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PcASTA in *Procambarus clarkii*, a novel astaxanthin gene affecting shell color

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The color of crayfish (*Procambarus clarkii*) shells not only affects their value, but also has a significant impact on individual size. Astaxanthin is a common carotenoid that has been found in many studies to have different functions, including regulating body color formation. In this study, the levels of different carotenoids were detected using high performance liquid chromatography (HPLC) and found a significant correlation between astaxanthin content and shell color. An astaxanthin gene (*PcASTA*) was identified from *P. clarkii*, and it was expressed in tissues of crayfish with dark-red shells and green shells. The highest expression levels of *PcASTA* were found in the hepatopancreas, followed by the muscle. The expression of *PcASTA* in all tissues of red shell crayfish was higher than that of green shell crayfish, indicating that *PcASTA* may be involved in shell color formation in crayfish. Moreover, high water temperature and feeding β -carotene can lead to an increase in tissue expression of *PcASTA* and astaxanthin content in crayfish shells. The findings indicate that *PcASTA* may affect shell color in *P. clarkii* by being involved in astaxanthin synthesis and transformation.

KEYWORDS

Procambarus clarkii, carotenoid, astaxanthin, shell, color

1 Introduction

The red swamp crayfish (*Procambarus clarkii*) is a high-quality economic freshwater shrimp that is native to south central USA and northeastern Mexico. It is popular among consumers because of its attractive color, delicious flavor, and high nutritional value (high-quality protein, low lipid content, and rich minerals) (Amine et al., 2008). The red swamp crayfish was introduced from Japan in the 1930s and it has become the most important cultured crustacean with the largest aquaculture area and the highest total yield in China (Bureau of Fisheries and Fishery Management, 2021).

Crayfish belong to crustacean aquatic animals, and the shells are usually composed of an epicuticle, pigmented layer, calcified layer, and uncalcified layer. The growth of crustaceans is

not continuous, but typically presents a discontinuous trapezoidal growth pattern, where the weight increases significantly after each molting (Freeman, 1990; Chengcong et al., 2019). As the number of molts increases during crayfish growth, the weight also gradually increases, the shell hardness increases, and the color becomes red. Once the crayfish shell turns dark red, it becomes strong, molting becomes difficult, and the individual size stabilizes as growth is hindered. In the market, individual shell color affects the price of crayfish; typically, red shell crayfish are most popular.

The shell color of aquatic crustaceans has a large impact on their commercial value. Such as greenish brown *Astacus pallipes*, orange-red *Carcinus maenas*, purple *Hyriopsis cumingii* and dark-red crayfish usually have higher market prices (Quarmby et al., 1977; Shahidi and Brown, 1998; Chen et al., 2021; Chen et al., 2022a). Studies have shown that the shell color of aquatic crustaceans is influenced by a variety of factors (e.g., pigments, metal elements, genetic, environmental factors) (Li et al., 2014b; Amaya and Nickell, 2015; Chen et al., 2017; Wang et al., 2020; Chen et al., 2021; Chen et al., 2022b).

Although there are many factors that affect the color of crustacean shells, some studies suggested that carotenoids are a key influencing factor (Shahidi and Brown, 1998; Niu et al., 2014). Carotenoids are a type of natural fat-soluble pigments that are synthesized by algae, plants, and microorganisms. Animals (including aquatic crustacean) cannot synthesize carotenoids on their own. They are obtained only by feeding and then converted into different types of carotenoids in the body (Miki et al., 1982; Yabuzaki, 2017). Carotenoids play a role in pigmentation, wound healing, and immune reactions in aquatic animals (Shahidi and Brown, 1998). More than 750 different carotenoids have been discovered, including canthaxanthin, echinenone, lutein, and astaxanthin, among others (Maoka, 2009). Shahidi and Brown suggested that astaxanthin is most widely distributed in aquatic animals, and is closely related to body color and shell color (Shahidi and Brown, 1998). Studies found that adding astaxanthin to feed is beneficial to improve shell color in aquatic crustaceans (Menasveta et al., 1993; Boonyaratpalin et al., 2001). Moreover, researchers have shown that aquatic crustaceans can convert a variety of carotenoids into astaxanthin, but the relevant regulatory genes and specific mechanisms by which that occurs are not clear.

