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RESEARCH

Effects of Dietary Cystine and Tyrosine Supplementation on Melanin Synthesis in the Pacific Oyster (*Crassostrea gigas*)

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Abstract

Melanogenesis is a multistep process to produce melanin for dark pigmentation in skin coloration. Previous studies in vertebrates demonstrated that cystine and tyrosine amino acids are involved in the melanin synthesis. However, very little is known about the melanogenesis in bivalve. In this study, cystine supplementation for 30 days significantly upregulated the expression of *CgB-aat1*, *CgCbs* and *CgTyr* and pheomelanin content in the Pacific oyster *Crassostrea gigas*. Transmission electron microscope (TEM) results revealed more melanosomes in the connective tissue and melanin granules were secreted in epithelium of mantle. In contrast, tyrosine supplementation had no clear effect on melanogenesis except the gene expression changes of *CgB-aat1* and *CgCbs*. In addition, prolonged supplementation of cystine or tyrosine for 60 days had a negative impact on melanogenesis. Indeed, after 60 days, expression of most of the melanin synthesis-related genes under study was decreased, and melanin content was significantly reduced, indicating that cystine and tyrosine might inhibit production of eumelanin and pheomelanin, respectively. In addition, in vitro analysis using primary cell culture from mantle tissue indicated that incubation with cystine, tyrosine, or B-AAT1 polypeptide, CBS/TYR recombinant proteins induced the increase of *CgB-aat1* and *CgCbs* expression in a dose-dependent manner, suggesting the presence of a regulatory network in response to cystine and tyrosine amino acids intakes in pheomelanin synthesis-related gene expression. Taken together, these data indicate that cystine-*CgB-aat1*-*CgCbs*-*CgTyr* axis is a potential regulator of the pheomelanin biosynthesis pathway, and thus plays an important role in the mantle pigmentation in *C. gigas*. This work provides a new clue for selective cultivation of oyster strains with specific shell colors in bivalve breeding.

Keywords Cystine · Tyrosine · Melanin biosynthesis pathway · Shell color · *Crassostrea gigas*

Introduction

Molluscan shellfish, such as oyster and scallop, are major aquaculture seafood products worldwide. Their colorful shells are one of the major contributing factors in enhancing seafood market values and also have an influence in shell collection for naturalists, in addition, the comprehension of shell color determination is also relevant for pearl oysters (Nell 2001; Williams 2017). Shell color is determined by multiple genetic, dietary and environmental factors (Liu et al. 2009; Zhu et al. 2022), among which genetic factors have the most significant influence. To date, various shell colorations have been found under independent genetic controls (Ky et al. 2019; Han and Li 2020). Traditional selective breeding has successfully produced strains of bivalves with various shell colors, like oysters, clams and scallops (Evans et al. 2009; Vu et al. 2020; Wang et al. 2017; Liang et al. 2019). However, the genetic and molecular mechanisms of

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shell color regulation remain unclear in bivalve. With the rapid advancement of next-generation sequencing, genome-wide selection (Williams et al. 2017), RNA sequencing (Feng et al. 2015; Hu et al. 2020; Li et al. 2021) and genome-wide association studies (GWAS) (Zhao et al. 2017) are used to identify candidate regulatory genes in shell coloration. Furthermore, gene functional analysis has been carried out to clarify the genetic basis of shell color (Saenko and Schilthuizen 2021). To date, most studies in bivalve were limited to a few key genes, while the molecular pathways and cellular mechanisms involved in shell color regulation are poorly understood. The limited knowledge on shell color determination has negatively impacted selective breeding of shell coloration lines of bivalves.

Melanin, carotenoid and porphyrin are important biological pigments in bivalve mantle tissue that determine shell color formation in bivalve. Among these pigments, melanin is one of the most studied, despite limited identification of melanin in shell of bivalve (Affenzeller et al. 2019; Yu et al. 2015). Significant effort has been made to uncover genes involved in pigment synthesis in bivalve. Candidate gene approach has implicated the melanin biosynthesis pathway involving *Pax3/7*, *Mitf*, *Tyr* and *Tyrp2* in shell pigmentation of scallop (Mao et al. 2019), clams (Zhang et al. 2018; Jiang et al. 2020) and oysters (Lemer et al. 2015; Yu et al. 2018; Ky et al. 2019; Zhu et al. 2022; Li et al. 2022). In addition, melanosomes have been recently identified in the mantle of the Pacific oyster (Han et al. 2022; Li et al. 2023b).

Melanin pigment in vertebrates consists of eumelanin and pheomelanin in various ratios. The pathway of eumelanin and pheomelanin biosynthesis, also known as "melanogenesis", is controlled by several genes expressed in pigment cells (Wakamatsu et al. 2021). Eumelanin biosynthesis starts with L-tyrosine as the substrate that is converted to eumelanin by several enzymes encoded by *Tyr*, *Mc1r*, *Mitf*, *Tyrp1* and *Tyrp2* (D' Mello et al. 2016). Pheomelanin synthesis differs from eumelanin synthesis in that pheomelanin incorporates cystine into its structure, catalyzed by an enzyme encoded by *Slc7a11* gene (Hoekstra 2006). Previous studies showed that melanin content varied with in vivo and in vitro dietary supplementation of tyrosine or cystine in vertebrates (Słominski et al. 1988; Yu et al. 2001; Morris et al. 2002; Chintala et al. 2005; Zhao et al. 2010; Park et al. 2018). Based on the type of melanin produced, melanosomes can be divided into eumelanosome and pheomelanosome. Structurally, eumelanosome is an elliptical shape contained a fibrillar matrix and is the most electron-dense structure in the melanocyte. In contrast, pheomelanosome appears as mostly rounded shape and contains a vesicular matrix, in which melanin is deposited irregularly in blotches (Słominski et al. 2004). In fish, transcriptome analysis evidenced conserved melanogenesis pathways (Jiang et al. 2014; Wang et al. 2014; Zhu et al. 2016). A gene function study has been reported to uncover the signal pathways of melanin synthesis in fish (Luo et al. 2021). Recently in fish, Wang et al. (2018, 2019) uncovered that the Slc7al1 gene was responsible for skin color determination through the melanogenesis pathway and that the dietary cystine and tyrosine could affect melanin level in red tilapia. The influence of cystine and tyrosine in melanin synthesis has yet to be investigated in bivalves.

