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**Integrating an algal β -carotene hydroxylase gene into a
designed carotenoid-biosynthesis pathway increases
carotenoid production in yeast**

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Abstract

The algal β -carotene hydroxylase gene *Crchyb* from *Chlamydomonas reinhardtii*, *Czchyb* from *Chlorella zofingiensis*, or *Hpchyb* from *Haematococcus pluvialis* and six other carotenoid-synthesis pathway genes were co-integrated into the genome of a yeast host. Each of these three algal genes showed a higher efficiency to convert β -carotene to downstream carotenoids than the fungal genes from *Phaffia rhodozyma*. Furthermore, the strain with *Hpchyb* displayed a higher carotenoid productivity than the strains integrated with *Crchyb* or *Czchyb*, indicating that *Hpchyb* is more efficient than *Crchyb* and *Czchyb*. These results suggest that β -carotene hydroxylase plays a crucial role in the biosynthesis of carotenoids.

Keywords: green algae; carotenoids; β -carotene hydroxylase; synthetic biology

1. Introduction

Carotenoids are mainly found in photosynthetic organisms and play an important role as light harvesting pigments that protect the photosynthetic apparatus from photooxidative damage (Sandmann, 1994; Bartley and Scolnik, 1995). Carotenoids can stimulate the immune system in human to confer protection against a broad range of diseases, including cancer (Machlin, 1995; Hussein et al., 2006). Thus, carotenoids have a great commercial potential in the pharmaceutical and food industries. Although chemically synthesized products from petrochemicals (Gordon et al., 1982) still occupy the major proportion of the market (Palozza and Krinsky, 1992), some chemically synthesized carotenoids, including astaxanthin, have been found harmful and prohibited from the pharmacy market (Miyawaki et al., 2008; Aoi et al., 2003), and a great demand for natural carotenoids has been created.

The green alga, *Haematococcus pluvialis*, provides the most concentrated natural source of astaxanthin known (1-4 % dry weight), as well as some other important carotenoids such as β -carotene, lutein and canthaxanthin (Paniagua-Michel *et al.*, 2012). However, cultivation of green algae requires a large area of land with strong sunlight and a large amount of fresh water. Therefore, either a more cost-effective process or a high-productivity host organism is needed.

Besides green algae, many other organisms, including fungi, bacteria, and plants, have the ability to produce carotenoids and carotenoid derivatives (Miki, 1991). To reduce the cost of natural carotenoid production, biosynthesis techniques in microorganism or plant provide a promising way for mass production (Misawa and Shimada, 1997). However, currently the total carotenoid productivity is still quite low for many reasons. In the case of transgenic tobaccos, it was probably because of the competition between foreign and endogenous carotenoid ketolases or other carotenoid hydroxylases (Gerjets et al., 2007; Mann et al., 2000; Zhong et al., 2011). The productivity of carotenoid can be improved by designing an efficient pathway by selecting genes from different organisms. Carotenoids are formed via the mevalonate pathway, which starts at acetyl-CoA and proceeds through mevalonate to isopentenyl-pyrophosphate (IPP), the general precursor of all isoprenoids. Eight molecules of IPP are subsequently condensed to form colorless carotenoids via several dehydrogenation and cyclization reactions (Fig. S1a in the Supplementary data). In order to increase the metabolic flux towards the isoprenoid precursors, an additional copy of *tHMG1* (a truncated 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene from *Kluyveromyces marxianus*) and the *crtE* (geranylgeranyl pyrophosphate synthase gene from *X. dendrorhous*) were co-transformed into the yeast host. The β -carotene biosynthesis pathway of *X. dendrorhous*, which has fewer steps compared to others and