In this study, the levels of different carotenoids in dark-red shell and green shell *P. clarkii* were determined. A new astaxanthin gene (*PcASTA*) was identified in *P. clarkii* for the first time. The expression levels of *PcASTA* in different tissues were also determined by qPCR. The content of astaxanthin in shells and *PcASTA* expression in different tissues was also determined in red shell *P. clarkii*. The findings suggested that *PcASTA* may affect shell color by affecting the synthesis and deposition of astaxanthin.

2 Materials and methods

2.1 Experimental animals

Two hundred red swamp crayfish (100 red shell crayfish and 100 green shell crayfish; weight = 24.67 ± 0.57 g; length = $6.57 \pm$

0.67 cm) were obtained from a crayfish breeding base in Chaohu City, Zhejiang Province, China. Before experimentation, all crayfish were acclimated for 1 week in tanks containing aerated fresh water.

2.2 Carotenoid content

The tail shell (TS) and cephalothorax shell (CS) were collected from six red shell crayfish (three males and three females) and six green crayfish (three males and three females), respectively. The TS and CS were then cleaned with $1 \times$ PBS (phosphate buffered saline). All shells were frozen at -80°C for 24 h, and then a vacuum freeze-drying machine (Model FD-ID-50, Shanghai, China) was used to freeze-dry shells. The lyophilized (48 h) shells were ground into fine powder at room temperature, and ground TSs and CSs of the same shell color were mixed separately. Each sample (0.4 g) was mixed with 2 mL of acetone and cleaned by ultrasound for 15 min. After centrifugation at 4000 rpm for 10 min at 4°C , supernatants were collected and the insoluble fraction was subjected to the procedures described above 4-5 times (until the supernatant is colorless). All supernatants were collected into fresh tubes and diluted to 15 mL with acetone. A spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA) was used to determine the O.D. of each sample at 470 nm. The extraction and determination processes were completed in the dark. Purified astaxanthin (purity: 95.2%, Dr. ehrenstorfer) was used as the standard with which a standard curve was established.

High performance liquid chromatography (HPLC) (Agilent 1260, Agilent Technologies, USA) with a YMC carotenoid separation column (YMC-carotenoid TM, YMC, Japan) was used to separate and quantify carotenoids in each sample. Briefly, 2.9 mL NaOH-methanol solution (0.02 mol/L) was added to 2 mL samples and let stand at 4°C for 12 h after sealing. Then, 0.6 mol/L phosphoric acid methanol solution and 100 mg PSA (Pressure Swing Adsorption) adsorbent was added and filtered using a $0.2 \mu\text{m}$ microporous filter membrane. Finally, 20 μL of treated sample was used for testing. Two mobile phases, methanol (A) and methyl tert butyl ether (B) were used for step-by-step elution, with a 1.0 mL/min flow rate and column temperature of 25°C . Five purified carotenoid standards (astaxanthin, zeaxanthin, lutein, echinenone and β -carotene) were used for qualitative and quantitative analysis.

