

Analysis of relationship between key genes and astaxanthin biosynthesis in *Phaffia rhodozyma* by transcript level and gene knock-out

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Abstract

Background *Phaffia rhodozyma* is a potential industrial source for production of natural astaxanthin. The synthetic mechanism of astaxanthin in *P. rhodozyma* is complex and unclear that blocked its development.

Results In this study, eight genes related to dicyclic and monocyclic pathway in three different strains of *P. rhodozyma* were analyzed, and the relationship between the expression and astaxanthin biosynthesis was explored. Among these genes, *crtYB* (R=0.75, P<0.05) and *asy* genes (R=0.74, P<0.05) showed the most closely correlation with astaxanthin biosynthesis. In order to further study exact relationship, *crtYB* and *asy* genes were knocked out by homologous recombination. After *crtYB* knock-out, astaxanthin was decreased to be under detected line. It suggested *crtYB* played a role in dicyclic and monocyclic pathway. Meanwhile, the *asy* gene was in dicyclic pathway of astaxanthin biosynthesis, and its knock-out would promote the astaxanthin biosynthesis in monocyclic pathway, resulting in a 25.04% increase in astaxanthin production.

Conclusion The possible rate-limiting enzymes were *asy* gene and *crtYB* illustrated by analysis of regression. Knock-out of *asy* and *crtYB* gene was great helpful to understand the synthetic pathway of astaxanthin, and significant to the industrial application of producing astaxanthin.

Background

Astaxanthin (3, 3'-c- β , β -carotene-4, 4'-dione; C₄₀H₅₂O₄) could be acted as protective agent against oxidative damage to cells in vivo [1, 2] to resist against many diseases, such as cancer, skin related illness [3], and heart disease [4]. Based upon its various biological functions, astaxanthin has a significant economic value and growing global commercial market. It is well established that *Phaffia rhodozyma* and *Haematococcus pluvialis* are important sources for natural astaxanthin [5–8]. Among them, the fermentation conditions of *P. rhodozyma* are easy to meet, and the fermentation medium is simple [5–7]. Therefore, *P. rhodozyma* has gradually became one of the strains with industrial astaxanthin production capacity.

Astaxanthin biosynthesis pathway in *P. rhodozyma* has been reported by researches [9–11]. It is mainly considered as two pathways: dicyclic and monocyclic pathway (Fig. 1) [5, 12]. The dicyclic pathway of astaxanthin in *P. rhodozyma* begin with isoprene pyrophosphate (IPP) isomerase encoding the *idi* gene [13, 14], then go through the geranylgeranyl pyrophosphate (GGPP) synthase (*crtE* gene)[15], phytoene- β -carotene synthase (*crtYB* gene)[16] and phytoenedesaturase enzyme (*crtl* gene) [17], and then forming β -carotene. In the last step, the formation of astaxanthin from β -carotene in *P. rhodozym* was performed by a single astaxanthin synthase (*asy*) gene that could own both ketolase and hydroxylase activities [11, 18]. It exists a great controversy about function of *asy* gene. The *asy* may still require an auxiliary enzyme of cytochrome P450 reductase (*crtR*) [19]. The monocyclic pathway is the differentiation of neurosporene

from the dicyclic pathway through crtYB and crtI, forming 3, 3'-didehydrogenated- β -carotene-4, 4'-dione (DCD), finally converted to astaxanthin [20]. Previous studies mainly focused on the functions of these genes, but few researches could pay close attention to the relationship between these genes and astaxanthin synthesis. However, the clear and definite of the relationship is more significant for regulating metabolism of synthetic astaxanthin.

Therefore, we investigated expression levels of the genes related to dicyclic and monocyclic pathway in three *P. rhodozyma* of high-yield, low-yield and wild strains, and their relationship with astaxanthin and carotene synthesis. Meanwhile, the possible rate-limiting enzymes in astaxanthin synthesis were speculated by regression analysis. Moreover, the mechanism of action of the speculated rate-limiting enzymes were further validated and analyzed by gene knock-out. It provided support for understanding the relationship between these key genes and astaxanthin synthesis process in *P. rhodozyma*.

Results

Relationship between total carotene content and genes expression level

We detected the relative expression of eight genes in three different yields of *P. rhodozyma* and analyzed their correlation with total carotene, β -carotene and astaxanthin. Figure 2 shows that four genes such as *asy*, mature mRNA of crtYB (mm crtYB), alternatively spliced mRNA of crtYB (am crtYB) and crtE were significantly correlated with total carotene content, while other genes showed a low correlation. The results indicated that the four genes have an impact on carotene synthesis.

The *asy* is presumed to be ending gene for the conversion of carotene to astaxanthin biosynthesis in the dicyclic pathway [6, 21]. Figure 2A shows that it had the highest positive correlation with total carotene content ($R = 0.68$, $P < 0.05$). It further confirmed that the high expression of *asy* gene promoted the accumulation of carotene.

In this study, am crtYB gene showed a positive linear correlation ($R = 0.675$, $P = 0.0011$, Fig. 2B) with total carotene content. The results suggested that the gene played an important role in the whole process of carotene synthesis. Interestingly, unlike am crtYB, mm crtYB showed negatively correlated ($R = 0.475$, $P < 0.05$, Fig. 2C). This was related to the functional complementarity between the two genes [16].