The Pacific oyster, *Crassostrea gigas*, is one of the most economically important marine bivalve species. Recently, it has been reported that the *CgB-aat1* (a homolog of *Slc7a11* in vertebrate)-*CgTyr*-*CgCbs* genetic axis was involved in the pheomelanin synthesis and mantle pigmentation in *C. gigas* (Li et al. 2023a). However, the effects of dietary cystine or tyrosine supplementation on the expression of the genes and on the melanin levels in *C. gigas* is not known. Here, we carried out in vivo and in vitro analyses to determine the effects of cystine and tyrosine on the expression of melanin synthesis-related genes, on the melanin content and on the melanosome formation. This work provides useful information for the understanding of molecular pathway of melanin synthesis and for selective breeding of various shell coloration lines of *C. gigas*.

Materials and Methods

Animals, Feeding Experiment and Sampling

One-year old Pacific oysters with orange shells were collected from Rongcheng City, Shandong province, China. Prior to the feeding experiment, the oysters were acclimatized in recirculating seawater at 23–25 °C for 7 days in the laboratory. The oysters were fed with *Chlorella vulgaris* twice a day (08:00 and 20:00) (Kuhn et al. 2013). Approximately 60% of water was exchanged with aerated water (24 ± 1 °C) before 08:00 and 20:00. After acclimatization, some oysters (shell length 51.34 ± 8.77 cm, shell height 24.79 ± 3.72 cm) were randomly chosen for the subsequent experiment.

During the feeding experiment, 280 oysters were cultured in 7 tanks (40 each) in 40 L seawater. Three tanks were used as cystine supplemented group (denoted as CS) and three tanks as the tyrosine supplemented group (denoted as TS). One tank was used as a control group (denoted as C). Oysters in the CS group were fed daily with one liter of *Chlorella vulgaris* containing 5 mg/L (denoted as CS-5), 10 mg/L (denoted as CS-10) and 15 mg/L (denoted as CS-15) of cystine (Sango Biotech, Shanghai, China), respectively (Wan et al. 2022; Chen et al. 2019; Nell and Wisely 1984). A similar feeding regimen was performed for the TS group (denoted as TS-5, TS-10, TS-15). Control oysters were fed with *Chlorella vulgaris* only.

Twenty oysters were randomly selected from each group after 30 and 60 days of cultivation. Mantle tissues were dissected and immediately frozen in liquid nitrogen and stored at -80 °C for RNA isolation and Elisa experiment. Additionally, mantle samples were fixed in 2.5% glutaraldehyde at 4 °C for later histological analysis.

Mantle Cell Culture and Treatment Assay

The whole mantle tissues were dissected from healthy oysters with orange shells. The tissues were washed with 1 × PBS (pH 7.4) six times, and then followed by incubation in sterile PBS solution containing penicillin (100 U/mL), streptomycin (100 μg/mL) and gentamicin (50 μg/mL) for 30 min. The samples were then washed with primary culture medium consist of L15 medium and M199 (V: V = 1:1, pH 7.2–7.4) plus 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL gentamicin. Finally, the tissues were cut into pieces with sterile scissors and cultured in a 12-well plate at 26 °C. During the culture, trypan blue staining was used to determine cell survival.

The cultured mantle primary cells were harvested at 48 h of culturing by centrifugation at 1200 rpm for 5 min. The cell pellets were resuspended in fresh culture medium for subsequent L-cystine and tyrosine incubation. L-cystine dihydrochloride (Merck, Germany), and tyrosine (Solarbio, China) were purchased from the respective companies. B-AAT1 polypeptide, purified proteins CBS and TYR were produced from our previous study (Li et al. 2023a). Amino acid stock solutions were prepared at 5 mg/ml and 2 mg/ml, respectively. The purified protein stock solutions were prepared at 1.5 mg/ml. To evaluate their effect, these additives were added to the primary culture medium with the final concentration of 0, 0.5, 2, 5, 10, 20 μg/mL and cultured cells were maintained at 26 °C for 12 h. Cells were collected and froze immediately in liquid nitrogen and stored at -80 °C for the subsequent quantification of gene expression and protein contents.

Gene Expression Analysis by Real-Time Quantitative PCR

Total RNA was isolated from cultured cells using Trizol Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Each RNA sample integrity was examined on 1.2% agarose gel and the concentration and purity was checked with Nanodrop 2000 (Thermo scientific, USA). First strand cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, China) following the manufacturer's protocol. qPCR was caried out on 5 melanin synthesis-related genes (Table 1) using QuantiNovaTM SYBR® Green PCR Kit (QIAGEN, Germany) on a Light Cycler 480 real-time PCR instrument (Roche Diagnostics, Burgess Hill, Switzerland). The specificity and amplification efficiency of PCR primers (listed in Table 1) were evaluated using melt curve and standard curve analyses qPCR reaction was performed in triplicates for each cDNA sample with the following thermal cycling: 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 5 s, annealing and extension at 60 °C for 10 s. Gene expression level was normalized to the housekeeping gene *ef1α* transcriptional level (Du et al. 2013). The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was used to calculate the relative gene expression in comparison with the control group.