2.3 RNA extraction and full-length cDNA cloning of the astaxanthin gene

The hepatopancreas (0.15 g) was collected from red shelled crayfish and immediately frozen in liquid nitrogen and stored at -80°C until use. Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Then, a spectrophotometer (NanoDrop 2000c, Thermo, USA) and electrophoresis with 1% agarose gels were used to determined quantity, purity and integrity of RNA. The hepatopancreas cDNA was obtained using the SMART RACE cDNA amplification kit (Clontech, Dalian, China). Based on the sequence characteristics of astaxanthin gene in relevant study and

combined with the genomic information of *P. clarkii*, we obtained the predicted astaxanthin sequence (XM_045755678.1) from the NCBI database (He-Ling et al., 2012). Then the full-length cDNA of *PcASTA* was cloned with 5'- and 3'-RACE, and specific primers (Table 1) were designed based on the predicted sequence. The PCR protocol used was as follows: 5 min at 94°C; 32 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, and a 10 min final extension at 72°C. The PCR products were sequenced using the Sanger method (Sangon, Shanghai, China).

2.4 Sequence analysis

The NCBI ORF Finder tool (<http://www.ncbi.nlm.nih.gov/projects/gorf/>) was used to identify the open reading frame (ORF) of *PcASTA* (Rong, 2006). The homology analyses of the *PcASTA* nucleotide and protein sequences were performed using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>) (Rong, 2006). The structural domain of the amino acid sequence was identified using the Simple Modular Architecture Research Tool (<http://smart.embl-heidelberg.de/>). Amino acid sequence composition, molecular weight, isoelectric point, and other physical parameter information were obtained using the online ProtParam (<http://www.expasy.org/tools/protparam>) tool (Gasteiger et al., 2005). Multiple sequence alignment of the astaxanthin gene was performed using ClustalX software (Thompson et al., 1997). MEGA 5.2 was used to construct a phylogenetic tree with the maximum likelihood method, and the aligned sequences were bootstrapped until 1000 replicates were performed (Kumar et al., 2008).

2.5 Tissue expression of *PcASTA*

First, different tissues (hepatopancreas, muscle, gonad, intestine, tail shell membrane and cephalothorax shell membrane) were obtained from eight red shell crayfish (four females and four males) and eight green shell crayfish (four females and four males). Matched tissues were then mixed and total RNA was extracted according to the methods described above. The PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Dalian, China) was used to

synthesize cDNA for qPCR analysis. Specific primers (Table 1) were designed based on the sequence of *PcASTA*. The 18s sequence (Table 1) was used as the internal reference. qPCR was carried out in a quantitative thermal cycler (Bio-Rad CFX-96; Bio-Rad, USA), with the following parameters: 3 min at 95°C; then 40 cycles of 95°C for 5 s, 57.5°C for 30 s and 72°C for 30 s. Finally, tissue-specific expression of *PcASTA* was analyzed using the comparative CT method ($2^{-\Delta\Delta Ct}$).

2.6 Impact of β -carotene

Sixty healthy red shell *P. clarkii* were divided into two groups (30 crayfish per group), where one group was fed a common commercial crayfish diet (3% of biomass), and the other was fed the commercial crayfish diet with added β -carotene (80 mg/kg). After two weeks of breeding at room temperature ($28 \pm 2^\circ\text{C}$), eight crayfish (four females and four males) were randomly selected from each group and the shells and tissues were taken according to the methods described above. The content of astaxanthin in shells and the relative expression of *PcASTA* in the tissues were also detected according to the methods described above.

2.7 Temperature stress

Sixty healthy red shell *P. clarkii* were divided into two groups (30 crayfish per group), and two groups were cultured at a water temperature of 20°C (group A) and 30°C (group B) for 7 days, respectively. The shells and tissues were collected according to the methods described above. The content of astaxanthin in shells and the relative expression of *PcASTA* in the tissues were detected according to the methods described above.

2.8 Statistical analysis

Data are shown as the mean \pm SD. One-way ANOVA was performed using SPSS 17.0 (SPSS, Chicago, IL, USA) to determine whether there were any significant differences between groups. A *p*-value < 0.05 was considered statistically significant.