The crtE gene encodes GGPP synthase that catalyzes the addition of three-molecule IPP to a DMAPP in turn to form GGPP. We have confirmed that there was a positive correlation between crtE and total carotene synthesis ($R = 0.44$, $P < 0.05$), that GGPP synthase could play a role in regulating carbon flow [22].

On the other hand, there were different trends of these genes for β -carotene (Fig.S1). The significant correlation genes with β -carotene were the am crtYB, crtE and the alternatively spliced mRNA of crtI (am crtI), while other genes had lower correlation and regression coefficient. The crtYB catalyzes the synthesis

of β -carotene from lycopene [21]. It was a direct gene involved in β -carotene synthesis. The correlation ($R = 0.61, P < 0.05$) between *am crtYB* and β -carotene further confirmed this result. The *crtE* could provide precursor GGPP for the synthesis of β -carotene. That was why its expression showed positively correlated. The *crtI* was an octahydrogen tomato dehydrogenase and a key synthetase for γ -carotene synthesis [23]. Fig. S1G shows that the expression of *am crtI* was positively correlated with β -carotene ($R = 0.57, P < 0.05$). Some studies have pointed out that there is a certain positive correlation between γ -carotene and β -carotene synthesis [22].

Relationship between astaxanthin content and genes expression level

Astaxanthin synthesis might be regulated by a variety of genes [24]. In this study, we focused on the relationship between astaxanthin production and these genes. Figure 3 shows that there were significant correlations of *am crtYB*, *asy*, *am crtI* and *crtE* gene with astaxanthin production ($p < 0.05$). There was a high correlation ($R = 0.74, P = 0.0042$, Fig. 3A) between *asy* gene expression and astaxanthin production. The expression of *crtYB* also showed high correlation with astaxanthin content ($R = 0.75, P = 0.0005$ Fig. 3B).

Overall, there were great different affection on carotene synthesis among different genes, and the most significant correlation for total carotene was *asy* gene ($R = 0.69, P = 0.003$). Moreover, the genes of the most significant correlation with astaxanthin were *am crtYB* ($R = 0.75, P = 0.0005$) and *asy* ($R = 0.74, P = 0.0042$).

Verification of genes knock-out in *P. rhodozyma*

In the above studies, we confirmed that *am crtYB* and *asy* genes were highly correlated with astaxanthin synthesis. Therefore, the two genes were selected for deletion in high-yield and wild strain *P. rhodozyma* in order to further analyze their mechanism of action in astaxanthin synthesis.

Single colonies grown on the transformed culture plate were randomly selected to form cell solution. The genome was identified by PCR using primers M-F/M-R and K-F/K-R designed according to exogenous gene fragments. Fig. S2A, B shows that exogenous gene fragments could not be amplified in the original strain JMU-MVP14 and JMU-VDL668, while the *crtYB* gene knock-out strains could be used as template to amplify the products of about 1200 bp. It suggested that the *crtYB* gene knock-out strains were successfully screened. The result of electrophoresis of *asy* gene was consistent with the expectation (Fig. S2C) that indicated *asy* gene also was knocked out. The strains JMU-MVP14 and JMU-VDL668 after knocking out the *asy* gene were named JMU-MVP Δ *asy* and JMU-VDL Δ *asy*, respectively. So was *crtYB* gene.

From the colony morphology, the strains with the *crtYB* gene knock-out could be preliminarily screened out (Fig. S3). The colony of the original strain JMU-MVP14 was dark red. The colony of the strain JMU-

VDL668 was pink. The colony of both strains turned white after the crtYB gene was knocked out. However, the color of asy knock-out strain did not change significantly.

Effect of genes knock-out on growth

The biomass curves of *P. rhodozyma* strains JMU-MVP14, JMU-VDL668 JMU-MVP Δ crtYB and JMU-VDL Δ crtYB are shown in Fig. 4A, B. The results showed that the growth trend of JMU-MVP Δ crtYB and JMU-MVP was similar, while the growth rate of JMU-VDL Δ crtYB was faster than that of original strain, and the final biomass was about 35% higher than that of original strain. The knock-out of crtYB gene had no significant effect on the growth of JMU-MVP14. It also promoted the growth of strain JMU-VDL668. On the other hand, the biomass curves of the original strains JMU-MVP14, JMU-VDL668, JMU-MVP Δ asy and JMU-VDL Δ asy are shown in Fig. 4C, D. The growth trends of strains JMU-MVP14 and JMU-MVP Δ asy were similar. After stabilization, the biomass of JMU-MVP Δ asy and JMU-VDL Δ asy strains did not differ significantly from that of their original strains.

Effect of genes knock-out on accumulation of pigmentation

The total carotenoid production of JMU-MVP14, JMU-VDL668 and crtYB gene knock-out strains JMU-MVP Δ crtYB, JMU-VDL Δ crtYB was shown in Fig. 5A, B. The carotene content of crtYB gene knock-out strains were always under detected line. It was almost negligible compared with the original strains.