Western Blot Analysis

Total proteins were extracted from cultured mantle cells by homogenizing in RIPA lysis buffer (Beyotime, China) with PMSF (Beyotime, China). The homogenate was centrifuged at 12,000 × g for 5 min to collect the supernatants. Protein concentration was measured by BCA Protein Assay Kit (Beyotime, China). The supernatants were mixed with loading buffer (1 ×) and denatured at 100 °C for 10 min. 20 μg total proteins were loaded for each lane in a SDS PAGE electrophoresis. Proteins in the gels were transferred onto PVDF membrane (Beyotime, China) using wet transfer (Bio-Rad, CA) at 220 mA. The membrane was blocked with 5%

Table 1 Sequences information of Specific Primers

Gene name	Primer	Sequence (5'-3')
B-aat1, b (0,+)-type amino acid transporter 1	B-aat1-F	GCTCTGGAATGGGGAGAAGTAG
	B-aat1-R	CCCGTTAGCAGCACCAAATG
Cbs, cystathionine beta-synthase	Cbs-F	TGGAGGAAATCCCCAATGCC
	Cbs-R	CGACCACAACCATGTCCACT
TYR, tyrosinase	Tyr-F	GTACGATTCTTGTGGTCGGC
	Tyr-R	GAGGTGAAGCGTCATCCAAAG
TYRP1, tyrosinase-related protein 1	Tyrp1-F	CGAGGCGTTTCCAGTTTGTG
	Tyrp1-R	TGGCAGTAGCCGGTGAATTT
TYRP2, tyrosinase-related protein 2	Tyrp2-F	TCGTCGATGAAAGGCAACCA
	Tyrp2-R	CATACACTGGACAAGCGGGT
Ef1α, α subunit of elongation factor 1	Ef1α-F	ACGAATCTCTCCCAGAGGCT
	Ef1α-R	GAAGTTCTTGGCGCCCTTTG

skimmed milk for 2 h at room temperature and then incubated with anti-*CgB-aat1*/*CgCbs* polyclonal antibody (Li et al. 2023a) or anti-β-actin antibody (Beyotime, China) overnight at 4 °C. The membrane was washed with 1 × PBST and then incubated with HRP-conjugated secondary antibodies (dilution 1:5000, ABclonal, China) at 37 °C for 1 h. The target proteins were detected using chemiluminescence and Gel Image System (JS-2000).

Enzyme-Linked Immunosorbent Assay

Mantle tissues (0.1 g) were homogenized in 1 × PBS buffer. The supernatants were collected by centrifugation at 12,000 × g for 5 min. Eumelanin and pheomelanin contents were measured using Elisa Kit (Yanzunbio, China). Briefly, 50 µL of supernatant were added to microwell plate coated in Elisa plate with anti-eumelanin or anti-pheomelanin antibody and incubated at 37 °C for 30 min. Following washing five times, catalyzed reaction with enzyme was conducted at 37 °C for 30 min, and then incubated with HRP-conjugated reagent at 37 °C for 30 min. Then color reaction was proceeded at 37 °C in dark and was terminated after 10 min. Multimode microplate reader (Synergy H1, BioTek, USA) was used to analyze the absorbance at 450 nm.

Transmission Electron Microscopy

Mantle tissues were dissected from oysters of the CS-5, TS-5 and C groups after 30 days of feeding. The mantle tissues were fixed in 2.5% glutaraldehyde for 12 h, and postfixed with 1% osmic acid for 2 h. The samples were dehydrated by gradient ethanol (30%, 50%, 70%, 90% and 100%), and then embedded in EPON 812 resin. The blocks were cut to produce 60 nm sections and stained with uranyl acetate. Ultrastructure of melanosome was observed using a transmission electron microscope (ME-1200EX, JEOL, Japan).

Statistical Analysis

All data were analyzed by one-way ANOVA using SPSS 20.0 and the results were presented as means ± standard error (SE). Significant differences were considered at $P < 0.05$.

Results

The Effects of Dietary Cystine and Tyrosine Supplementation on Melanin Synthesis-Related Gene Expression

Pheomelanin is a red-yellow color pigment found in pigment cells. Pheomelanin biosynthesis is initiated from cystine. To determine whether dietary cystine supplementation could affect the expression of melanin synthesis-related genes in oysters, we analyzed the expression of *CgB-aat1*, *CgCbs, CgTyr, CgTyrp1* and *CgTyrp2* in mantle tissues of oysters after 30 days and 60 days of cystine supplementation. After 30 days, data from qPCR analysis showed that both *CgB-aat1* and *CgCbs* were significantly upregulated in the CS-5 group ($P < 0.05$) (Fig. 1a). In addition, a dramatic upregulation of *CgTyr* expression was detected in the CS-10 group ($P < 0.05$). *CgTyrp2* expression was also increased after feeding cystine and reached the highest levels in the CS-15 group ($P < 0.05$). Interestingly, there was no significant difference of *CgTyrp1* expression (Fig. 1a). To our surprise, after 60 days of cystine supplementation, *CgB-aat1*, *CgCbs*, *CgTyr* and *CgTyrp1* expression was significantly down-regulated, while *CgTyrp2* expression was upregulated with the most significant upregulation in the CS-15 group (Fig. 1b).

To test whether supplementation with tyrosine could alter expression of melanin synthesis-related genes, we analyzed *CgB-aat1*, *CgCbs, CgTyr, CgTyrp1* and *CgTyrp2* expression in mantle tissue by qPCR. The data showed that after tyrosine supplementation for 30 days, significant decrease of *CgB-aat1* expression was detected in the TS-10 group while significant increase of *CgCbs* expression was observed in the TS-10 and TS-15 groups. Interestingly, tyrosine supplementation did not affect the gene expression of *CgTyr*, *CgTyrp1* and *CgTyrp2* (Fig. 1c). After 60 days of tyrosine supplementation, we observed a significant down regulation of *CgB-aat1* and *CgTyrp1* expression in the TS-10 and TS-15 groups, but significant upregulation of *CgTyr* and *CgTyrp2* expression in the TS-15 group (Fig. 1d).