3 Results

3.1 Analysis of carotenoid content in different color shells

As shown in Figure 1, five different carotenoids were detected in red and green shell crayfish. The total carotenoid content in red shells (TS and CS) was significantly higher than that in green shells (TS and CS). The content of astaxanthin in all shrimp shells was significantly higher than in other carotenoids. The content of lutein and β -carotene was also higher, while the content of zeaxanthin and echinenone were the lowest. Moreover, the content of various carotenoids in the TS was higher than that in the CS of the same-color crayfish.

TABLE 1 Primers used in the study.

Primer name	Sequence (5'-3')	Application
PcASTA-3'	CTCGCGCTATGTGGGTGAAGG	3'RACE
PcASTA-5'	CTCGGTCCATACGGGTGTGC	5'RACE
PcASTA-RTF	CGGCTCAGCCATCTGGGAG	qPCR
PcASTA-RTR	ACCTGCTGCGCTCCTCCTAT	qPCR
18S-F	CTGTGATGCCCTTAGATGTT	qPCR internal control
18S-R	GCGAGGGGTAGAACATCCAA	qPCR internal control

3.2 Cloning and sequence analysis of *PcASTA*

The full-length cDNA of *PcASTA* (Accession No. OR514599) was obtained by 3'- and 5'- RACE (Figure S1). The sequence contained a 27-bp 5'-UTR, a 556-bp 3'-UTR, and a 747-bp open reading frame (ORF). The ORF encoded a polypeptide of 248 amino acids with a calculated molecular weight of 27.6 kDa and a theoretical isoelectric point of 4.67. It also contained a conserved zinc metalloproteinase domain (HEXXHXXGFXHEXXRXDRD). A phylogenetic tree was constructed and indicated that the amino acid sequence of *PcASTA* belonged to the same clade as astaxanthin from *Astacus astacus*, with a confidence level of 100% (Figure 2).

3.3 Tissue expression analysis

qPCR was used to detect the expression levels of *PcASTA* in different tissues of red and green shell crayfish. As shown in Figure 3, *PcASTA* was expressed in all tissues from both types of crayfish. In green shell crayfish, the highest level of *PcASTA* was in hepatopancreas, followed by muscle. Expression in those tissues was significantly higher than that in other tissues ($p < 0.05$). The levels of expression in the remaining tissues followed the order: tail shell membrane < gonad < intestine < cephalothorax shell membrane. In red shell crayfish, the hepatopancreas also had the highest level of expression, followed by muscle. Expression in both tissues was significantly higher than that in the other tissues ($p < 0.05$). The levels of expression in the remaining tissues were similar to that in green shell crayfish (tail shell membrane < intestine < gonad < cephalothorax shell membrane).

Comparing the expression levels of *PcASTA* in different tissues of the two colors of crayfish, it was observed that expression was significantly higher in all tissues from red crayfish compared to green crayfish ($p < 0.05$). Moreover, the most significant difference in expression was in tail shell membrane.

3.4 The impact of β -carotene

The astaxanthin content in shells and the expression levels of *PcASTA* were detected in crayfish fed a commercial diet containing β -carotene. As shown in Figure 4A, the astaxanthin content of CS and TS among crayfish fed a diet containing β -carotene was significantly higher than in crayfish fed a commercial diet ($p < 0.05$).

As shown in Figure 4B, the expression of *PcASTA* in all tissues tested (except gonad) in crayfish fed a diet containing β -carotene was significantly higher than in crayfish fed a commercial diet ($p < 0.05$).

3.5 The impact of temperature

The astaxanthin content in shells was evaluated in crayfish cultured at different water temperatures. As shown in Figure 5A, the astaxanthin content of the TS in crayfish from group B was significantly higher than that of group A ($p < 0.05$). However, the astaxanthin content in the CS of group B crayfish was higher than that of group A crayfish, but the difference was not significant.