By comparing the total carotenoid production of strains JMU-MVP14 and JMU-MVP Δ asy (Fig. 5C, D), it can be found that the trends of total carotenoid accumulation of the two strains were similar in the whole fermentation process. The total carotenoid increased slowly in 0 ~ 48 h period with low yield, it showed rapid accumulation in 48 ~ 80 h period, and then the yield increased slowly and tended to increase gradually. However, the total carotenoid accumulation of the original strain JMU-MVP14 was higher than that of the strain JMU-MVP Δ asy (Fig. 5C, D). It further indicated that asy gene had a significant effect on the carotenoid accumulation.

Furthermore, the production of β -carotene and astaxanthin in different strains of *P. rhodozyma* was determined by HPLC. The curve of astaxanthin production in the strain of asy gene knock-out was drawn according to the experimental results, as shown in Fig. 6. Because the knock-out of crtYB led to the total carotene content under the detected line (Fig. 5A, B), astaxanthin was not detected in them.

The astaxanthin production of strains JMU-MVP 14 and JMU-MVP Δ asy were relatively low and no significant difference was found during 0 ~ 24 h. At 24 ~ 56 h, astaxanthin accumulation of both strains were relatively slow, and astaxanthin production of strain JMU-MVP 14 was higher than that of strain JMU-MVP Δ asy. After 56 h of fermentation, the astaxanthin production of strain JMU-MVP Δ asy was higher than that of original strain. The astaxanthin production of strain JMU-MVP Δ asy was slightly higher than that of strain JMU-MVP (12.32 ~ 15.12%) at most (Fig. 6A).

The trend of astaxanthin production of strains JMU-VDL668 and JMU-VDL Δ asy with time was similar to that of strains JMU-MVP14 and JMU-MVP Δ asy with time. Astaxanthin could not be detected in the

cell extracts of both strains at 0 ~ 8 h, and the astaxanthin yield of strain JMU-VDL668 was higher than that of strain JMU-VDL Δ asy at 8 ~ 40 h. After 40 h, the astaxanthin production of strain JMU-VDL Δ asy exceeded that of strain JMU-VDL 668, and was 25.04% higher than that of strain JMU-VDL 668 (Fig. 6B).

Discussion

The crtYB was considered to be a variable expression protein that belongs to a class of regulatory proteins [16]. It can be transcribed to produce two kinds of products: mature mRNA (mmRNA) and alternatively spliced mRNA (amRNA) [16]. In the nematode *C. elegans*, splicing of the RNA encoding the ribosomal protein was a regulated process for productive or unproductive transcripts [25]. In this study, when the synthesis of mm crtYB was reduced, am crtYB was accumulated. These results indicated that a similar regulatory pattern of functional complementarity might exist between the two splicing products in *P. rhodozyma*. Moreover, the knock-out result further confirmed the important role of crtYB in astaxanthin and total carotene synthesis. Therefore, its positive effect on the synthesis of total carotene was beyond doubt.

The direct gene for astaxanthin synthesis is asy gene (Fig. 7). The previous studies of Lodato, Miao and Marcoleta et al. pointed that the expression of asy gene in high-yielding astaxanthin-producing strains was higher than that in low-yielding strains [17, 26, 27]. Contreras et al. also found that asy gene was overexpressed in *P. rhodozyma* [28], the total carotenoid production of the strain did not change significantly, and the astaxanthin ratio increased. In this study, the asy gene expression showed high correlation with astaxanthin content and total carotene content. Therefore, asy was an important gene to regulate the accumulation of pigments, especially astaxanthin.

As secondary metabolites [29], carotene and astaxanthin have no significant effect on biomass of primary production in *P. rhodozyma*. That was why the biomass of the knock-out strain did not decrease. Due to substance conservation, secondary metabolism was weakened, while primary metabolism would be strengthened. The knock-out of crtYB gene promoted the growth of JMU-VDL668. Compared with asy gene, crtYB gene played a more significant role as a key gene in the synthesis of secondary metabolites such as astaxanthin and carotene. The JMU-VDL668 strain was a low-yield carotene strain (Table 1), and the asy gene presumed to be the terminal pathway of its carotene [18, 19], the effect of asy gene knock-out was lower than that of crtYB.

Table 1
Pigment profiles of *P. rhodozyma* strains

Strains	Biomass(g/L)	Total Carotene(mg/g)	β -Carotene(mg/g)	Astaxanthin(mg/g)
<i>P. rhodozyma</i> MVP14	5.12 ± 0.15	5.15 ± 0.23	1.89 ± 0.32	2.23 ± 0.14
<i>P. rhodozyma</i> VDL668	12.22 ± 0.43	0.83 ± 0.0050	0.045 ± 0.0023	0.28 ± 0.015
<i>P. rhodozyma</i> N3	15.29 ± 0.23	0.30 ± 0.032	0.023 ± 0.0016	0.019 ± 0.0017

