evaluate the pearl value. The nacre, secreted by mantle, is regarded as “mother of pearl”, whose color decides the color of pearl (Chen et al., 2017). The melanin is the major pigment in nacre of *P. penguin* and significantly affects the color of nacre (Yu et al., 2016). Inhibiting the synthesis and secretion of melanin in mantle could weaken the color of nacre and pearl in *P. penguin*.

Melanin has attracted considerable interest because of their involvement in pigmentation and protection against ultraviolet (Ito et al., 2013). There is a complex pathway to regulate the melanin synthesis in vertebrates (Cheli et al., 2009). Tyrosinase (TYR) is a key rate-limiting enzyme for melanogenesis in this pathway (Hofreiter and Schoneberg, 2010; Cieslak et al., 2011), because tyrosinase catalyzes three different reactions in biosynthetic pathway of melanin: 1) the hydroxylation of tyrosine to L-DOPA; 2) the oxidation of L-DOPA to L-dopaquinone; and 3) the oxidation of 5, 6-dihydroxyindole (DHI) to indole-quinone (Inoue et al., 2013). The abnormality of TYR inhibited melanin production and caused oculocutaneous albinism type I (Bennett and Lamoreux, 2003). Recently, tyrosinases from several shellfish were characterized and studied (Chen et al., 2017; Feng et al., 2015; Takgi and Miyashita, 2014; Nagai et al., 2007). TYR was considered to play an important role in melanin synthesis, formation of shell matrix, and pigmentation of prismatic layer in *Pinctada fucata* (Takgi and Miyashita, 2014; Nagai et al., 2007), *Hyriopsis cumingii* (Chen et al., 2017) and *Crassostrea gigas* (Feng et al., 2015). We speculated that tyrosinase also worked as an important regulator to affect the melanin synthesis in *Pteria penguin*.

Besides tyrosinase, some important factors were involved in melanin synthesis in mammalian (Rzepka et al., 2016; Lang et al., 2005; Kunwar et al., 2012). The cyclic-AMP responsive element-binding protein (CREB) was well known to regulate the expression of microphthalmia-associated transcription factor (MITF) (Rzepka et al., 2016), which regulated tyrosinase expression by phosphorylation (Cheli et al., 2009). The paired-box 3 (PAX3) was a transcription factor containing a

*Abbreviations:* RNAi, RNA interference; UTR, untranslated region; LC-MS/MS, liquid chromatograph-tandem mass spectrometer; PDCA, pyrrole-2, 3-dicarboxylic acid; PTCA, pyrrole-2,3,5-tricarboxylic acid; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; CREB, cyclic-AMP responsive element-binding protein; MITF, microphthalmia-associated transcription factor; Tyr, tyrosinase; PAX3, paired-box 3; BCL-2, B-cell lymphoma 2

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**Table 1**
Primers used in the study.

| Primer | Sequence(5′–3′) | Application |
|---|---|---|
| *Pp*tyr-outer-F | TGGATCTCCAGGCAGCAGTTTAATGAG | 3′RACE |
| *Pp*tyr-inner-F | GATGCCATTATGTACGAT | Nest-3′RACE |
| *Pp*tyr-outer-R | GACTCCACCAACCCAGACATGAGG | 5′RACE |
| *Pp*tyr-inner-R | GATTACGCCAAGCTTGGGATTCTGCTGACTGGTG | Nest-5′RACE |
| UPM | TAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT | RACE universal primer |
| NUP | AAGCAGTGGTATCAACGCAGAGT | Nest-RACE universal primer |
| *Pp*tyr-test-F | AGATGGGTCTCATGTGGGGAT | cDNA test |
| *Pp*tyr-test-R | CAATAACGTTTACGGAGCATTC | cDNA test |
| *Pp*tyr-qPCR-F | GTTTGGTAATGGCAGAGGGTC | qRT-PCR |
| *Pp*tyr -qPCR-R | TCGATAAAGGTATGGTGGAACC | qRT-PCR |
| *PpCreb2*-qPCR-F | AACTCCCAGTGAAGCAGACA | qRT-PCR |
| *PpCreb2*-qPCR-R | GCTCCCCAACAGTAGCCAAT | qRT-PCR |
| *PpPax3*-qPCR-F | TCCGTGCGTCATCAGTAGAC | qRT-PCR |
| *PpPax3*-qPCR-R | CCCTTGGTTTACTTCCGCCA | qRT-PCR |
| *PpBcl2*-qPCR-F | TGAGGCACAGTTCCAGGATT | qRT-PCR |
| *PpBcl2*-qPCR-F | ACTCTCCACACACCGTACAG | qRT-PCR |
| 18S rRNA-F | CGTTCTTAGTTGGTGGAGCG | qRT-PCR |
| 18S rRNA-R | AACGCCACTTGTCCCTCTAA | qRT-PCR |
| *Pp*tyr-siRNA1-F | GCGTAATACGACTCACTATAGGGCTGGCAATCCTATCGAGTG | RNAi |
| *Pp*tyr- siRNA1-R | GCGTAATACGACTCACTATAGGGATGACAGACCCTCTGCCA | RNAi |
| *Pp*tyr-siRNA2-F | GCGTAATACGACTCACTATAGGGGGGATCAGCTTATGTTATTGGGACT | RNAi |
| *Pp*tyr- siRNA2-R | GCGTAATACGACTCACTATAGGGATCTCTTGGGGACTGAGCAATGTTA | RNAi |
| GFP-siRNA-F | GATCACTAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGA | RNAi |
| GFP-siRNA-R | GATCACTAATACGACTCACTATAGGGTTACTTGTACAGCTCGTCCA | RNAi |