The expression of *PcASTA* in different tissues from crayfish in groups A and B were also detected (Figure 5B). *PcASTA* was expressed in all tissues, but the expression levels in each tissue

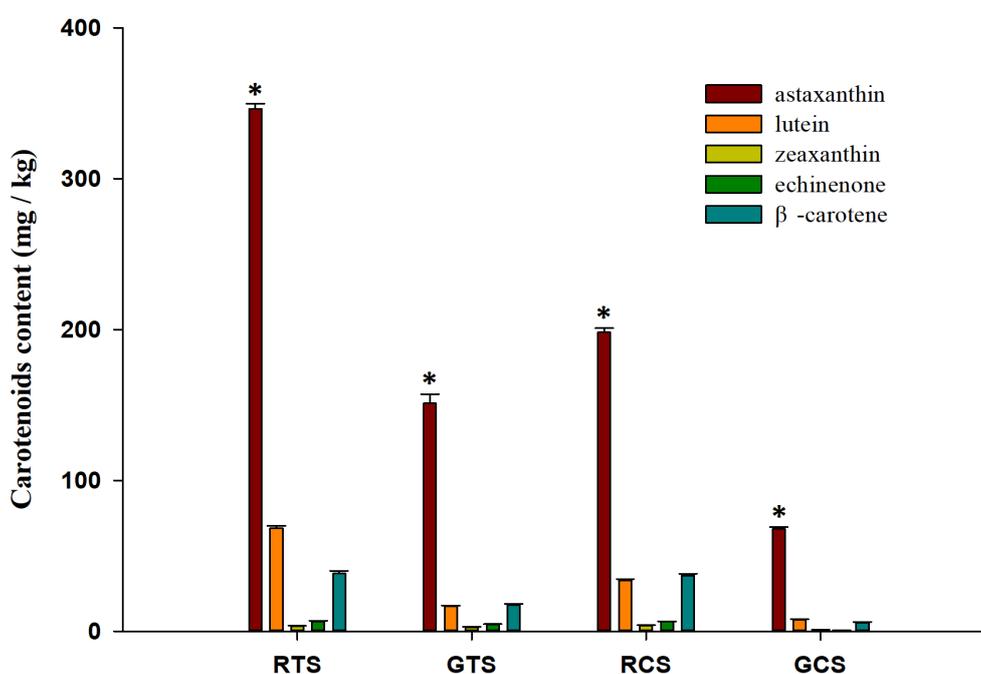


FIGURE 1

Different carotenoids contents in shells. RTS, red tail shell; GTS, green tail shell; RCS, red cephalothorax shell; GCS, green cephalothorax shell. "*" indicate significant differences in astaxanthin content compared to other carotenoids in each shell ($p < 0.05$).

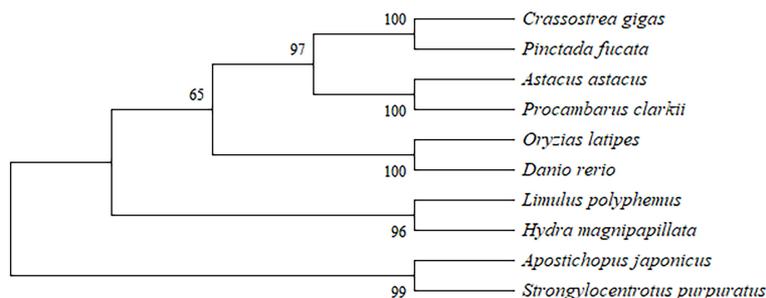


FIGURE 2
Phylogenetic analysis based on multiple sequence alignment. GenBank accession numbers: *Crassostrea gigas* (AAQ13464), *Pinctada fucata* (AAX56337), *Astacus astacus* (CAA64981), *Procambarus clarkii* (OR514599), *Oryzias latipes* (NP_001098208), *Danio rerio* (XP_001342763), *Limulus polyphemus* (CAQ16892), *Hydra magnipapillata* (XP_002164184), *Apostichopus japonicus* (HQ655812), and *Strongylocentrotus purpuratus* (XP_786540). Numbers are the bootstrap values for 1000 trials.

from group B crayfish was significantly higher than that of group A crayfish ($p < 0.05$).