The Effects of Dietary Cystine and Tyrosine Supplementation on Melanin Content in Mantle of Oysters

L-tyrosine can enhance the melanin synthesis (Park et al. 2018). To determine whether cystine and tyrosine supplementation affects the melanin content in the mantle tissue, we compared pheomelanin and eumelanin levels in control and treated groups. The data showed that pheomelanin content was significantly increased in the CT-5 group (Fig. 2a) after 30 days of dietary supplementation. However, dietary cystine had no effect on eumelanin synthesis, and feeding tyrosine slightly decreased eumelanin content (Fig. 2b). Notably, significant decreases of pheo-/eumelanin content were observed in both CS and TS groups 60 days after the dietary supplementation (Fig. 2c–d).

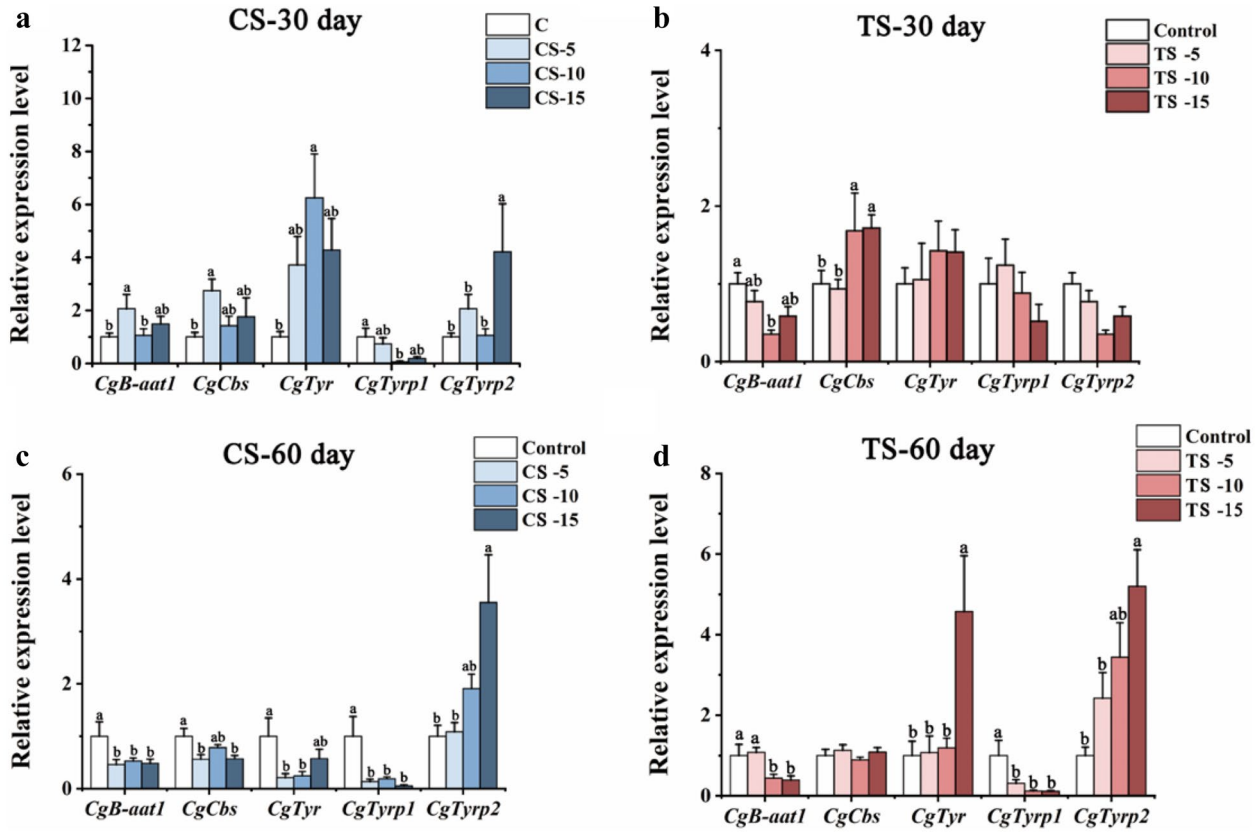

Fig. 1 Gene expression analysis in the mantle of *C. gigas* after amino acid feeding. a and b, Gene expression analysis after 30 days and 60 days of dietary cystine supplementation. CS-5, CS-10, and CS-15 represent oysters dieted with *Chlorella vulgaris* containing 5 mg/L, 10 mg/L and 15 mg/L cystine, respectively. The same is true for the tyrosine supplementary group (TS). **c and d**, Gene expression analysis after dieted with tyrosine for 30 days and 60 days, respectively. The control group was dieted with *Chlorella vulgaris* normally. Relative fold change in gene expression was compared to the control group. Values were expressed as the mean relative expression ± SE ($n = 6$), the different letters indicate significant differences ($P < 0.05$)