homeodomain and a paired domain. PAX3 combined with the promoter of *Mitf* gene and activated the expression of *Mitf*, while at the same time, it competed with MITF for occupancy of the enhancer of dopachrome tautomerase (DCT), an downstream enzyme that functioned in melanin synthesis, thus preventing the expression of terminal markers of melanin synthesis (Lang et al., 2005). So PAX3 was shown to both promote and inhibit melanin synthesis. The B-cell lymphoma 2 (BCL-2), an oncoprotein involved in the regulation of apoptosis, had been shown to influence the melanin synthesis in mammal (Kunwar et al., 2012). However, there were few reports showing that these proteins above had participated in melanin synthesis in bivalves.

There are two major chemically distinct melanin pigments, brown-black eumelanin and red-yellow pheomelanin (Szekely-Klepser et al., 2005). The shell color of *P. penguin* is deep black, therefore eumelanin is considered to be the main pigment in *P. penguin*. Eumelanin is mainly composed of the monomer units 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), which are insoluble in both acidic and alkaline solutions (Szekely-Klepser et al., 2005). However, the alkaline hydrogen peroxide oxidation products of DHI and DHICA, respectively named as pyrrole-2, 3-dicarboxylic acid (PDCA) and pyrrole-2,3,5-tricarboxylic acid (PTCA), could be detected by high-performance liquid chromatography (HPLC). PDCA and PTCA, as main markers for eumelanin, were identified and extensively used to evaluate the amount of eumelanin in sample using LC-MS/MS (Ito et al., 2011).

In this study, we obtained a partial sequence of tyrosinase gene from the transcriptome data of *P. penguin*. A new *Tyr* gene from *P. penguin* was identified and its expression profile was analyzed. The exact effect of tyrosinase on melanin synthesis was deliberated by RNA interference (RNAi) technology and liquid chromatograph-tandem mass spectrometer (LC-MS/MS) analysis.

## 2. Materials and methods

### 2.1. Experimental animals

The *Pteria penguin* (about 350–400 g, shell length ranging between 10 and 12 cm) were obtained from Weizhou Island in Beihai, Guangxi Province, China. They were cultivated with the recirculating seawater at 25–26 °C in lab. *Isochrysis zhanjiangensis* and *Platymonas subcordiformis* were main food of these experimental animals.

### 2.2. RNA isolation and cDNA synthesis

Total RNA from mantle (pallial zone), gill, adductor muscle, digestive diverticulum, foot, testis and ovary of *P. penguin* were isolated using RNeasyMini Kit according to the manufacturer's instructions (Qiagen, Gaithersburg, MD, USA). The quality of RNA was measured by electrophoresis on 1% agarose gels. The quantity of RNA was determined by measuring OD260/OD280 with NanoDrop ND1000 Spectrophotometer. The single strand cDNA was prepared from total RNA of mantle with M-MLV reverse transcriptase (Promega, Madison, WI, USA) and random primer. The transcriptome of *P. penguin* was constructed using RNA.

### 2.3. Cloning of Tyr cDNA and sequence analysis

5′RACE (Rapid Amplification of cDNA Ends) and 3′RACE reactions were conducted using SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) and Advantage 2 cDNA Polymerase Mix (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. The specific primers (*Pp*tyr-outer-F and *Pp*tyr-outer-R) were designed based on the partial sequence from the transcriptome of *P. penguin*. To increase the specificity of the amplification, nested-PCR was applied using *Pp*tyr-inner-F and *Pp*tyr-inner-R. All PCR products with expected size were subcloned into the PMD18-T vector (Takara, Dalian, China) and sequenced. The *Pp*tyr-test-F and *Pp*tyr-test-R were designed according to the linked nucleotide sequence to detect the correctness of sequence. All PCR primers were listed in the Table 1.

The full-length cDNA of *PpTyr* was analyzed using the BLAST program available from (http://www.ncbi.nlm.nih.gov/). ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to characterize the open reading fragment (ORF). Signal 4.1 program (http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptide of *PpTyr*. Transmembrane prediction was created by TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/). Multiple sequence alignments were created using the Clustal W program, and phylogenetic tree was constructed using MEGA 6.