4 Discussion

Carotenoids are natural pigments, and play important roles in color formation in aquatic animals. Some studies have found that carotenoids not only affect muscle color, but also affect the shell

color of shellfish (Li et al., 2010; Li et al., 2014b). In this study, different carotenoids were detected in red and green shell crayfish. We found that all types of carotenoids in the TS and CS of red shell crayfish were higher than in green shell crayfish, which means that carotenoids may affect shell color. Although animals can only obtain carotenoids (including astaxanthin) through their diet, Wade et al. indicated that crustaceans can convert one carotenoid to another (Wade et al., 2017). Some studies found that β -carotene and zeaxanthin were converted to astaxanthin through a complex

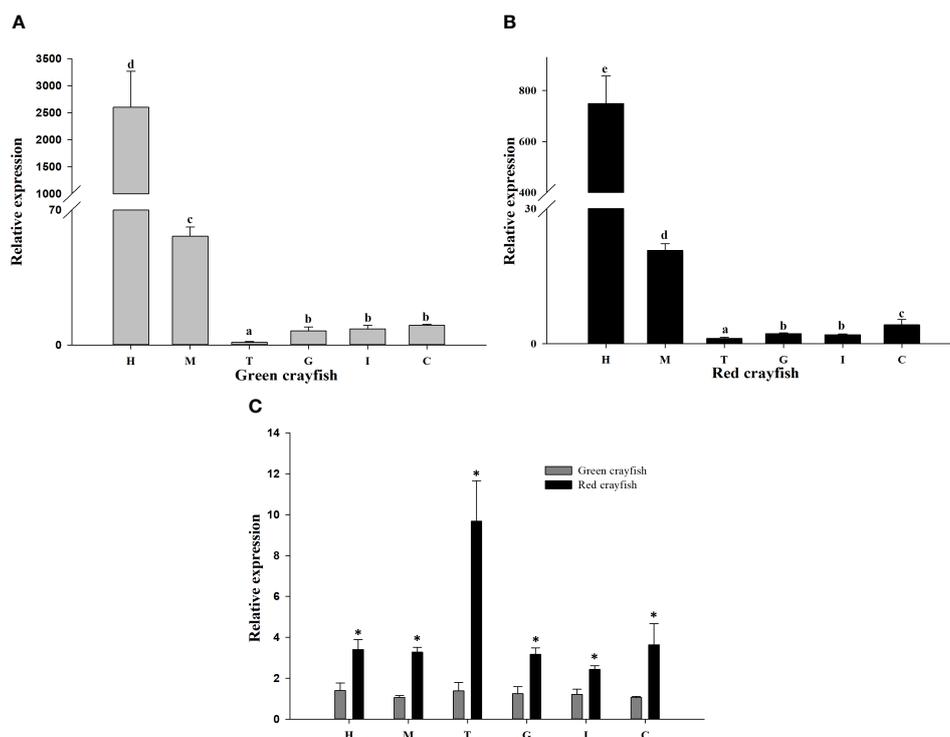


FIGURE 3
Relative expression of *PcASTA*. The relative expression of *PcASTA* in various tissues from green (A) and red (B) crayfish. Comparisons of *PcASTA* expression in green and red crayfish (C). H, hepatopancreas; M, muscle; T, tail shell membrane; G, gonad; I, intestine; C, cephalothorax shell membrane. Bars with different letters indicate significant differences in *PcASTA* expression levels between different tissues ($p < 0.05$). "*" indicates significant differences in *PcASTA* expression levels between the same tissues of two color crayfish ($p < 0.05$).