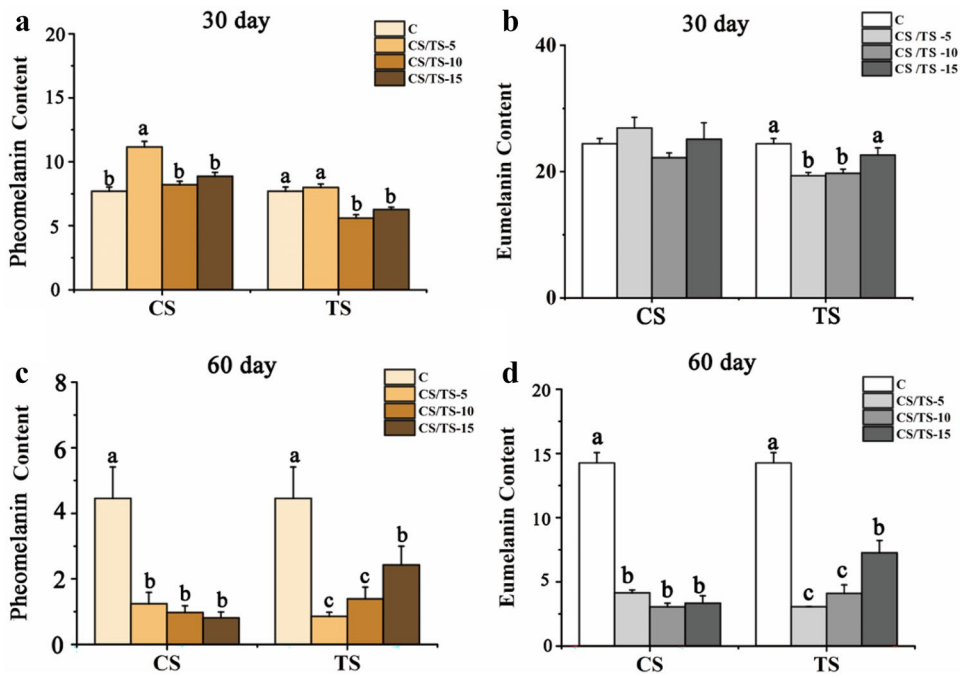

Fig. 2 The changes of melanin content in the mantle of *C. gigas* after amino acid feeding. a and c, Pheomelanin content in mantle tissue after dietary supplementation with cystine or tyrosine for 30 days and 60 days, respectively. **b and d**, Eumelanin content in mantle tissue after dietary supplementation with cystine or tyrosine after 30 days and 60 days, respectively. The content was calculated as the mean ± SE ($n = 6$), the different letters indicate significant differences ($P < 0.05$)

The Effects of Cystine and Tyrosine Incubation on Melanosome Formation in Cultured Mantle Cells of Oysters

To determine whether supplementation of cystine and tyrosine could affect melanosome formation in mantle tissue of oysters, we performed TEM analysis in cultured oyster mantle cells treated with cystine and tyrosine. The data showed that cystine supplementation significantly activated melanogenesis in mantle tissue of oyster (Fig. 3a–k). Increased number of cells were significantly melanized in the connective tissue. It appears that the cytosol was packed with melanized, granular melanosomes of various stages (Fig. 3a–g). In addition to dark and rounded melanosomes (Fig. 3c, f–g), small and lightly black granules were also detected, likely representing partially melanized organelles (Fig. 3d, f–g). Moreover, scattered melanin granules were also observed in epithelial tissues (Fig. 3h–k) and the melanin granules were deposited irregularly in blotches (Fig. 3j, l). In contrast, mantle cells cultured with tyrosine had less identifiable melanosomes and premelanosomes in connective tissues