The astaxanthin biosynthesis system in *P. rhodozyma* has dicyclic and monocyclic pathways (Fig. 7) [5]. The crtYB gene knock-out could interrupt the whole carotene pathway, resulting in the absence of carotene (Fig. 5A, B). The crtYB is distributed in both pathways. Meanwhile, asy gene was considered as terminal enzyme of forming astaxanthin from β -carotene [11, 18] in the dicyclic pathway (Fig. 7). When asy in the dicyclic pathway was disrupted, the efficiency of the monocyclic pathway might be enhanced. Under some specific conditions, the monocyclic pathway is more efficient than the dicyclic pathway for astaxanthin synthesis [20]. Figure 6 also showed that *P. rhodozyma* could synthesize more effectively astaxanthin after asy gene knock-out. Previous studies have also confirmed that there is no necessary relationship between asy and astaxanthin synthesis [30]. In addition, β -carotene was significantly reduced in asy knock-out strains (Fig. S4). Lei et al. pointed that carotene synthetase in *Haematococcus pluvialis* was restrained by increased product such as polyunsaturated fatty acid [31]. These results suggested that astaxanthin as terminal product might also restrain β -carotene synthetase. It further illustrated knock-out of asy gene might regulate down the dicyclic pathway. Knock-out of asy and crtYB gene was great helpful to understand the synthetic pathway of astaxanthin, and significant to the industrial application of producing astaxanthin.

Conclusions

We analyzed expression of the genes related to astaxanthin biosynthesis in *P. rhodozyma*, and explored the relationship between the expression and astaxanthin biosynthesis by analysis of regression. From the results of regression analysis, crtYB and asy genes were the most closely related to astaxanthin and carotene biosynthesis. And then crtYB and asy genes were further knocked out by homologous recombination for investigation of their action mechanism in dicyclic and monocyclic pathways. After crtYB gene knock-out, the synthesis of carotene and astaxanthin was decreased to be under detected line that indicated crtYB played a role in both dicyclic and monocyclic pathways. At the same time, asy gene knock-out would regulate down the dicyclic pathway of astaxanthin biosynthesis and promote another monocyclic pathway, resulting in a 25.04% increase in astaxanthin production. It is of great significance to understand the mechanism of astaxanthin synthesis and improve the industrial production of astaxanthin.

Methods

Yeast strains

P. rhodozyma strains JMU-MVP14, JMU-VDL668, JMU-N3 were used in the experiments. *P. rhodozyma* JMU-VDL668 strain in our laboratory originated from *P. rhodozyma* Past-1 (generously provided by professor Ulf Stahl, Berlin Industrial College, Germany). The astaxanthin overproducing mutant *P. rhodozyma* JMU-MVP14 and JMU-N3 were established through ethyl methylsulfonate mutagenesis from JMU-VDL668. *P. rhodozyma* strain JMU-N3 was a strain of low astaxanthin production established by our laboratory, while *P. rhodozyma* JMU-MVP14 was a strain with high astaxanthin production. The profiles of the three strains were shown in Table 1.

Culture conditions

P. rhodozyma were grown in YPD culture at 22 °C. 2% of the cells were inoculated in 250 mL flasks with 30 mL medium and cultivated for 48 h. After two generations of cultivation, the YPD culture was then transferred to fermentation medium. The fermentation medium and culture condition were the same as our previous study [32] with 12-hour intervals sampling. Three paralleled samples were performed and 5 mL fermentation broths were sampled and stored at -20 °C until detection.

Cell of dry weight, total carotenoid extraction and analysis of carotenoids

For dry weight determinations, 2 mL samples were centrifuged for 5 min at 3500 × g and cells were washed twice by distilled water. Then the washed cells were dried to constant weight at 105 °C to analyze the total weight.

Carotenoid was extracted by the method of dimethylsulfoxide [33]: 2 mL of samples were centrifuged for 5 min at 3500 × g, and the cells were washed twice with distilled water. After decanting the water, the cells were then treated with 2 mL of dimethylsulfoxide at 75 °C. Next, 5 mL of ethanol was used to extract carotenoid. Total carotenoid concentration was detected by spectrophotometer at a wavelength of 474 nm [34]. The analyses of total carotenoid were performed in triplicate, and pigments were normalized by the dry weight of the yeast. Profiles of carotenoid family were analyzed by RP-HPLC using a reverse phase Nova-Pak C18 column. The gradient conditions were shown in Table S1. The elution spectra were recuperated using a diode array detector. Peaks were identified by comparison to the prepared standards (Sigma, USA) and integration of the peak areas was used to quantify carotenoids from obtained standard curves.

Total RNA extraction

The cellular pellets obtained from 40 mL of culture were utilized for the extraction of total RNA by a modified protocol of TRI REAGENT RNA extraction kit (TRI, Sigma, USA). The total RNA concentration was quantified at 260 nm and the purity was determined by the ratio at 260: 230 nm and 260: 280 nm

greater than 1.8. The integrity of the RNA was checked by denaturant agarose gel electrophoresis. The cDNA was prepared using an EasyScript First-Strand cDNA Synthesis SuperMix (Transgen Biotech, China) according to the manufacturer's protocol.