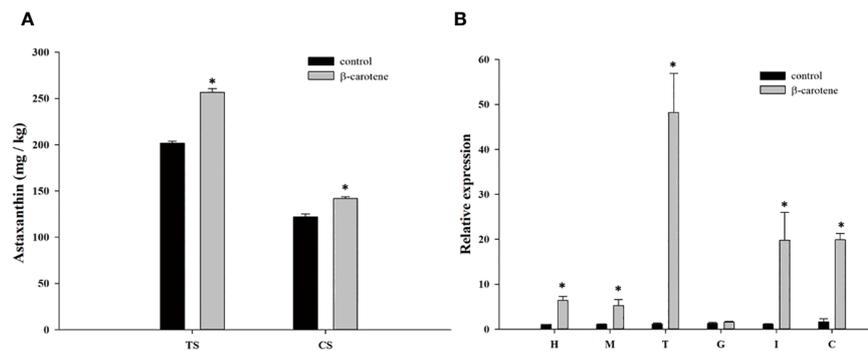


FIGURE 4

Astaxanthin content in shells (A) and the expression level of *PcASTA* in different tissues (B) following fed β-carotene. TS, tail shell; CS, cephalothorax shell; H, hepatopancreas; M, muscle; T, tail shell membrane; G, gonad; I, intestine; C, cephalothorax shell membrane. "*" indicates significant differences in astaxanthin content between the same shells and *PcASTA* expression levels between the same tissues of two groups ($p < 0.05$), the same as below.

process (Tanaka et al., 1976; Boonyaratpalin et al., 2001). The results also showed that astaxanthin was the carotenoid with the highest levels in shells, and its content in different color shells was also significantly different. Astaxanthin is a common carotenoid, which mainly exists in crustaceans and algae (Shahidi and Brown, 1998). Some studies indicated that astaxanthin is not only an antioxidant, but also a colorant (Shimidzu et al., 1996; Boonyaratpalin et al., 2001). Vogt et al. revealed that astaxanthin is the main colorant in aquatic animals, and the astaxanthin gene was first detected in the hepatopancreas of crayfish (Vogt et al., 1989). Those data indicated that astaxanthin may be the most important factor affecting the shell color of crayfish.

Although many studies have shown that the astaxanthin plays a role in the shell color formation of aquatic crustaceans, the specific molecular mechanisms by which that occurs are unclear. In this study, the astaxanthin gene (*PcASTA*) from *P. clarkii* was successfully cloned. The amino acid sequence contained a zinc metallopeptidase domain, which belongs to the conserved sequence of the astaxanthin superfamily. The 'HEXXH' motif is essential for zinc binding, and the biological function of zinc metallopeptidase requires the binding of zinc to this site (Bode et al., 1992). Moreover, phylogenetic analyses showed that the *PcASTA* amino

acid sequence belonged to the same clade as astaxanthin sequences from *A. astacus*. Therefore, the results suggested that *PcASTA* belongs to the astaxanthin superfamily. Astaxanthin genes have been found in many species, but their role in color formation remains unclear.

One study suggested a close relationship between the astaxanthin gene and sea cucumber body color (He-Ling et al., 2012). In this study, astaxanthin gene expression was the highest in the hepatopancreas of crayfish of two different colors. Studies have shown that the hepatopancreas is an important organ for metabolism and the storage of carotenoids (Schumaker et al., 1994; Vershinin, 1996). Some researchers also suggested that the expression levels of carotenoid-related genes were closely related to the levels of carotenoids in tissues (Schumaker et al., 1994; Li et al., 2014a). The high levels of *PcASTA* expression might be due to its involvement in metabolism and the storage of carotenoids in the hepatopancreas. Further comparing the expression levels of astaxanthin genes in the tissues of two different color crayfish, it was found that the expression levels of *PcASTA* in tissues from red shell crayfish were significantly higher than in green shell crayfish. The difference was greatest in the CS membrane, and there was also a significant difference in the TS membrane.