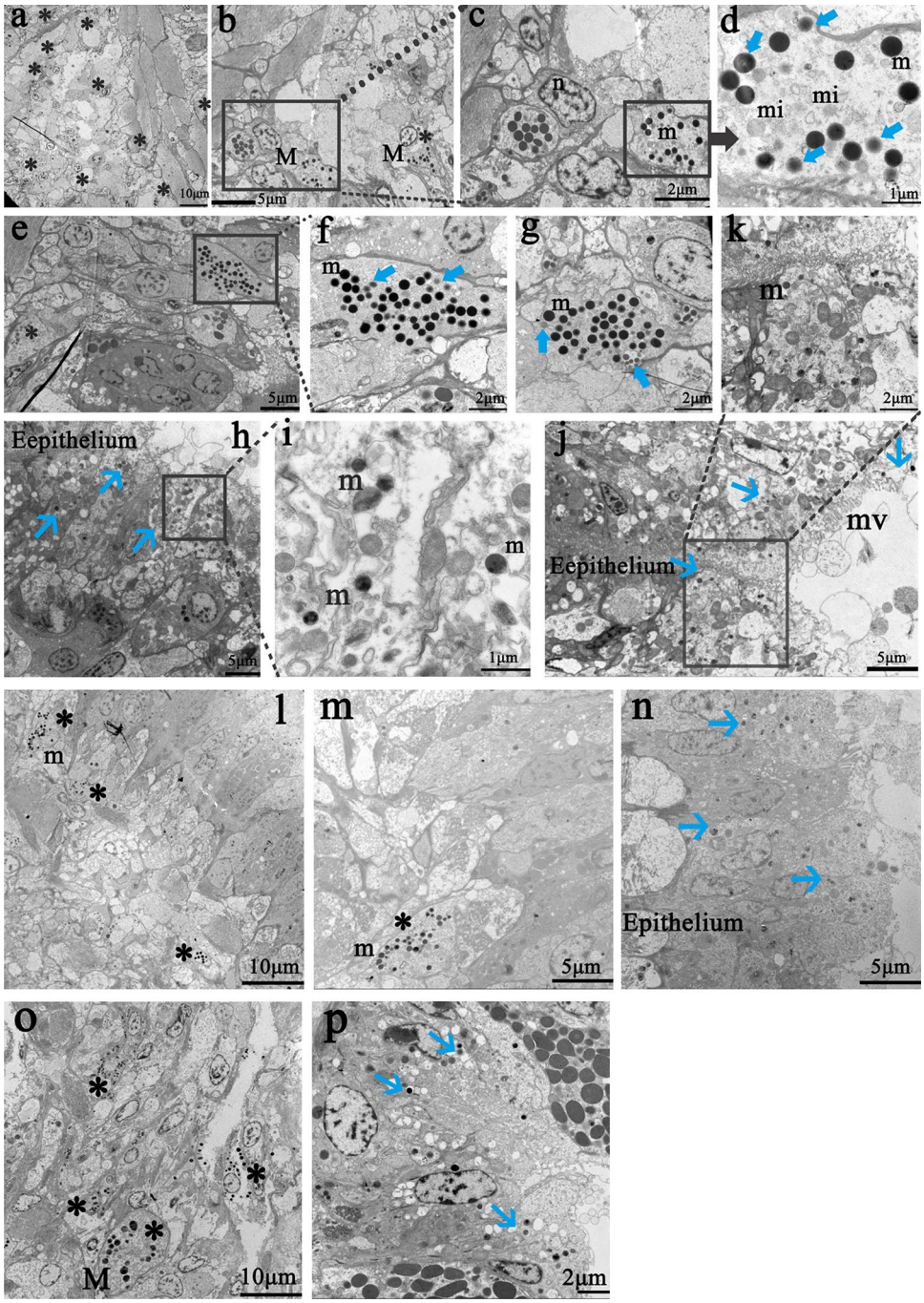

Fig. 3 Effect of cystine and tyrosine on melanosome formation in the mantle of *C. gigas*. TEM examination of mantle in TS (**a**–**k**), CS (**l**–**n**) and C (**o**–**p**) groups. m, melanin; n, nucleus; M, melanosome; mi, mitochondria. Asterisk point the melanin. Blue arrows in d, f and g indicate the partially melanized organelles. Blue arrows in h, g, n and p refer to the melanin granules distributed in the epithelium

(Fig. 3l–m), and there was no significant difference between TS group and C group (Fig. 3o–p).

The Effects of Cystine and Tyrosine Supplementation on Melanin Synthesis-Related Gene Expression In Vitro

To assess if cystine and tyrosine supplementation could alter melanin synthesis-related gene expression in mantle cells in culture, we performed qPCR analysis of *CgB-aat1*, *CgCbs*, *CgTyr*, *CgTyrp1* and *CgTyrp2* expression. The qPCR results showed a dose–effect on *CgB-aat1* and *CgCbs* expression in response to the treatment (Fig. 4). Specifically, with the increase concentration of cystine, *CgB-aat1* or *CgCbs* gene expression was upregulated and reached to the peak level at 5 μg/ml of cystine. Higher concentrations induced a down-regulation of these genes (Fig. 4a). Similar gene expression trend was also found in the samples treated with B-AAT1 and CBS (Fig. 4c, e), and their peak levels occurred at 5 μg/ml or 10 μg/ml of cystine treatment, respectively. When supplied with tyrosine, the transcriptional levels of *CgB-aat1* or *CgCbs* in cultured mantle cells were rapidly upregulated at 0.5 μg/ml and then gradually reduced to the base levels (Fig. 4b). A similar trend was also observed in *CgCbs* gene expression when incubated with TYR recombinant protein (Fig. 4d). Moreover, *CgB-aat1* gene expression of (Fig. 4d) was different with the results in Fig. 4a. And the original WB results was shown in Fig. S1. Finally, the trend of protein expression level was in agreement with the trend of corresponding gene expression.

Discussion

Cystine is involved in pheomelanin synthesis. It has been well documented that the cystine-glutamate antiporter SLC7A11 encoded by the *Slc7a11* gene directly regulates