Real-time quantified PCR

RT-qPCR analyses were performed using cDNA samples as a template, with an ABI 7900HT apparatus (Applied Biosystems, Norwalk, CT). Dissociation curves were constructed to test amplification validity. All sequences of target genes were obtained from NCBI. Database accession numbers and the corresponding primer sets in RT-QPCR are shown in Table S2. The β -actin was used as the control gene. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ (cycle threshold) method with SPSS software. Each RT-QPCR analysis was running with triplicate or quadruplicate. The data of relative expression of RT-QPCR were further normalized: relative gene expression on the first day was calibrated as a unit, and all the others were normalized. The processed data are used for correlation regression analysis and plotting by SigmaPlot 14 (Systat Software, Inc).

Gene knock-out

Construction of knock-out vector

Firstly, the long homologous arm fragments of crtYB genes and pMD-18T vector were digested by restriction endonuclease Hind III and Pst I, respectively. The two fragments were ligated by T_4 DNA ligase as pMD-YB. Then, the short homologous arm fragments of the plasmid pMD-YB and crtYB genes were treated with restriction endonucleases BamH I and Kpn I, and the short arm fragments were connected to the plasmid pMD-YB. Finally, the knock-out vector pMD-crtYB of crtYB gene was obtained. The knock-out vector construction method is similar as crtYB. Detailed information of plasmid construction was shown in Fig. S5.

Preparation of competent cells:

The *P. rhodozyma* strains named JMU-MVP14 and JMU-VDL668 were transferred to seed YPD culture medium at 22°C, 180 rpm/min for two days. After three generations of cultivation, 5 mL of culture in exponential growth was collected in a sterile centrifugal tube, centrifuged at 22°C for 5 min at 3000 × g, and the supernatant was discarded as far as possible to collect cells. 25 mL of phosphate buffer (containing dithiothreitol with final concentration of 25 mmol/L) was used to suspend cells at 22°C, for 15 minutes at 180 × g concussion. Then cells were collected at 4°C, 3000 × g centrifugation for 5 minutes, discarding supernatant. They were suspended with 25 mL of pre-cooled 1 mol/L sucrose Tris MgCl₄ (STM) solution at 4°C, and then 3000 × g centrifugation for 5 minutes, discarding supernatant. Repeat once, the cells were suspended with 500 μ L of precooling 1 mol/L STM solution, light rotation and mixing, and then placed on ice for use on the same day.

Electric transformation and screening:

The competent cell were mixed with 3 ~ 5 μg (< 10 μL) linearized plasmids and transferred to a 0.2 cm shock cup for 5 min in ice bath. The shock cup was placed on the electrolytic cell of the gene transducer. The electrotransformation parameters were set as voltage 1500 V, capacitance 25 $\mu\text{g}/\text{F}$ and resistance 200. 1 mL cold 1 mol/L STM solution was immediately added to the shock cup and mixed with the bacterial solution evenly. The mixture was transferred to a 15 mL sterile centrifugal tube (to ensure adequate oxygen supply) with 22°C incubation for 2.5 hours (not shaking). Appropriate amount of transformed solution was taken and coated on the YPD medium plate, and placed in a 22°C incubator at constant temperature. After the growth of the colony, the preliminary screening was carried out according to the colony morphology, and then the cell solution was selected as the template. The original strain was used as the blank control, and the primers designed according to the plasmid skeleton gene fragment were used for PCR amplification. The product was analyzed by agarose gel electrophoresis to identify the gene knock-out of the strain.

Abbreviations

astaxanthin synthase: *asy*; isoprene pyrophosphate: IPP; cytochrome P450 reductase: *crtR*; phytoene- β -carotene synthase: *crtYB*; geranylgeranyl pyrophosphate: GGPP; 3, 3'-didehydrogenated- β -carotene-4, 4'-dione: DCD; phytoenedesaturase enzyme: *crtI*; mature mRNA: mmRNA; alternatively spliced mRNA: amRNA; sucrose Tris MgCl_4 :STM

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

Not applicable.

Data and materials availability

All data needed to evaluate the conclusions are present in the paper and/or Supplementary file.

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Authors' contributions

Conceptualization, LL; Data curation, ZL; Formal analysis, LC, TL; Funding acquisition, ZL and XD; Investigation, ZJ, NH, HN; writing—original draft, ZL and LL. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