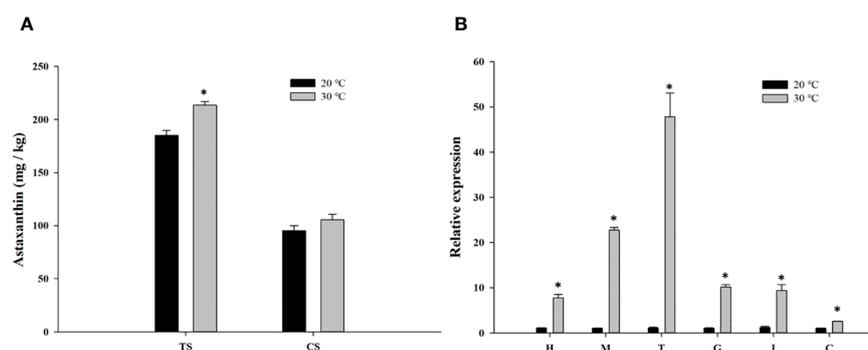


FIGURE 5

Astaxanthin content in shells (A) and the expression level of *PcASTA* in different tissues (B) following cultured in different temperature. TS, tail shell; CS, cephalothorax shell; H, hepatopancreas; M, muscle; T, tail shell membrane; G, gonad; I, intestine; C, cephalothorax shell membrane. "*" indicates significant differences in astaxanthin content between the same shells and *PcASTA* expression levels between the same tissues of two groups ($p < 0.05$).

Simkiss et al. indicated that the shell membrane provided a mineralization template and participates in calcification during the formation of crustacean shells (Simkiss and Wilbur, 1989). Shechter et al. found a large amount of uncalcified chitin in the shell membranes of red claw crayfish (Shechter et al., 2008). Chitin is synthesized in crustaceans, and then forms a hard shell through a series of complex processes such as transportation, deposition, and calcification. Thus, chitin is indispensable for the formation of crustacean shells (Muzzarelli, 2013). These results suggested that *PcASTA* may be involved in shell color formation in *P. clarkii*.

Crustaceans can convert one carotenoid to another, and Matsuno indicated that the major conversion pathway of carotenoids to astaxanthin in crustaceans was the conversion of β -carotene to astaxanthin (Matsuno, 2001; Wade et al., 2017). Some studies have found a significant increase in astaxanthin content in shrimp and crab after feeding on β -carotene (Campbell, 1969; Boonyaratpalin et al., 2001). In this study, the astaxanthin content in the CS and TS increased after feeding on β -carotene, and the expression levels of *PcASTA* also increased. Those results suggest that crayfish can also convert β -carotene to astaxanthin, and *PcASTA* may play an important role in that process. Moreover, we also found that high temperatures caused an increase in the expression of *PcASTA* and the levels of astaxanthin in shells. Cheesman et al. found that high temperatures cause esterified astaxanthin to transform into free astaxanthin in crustaceans, thus, darkening the shell color (Cheesman et al., 1967). Carla et al. found that the absorption and metabolism of carotenoids in animals were also affected by temperature (De Carvalho and Caramujo, 2017). Therefore, we hypothesize that *PcASTA* may affect shell color in *P. clarkii* by participating in the synthesis and transformation of astaxanthin.

In conclusion, this study for the first time indicated that *PcASTA* may be an important gene in the synthesis of astaxanthin, affecting the shell color of *P. clarkii*. These findings will help to understand the mechanisms of shell color formation, and thus, improve the crayfish farming industry.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary Material](#).

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee (IACUC) of Anhui Academy of Agricultural Sciences, Hefei, China. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XC: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. JC: Investigation, Project administration, Software, Writing – original draft. LH: Investigation, Resources, Software, Writing – review & editing. BW: Methodology, Software, Writing – review & editing. CW: Investigation, Validation, Writing – original draft. JH: Conceptualization, Project administration, Supervision, Writing – review & editing. ZB: Project administration, Supervision, Writing – review & editing.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1343126/full#supplementary-material>

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