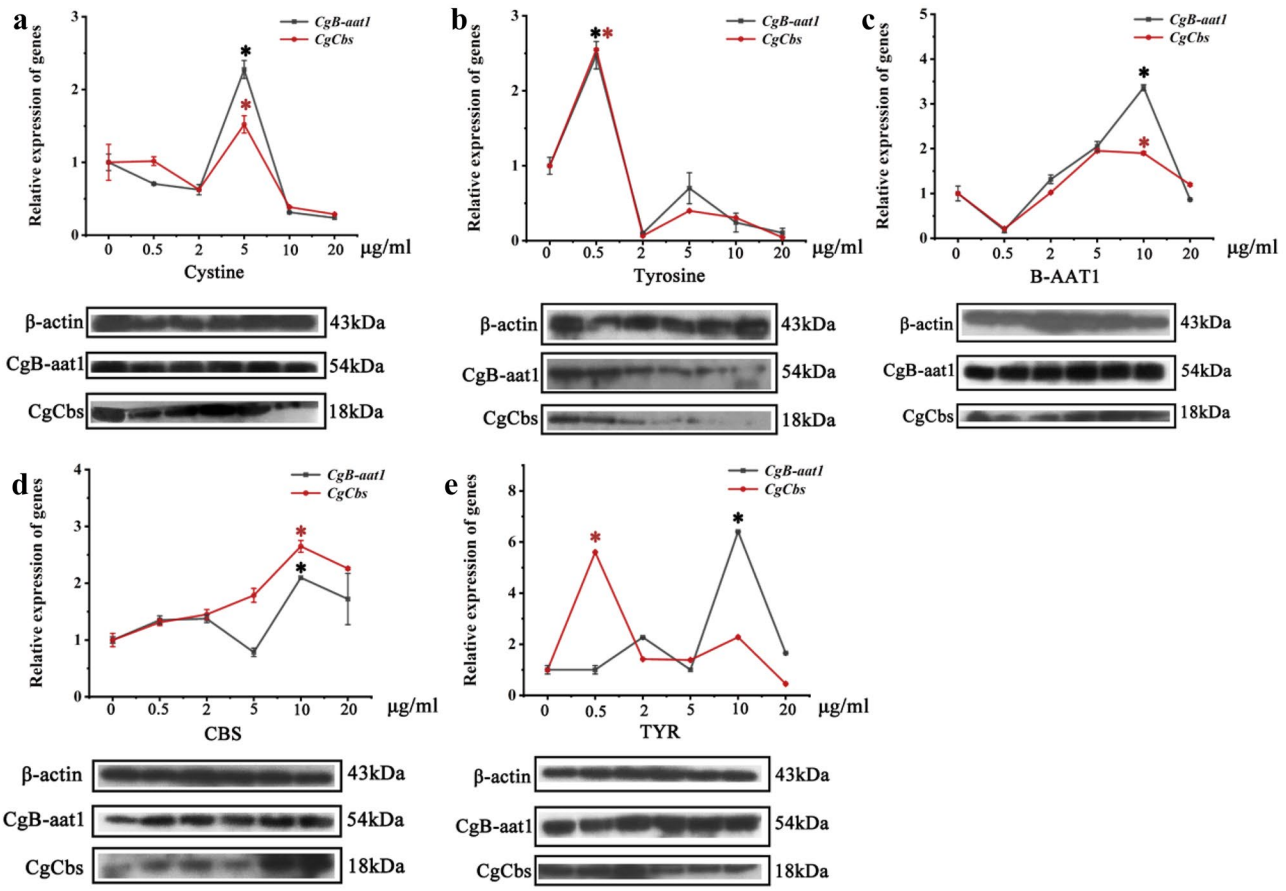

Fig. 4 CgB-aat1 and CgCbs expression analysis in vitro. Mantle cells of *C. gigas* were cultured in medium containing different concentrations of L-cystine dihydrochloride (**a**), tyrosine (**b**), purified protein B-AAT1(**c**), CBS(**d**) and TYR(**e**), respectively. The line chart was the *CgB-aat1* and *CgCbs* gene expression analysis within five gradients in five treatments, respectively. Relative fold change in expression was compared to that of 0 μg/mL. Values were expressed as the mean relative expression ± SE. "*" means the significance of the differences. the The corresponding protein expression analysis by WB was displayed under the line chart

pheomelanin synthesis by increasing intracellular cystine levels (Chintala et al. 2005; He et al. 2012; Tian et al. 2015). Ito and Wakamatsu (2008) suggested that depletion of cystine and cysteinyl-DOPA promoted pheomelanin synthesis. In our previous study, we showed the important roles of *CgB-aat1* and *CgCbs* in pheomelanin synthesis of *C. gigas* (Li et al. 2023a). Here, we further demonstrated that dietary cystine supplement had a positive effect on *CgB-aat1*, *CgTyr* and *CgCbs* gene expression, suggesting that they are involved in cystine metabolism in *C. gigas*. Our data also indicate that cystine supplementation was responsible for the pheomelanin biosynthesis in the mantle tissue, consistent with the previous study of Ito and Wakamatsu (2008). In Malaysian red tilapia, it has been shown that feeding fish with cystine and tyrosine supplement also affected the expression of genes involved in melanin synthesis pathway (Wang et al. 2018).

On the contrary, it has been reported that cystine inhibits tyrosinase-mediated dopachrome formation and eumelanin synthesis (Barek et al. 2018; Lee et al. 2021). Although the cystine-*CgB-aat1r-CgCbs-CgTy* axis mediated pheomelanin biosynthesis is conserved in *C. gigas* as in other animals (Morgan et al. 2013; Orhan and Deniz 2021), the competitive relation between eumelanin and pheomelanin synthesis in *C. gigas* is unclear. Emaresi et al. (2013) suggested that *Tyr*, typically involved in eumelanin synthesis, has a negative correlation with *Slc7al1* and *Cbs*, typically involved in pheomelanin synthesis in the tawny owl (*Strix aluco*). However, in this study, the *CgTyr* gene expression pattern was synchronous with *CgB-aat1* and *CgCbs* expression in cystine feeding experiment and no obvious changes have been observed by providing elevated tyrosine in 30 days. The discrepancy is not clear. It might be caused by the possibility that *CgTyr* primarily regulates biosynthesis of pheomelanin rather than eumelanin in mantle tissue of orange-shell-color oyster. In addition, *CgTyrp1* is a downstream gene of *CgTyr* in eumelanin biosynthesis, thus decrease in *CgTyrp1* gene expression may have no effect on eumelanin content in cystine supplement experiment.

The involvement of tyrosine in melanin biosynthesis has been extensively reported (Morris et al. 2002; Wang et al. 2018; Yu et al. 2001). In the present study, the dietary tyrosine supplementation for 30 days caused the downregulation of *CgB-aat1* gene expression and upregulation of *CgCbs* expression while no significant effect on *CgTyr*. One plausible explanation is that additional tyrosine promoted eumelanin synthesis and *CgTyr* participated in both biosynthesis pathway simultaneously in *C. gigas*. Because in the conserved melanin synthesis pathway, cystine was produced upstream of *CgB-aat1* while *CgTyr* was involved in both biosynthesis (Morgan et al. 2013; Orhan and Deniz 2021). Moreover, it has been reported that melanin level increases with cystine or tyrosine supplementation. However, too high levels of cystine or tyrosine could inhibit production of melanin (Schwahn et al. 2002; Słominski et al. 1988). This is consistent with our finding from this study, where excessive or prolonged supplement of cystine or tyrosine led to the decrease of melanin content. It was speculated that melanin synthesis has negative feedback regulation in *C. gigas*.

Brake et al. (2004) suggested that shell color has a positive correlation with the pigmentation at the edge of mantle tissue in oyster. Kang et al. (2013) also found the strong correlation between pigmentation on the edge of mantle and the black shell color after many generations of breeding. Hence, mantle was considered the main organ for shell color formation in bivalves. In this study, we showed that cystine supplementation led to a significant variation of pheomelanin content and stimulated more melanosome and melanin granules distribution in the mantle. This effect was specific because tyrosine supplementation had no obvious effect. It has also been reported that the dietary cystine results in increased melanin content in the skin and fur color variation in vertebrates (Wang et al. 2018; Yu et al. 2001). Collectively, these data indicate that dietary cystine supplementation is effective in increasing pheomelanin synthesis and thus plays an important role in pigment deposition.