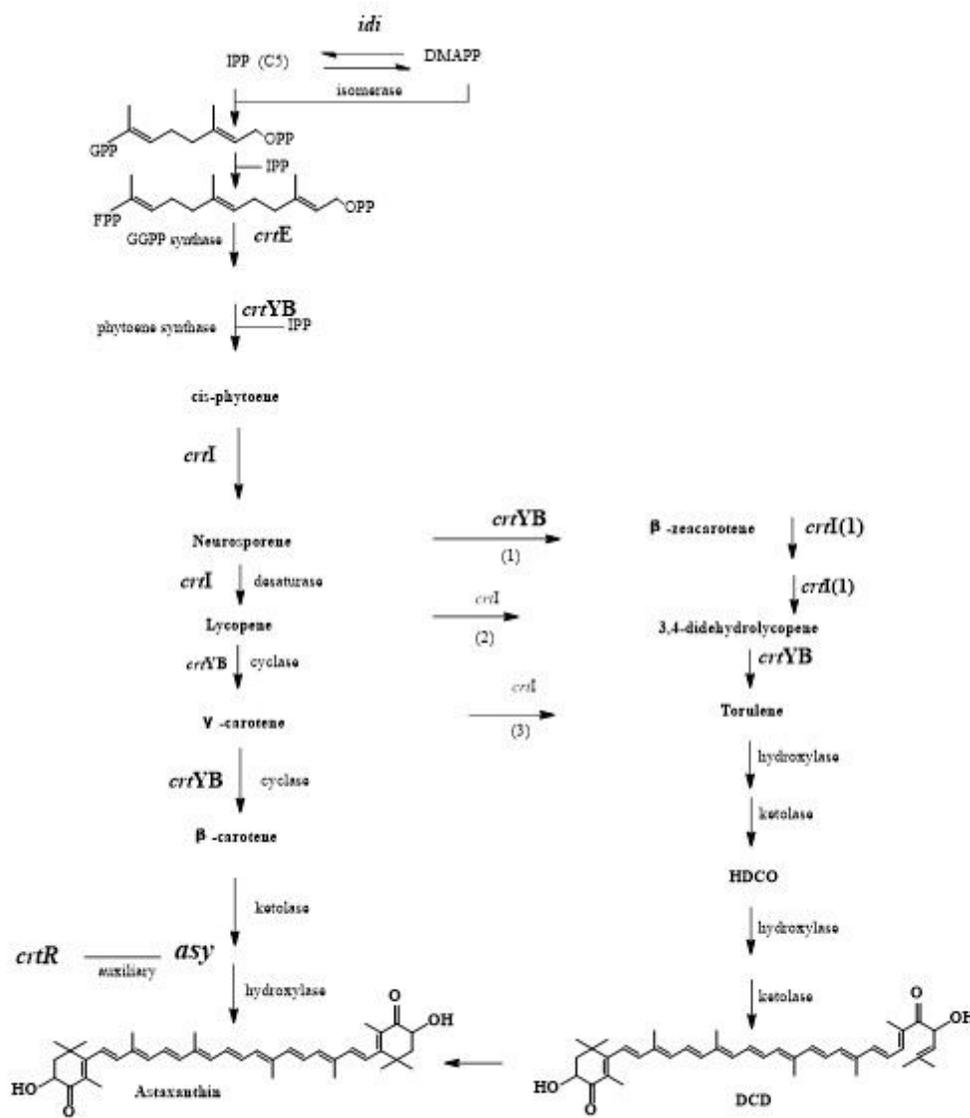


Fig. 1

Figure 1

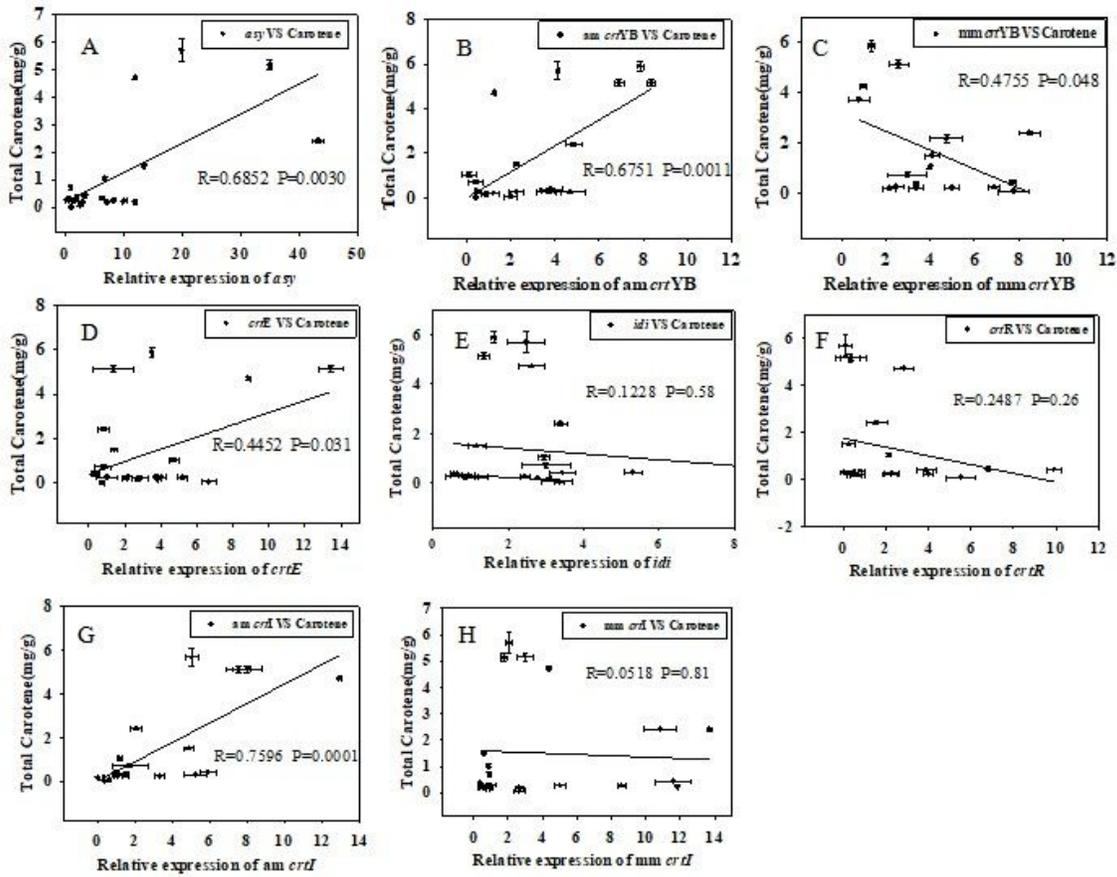


Fig. 2

Figure 2

The regression correlation of total carotene content with expression of gene *asy* (A), *am crtYB* (B), *mm crtYB* (C), *crtE* (D), *idi* (E), *crtR* (F), *am crtI* (G) and *mm crtI* (H)

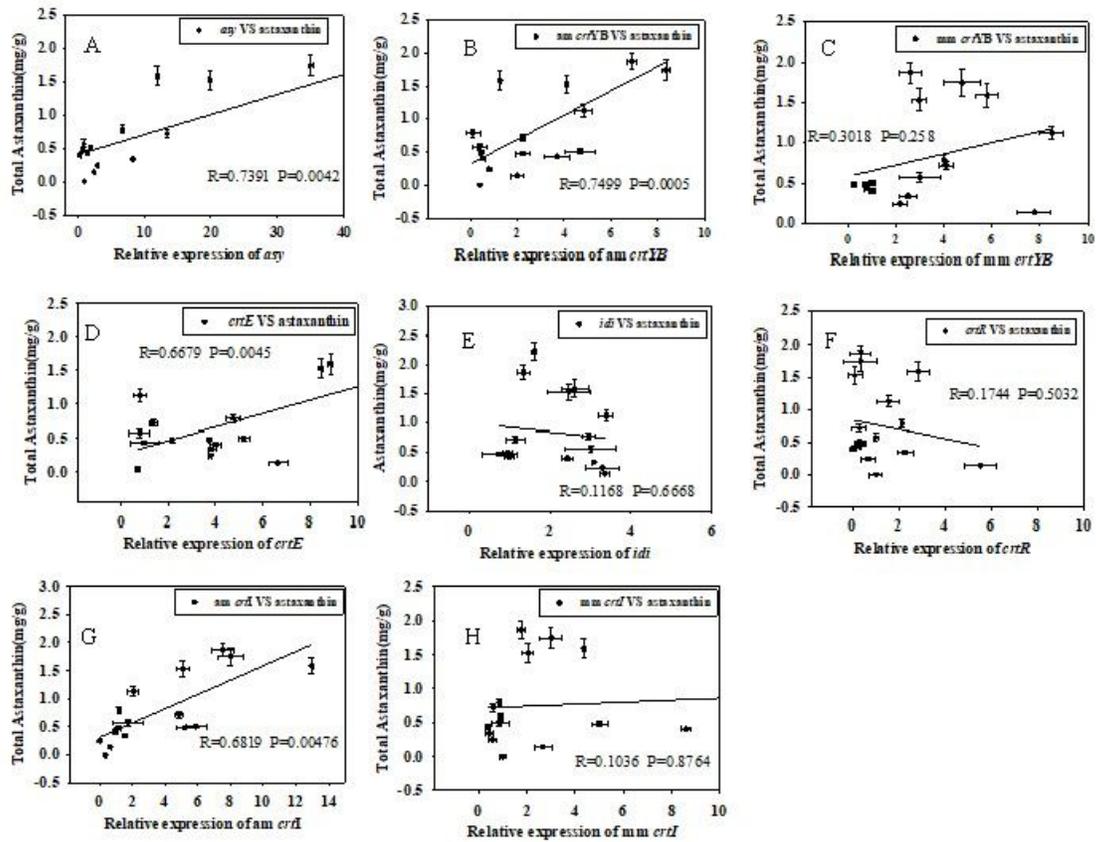


Fig. 3

Figure 3

The regression correlation of astaxanthin with expression of gene *asy* (A), *am crtYB* (B), *mm crtYB* (C), *crtE* (D), *idi* (E), *crtR* (F), *am crtI* (G) and *mm crtI* (H)

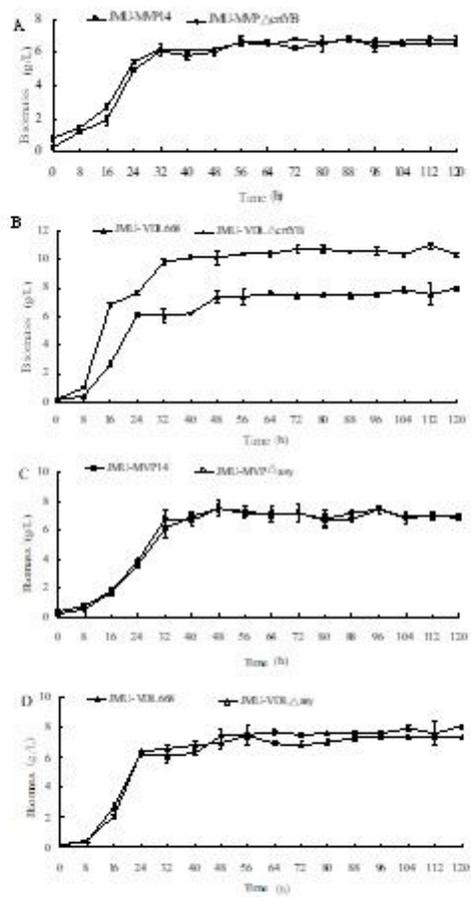


Fig. 4

Figure 4

Growth curves of original strains and the mutants with *crtYB* (A, B) and *asy* (C, D) gene knocking-out