Previous in vitro studies also examined the function of amino acid supplementation in melanin biosynthesis. Medium addition of L-tyrosine, L-dopa or D-tyrosine in culture medium influenced melanin formation in cultured cells (Słominski et al. 1988; Rzepka et al. 2016; Fernandez-Julia et al. 2021). The regulatory roles of cystine in antimelanogenic activity has been reported in human pigment cells (Del Marmol et al. 1996; Benathan et al. 1999; Galván et al. 2019). These findings suggested that such amino acids were likely involved in regulating L-tyrosine-mediated eumelanin synthesis and cystine-mediated pheomelanin at the cellular level. In this study, we showed that addition of cystine, tyrosine, B-AAT1 polypeptide and purified protein CBS/TYR in medium, could induce *CgB-aat1* and *CgCbs* gene expression and exhibited a dose dependent effect. Taken together with previous studies on melanin biosynthesis in insect and fish (Barek et al. 2018; Luo et al. 2021; Wang et al. 2018), we highlighted the amino acid and key genes in pheomelanin synthesis pathway of *C. gigas* (Fig. 5). Pheomelanin synthesis maybe mediated by cystine-*CgB-aat1-CgCbs-CgTyr* axis. Extracellular cystine was transported by CgB-AAT1 and formed intracellular cystine. In addition, tyrosine could also initiate biosynthesis of pheomelanin by providing dopaquinone for condensation with cystine, and with the help of CgCBS, these compounds eventually produce polymeric yellow to red pheomelanin pigments.

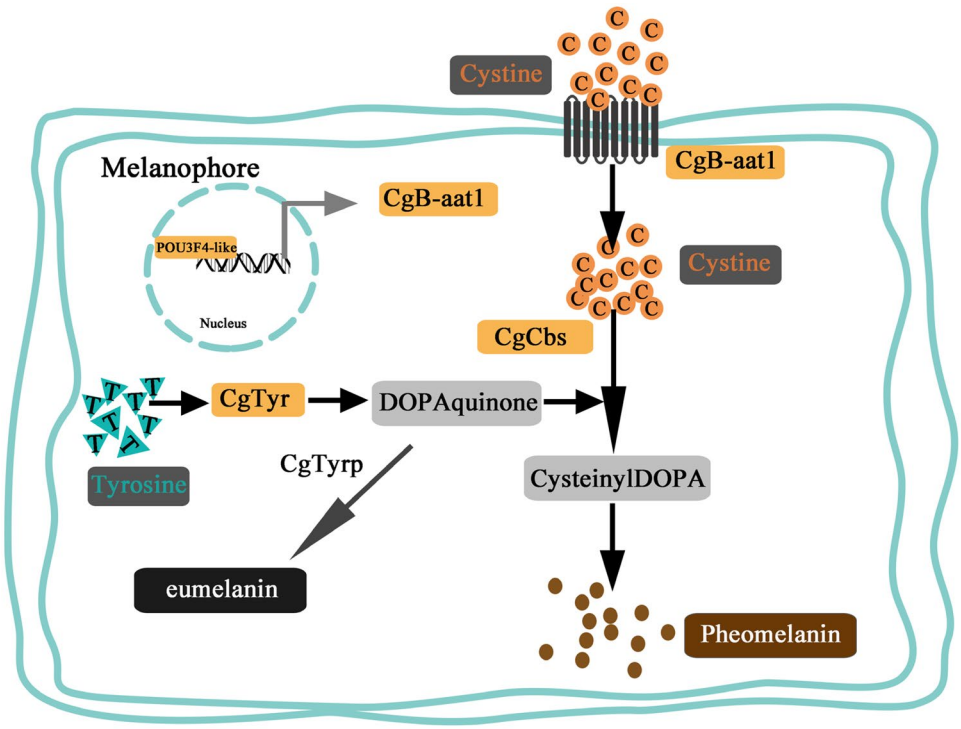

Fig. 5 The putative pheomelanin synthesis pathway in the *C. gigas*

Conclusion

We showed in this study that dietary 5 mg/L cystine supplementation had a significant impact on *CgB-aat1*, *CgCbs* and *CgTyr* gene expression and pheomelanin content as well as melanosome formation in the mantle of *C. gigas*. However, tyrosine supplementation seemed to be less effective. In vitro studies with cell culture, we further showed that addition of cystine/tyrosine/B-AAT1/CBS/TYR protein significantly altered *CgB-aat1* and *CgCbs* gene expression in a dose expression pattern. These findings suggest that cystine-*CgB-aat1*-*CgCbs*-*CgTyr* axis contributes to pheomelanin synthesis and has potential roles in mantle pigmentation and shell color formation, thus providing a new clue for cultivating strains with predictable shell color in bivalve breeding programs.

Abbreviations *B-aat1*: B(0, +)-type amino acid transporter 1; *Cbs*: Cystathionine beta-synthase; *Ef1α*: α Subunit of elongation factor 1; PMSF: Phenylmethanesulfonyl fluoride; RIPA: Radio immunoprecipitation assay; *Tyr*: Tyrosinase; *Tyrp1*: Tyrosinase-related protein 1; *Tyrp2*: Tyrosinase-related protein 2; WB: Western blot

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Authors Contributions Qi Li: Experimental design and coordination, manuscript revision and funding acquisition. Zhuanzhuan Li: Completion of the experiment, data analysis and manuscript drafting. Chengxun Xu: Resources. Hong Yu: Investigation. Lingfeng Kong: Data curation. Shikai Liu: Supervision.

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Data Availability The datasets generated in the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval Statement The pacific oyster is neither an endangered nor protected species. All experiments in this study were conducted according to national and institutional guidelines.

Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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