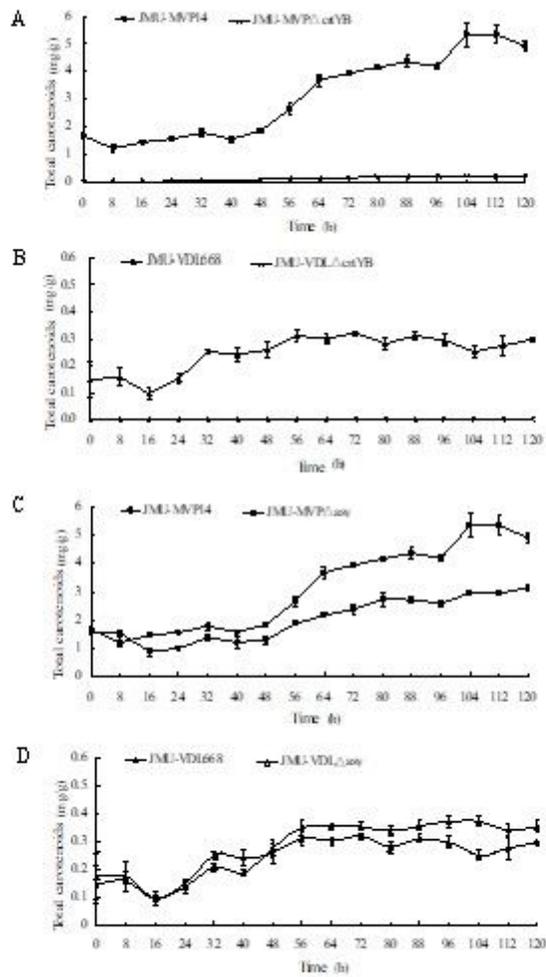


Fig. 5

Figure 5

The curves of total carotenoids in original strains and the mutants with *crtYB* (A, B) and *asy* (C, D) gene knocking-out

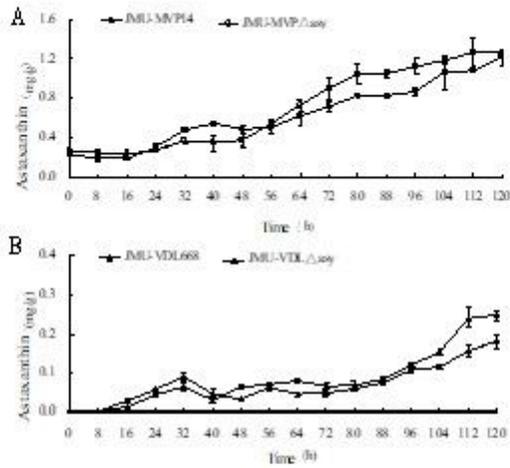


Fig. 6

Figure 6

The curves of astaxanthin in original strains and the mutants with asy gene knocking-out

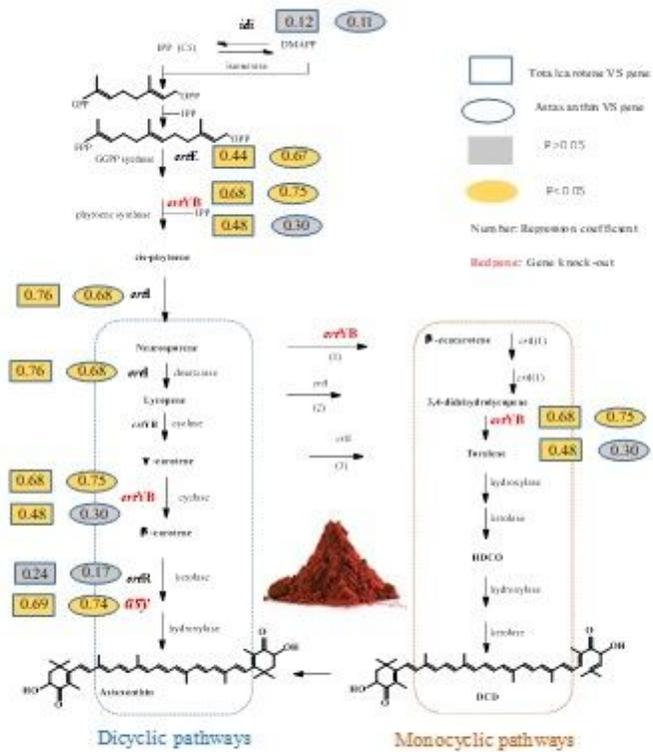


Fig. 7

Figure 7

Presumed pathway of astaxanthin synthesis and regression relationship of gene transcript and astaxanthin production in *P. rhodozyma*

Supplementary Files

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