showed a high productivity in engineered *Saccharomyces cerevisiae* (Verwaal et al., 2007; Ukibe et al., 2009), includes the bifunctional phytoene synthase/lycopene cyclase gene (*crtYB*) and the cytochrome P450 gene (*crtI*) were selected for optimal productivity. The algal β -carotene ketolase gene (*Crbkt*) from *Chlamydomonas reinhardtii*, which showed increased production of total carotenoids in engineered tobacco (Gerjets et al., 2007; Mann et al., 2000) was employed to overcome the limiting steps on *X. dendrorhous*, which possesses the astaxanthin synthase gene (*crtS*) and the cytochrome P450 gene (*crtR*). However, the last hydroxylation step that converts canthaxanthin to astaxanthin or the step that converts β -carotene to zeaxanthin may act as a crucial step. As the green algae can produce more astaxanthin than *X. dendrorhous*, the last hydroxylation related genes of green algae likely have a higher efficiency than those of *X. dendrorhous*. To verify this hypothesis, three algal β -carotene hydroxylase genes (*chyb*), *Crchyb* (*C. reinhardtii*), *Czchyb* (*Chlorella zofingiensis*), or *Hpchyb* (*H. pluvialis*), were characterized individually in the designed carotenoid biosynthesis pathway in *K. marxianus*, which has no endogenous carotenoid ketolases or hydroxylase. The performances of these three engineered strains were carried out to identify the most efficient hydroxylase. The synthetic biology technique, called “Promoter-based Gene Assembly and Simultaneous Overexpression” (PGASO) (Chang et al., 2012; Chang et al., 2013), was used for all of the genetic constructions.

2. Materials and Methods

2.1. Strains and media

In this study, the yeast *K. marxianus*, which has many advantages for a cell factory construction over *S. cerevisiae*, was used as the host (Chang et al., 2014). The fungus, *X. dendrorhous* (formerly *Phaffia rhodozyma*), which is a promising producer of astaxanthin, was used for DNA extraction and gene cloning (Johnson, 2003; de la Fuente et al., 2010; Rodríguez-Sáiz et al., 2010; Kim et al., 2005; Visser et al., 2003). The transgenic yeast strains, Xd7-3, Cr1, Cz5, and HP9, were genetically engineered using PGASO and will be described below. The media for the transformation and carotenoid producing tests were prepared in YPG medium (10 g L⁻¹ yeast extract and 20 g L⁻¹ peptone) with a 2% galactose as the carbon source.

2.2. Carotenoid biosynthesis pathway genes

All of the carotenoid biosynthesis pathway genes in this study are listed in Table 1. The *tHMG1* (a truncated 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene) was cloned from *K. marxianus*. The *crtE* (geranylgeranyl pyrophosphate synthase gene), *crtYB* (bifunctional phytoene synthase/lycopene cyclase gene), *crtI* (phytoene desaturase gene), *crtS* (astaxanthin synthase gene), and *crtR* (cytochrome P450 gene) genes were cloned from *X. dendrorhous*. The *bkt* gene (β -carotene ketolase gene) of *C.*

reinhardtii, and three algal β -carotene hydroxylase genes from *C. reinhardtii* (*Crchyb*), *Ch. Zofingiensis* (*Czchyb*), and *H. pluvialis* (*Hpchyb*) were synthesized by The GeneArt® Gene Synthesis (GENEART, Germany). All the synthesized gene sequences were subjected to multi-parameter gene optimization by GeneOptimizer® Process based on the codon usage of the host, *K. marxianus*.

2.3. Construction of the engineered strains

The gene cassettes were amplified by HiFi-PCR (polymerase chain reaction with high fidelity enzyme, PrimeSTAR MAX DNA Polymerase, TaKaRa, Japan) and introduced into the host genome via homologous recombination using PGASO (Chang et al., 2012). The neomycin phosphotransferase gene essential for G418 resistance (*KanMX*) was used as a marker gene for clone screening. A Long-PCR method (EmeraldAmp MAX PCR Master Mix, TaKaRa, Japan) was used to check the order of these gene cassettes via electrophoresis analysis. The primer pairs used in this study are listed in Table 2.

2.4. Carotenoid analysis

For all carotenoid analyses, acetone was employed for extraction of these pigments from the lyophilization of yeast cells. Based on the full-spectrum UV/Vis spectrophotometry (220nm-750nm) analysis by Nanodrop ND-1000 spectrophotometer

(Thermo Fisher Scientific Inc, USA), the total carotenoid accumulation of these engineered strains could be estimated with the total amounts of carotenoids (ug/mL):

$$(1000 A_{470} - 2.27 C_a - 81.4 C_b)/227$$

(Türlerinde et al., 1998). The predominant carotenoids and its derivatives were analyzed by High Performance Liquid Chromatography (HPLC) assay with a Nomura Chemical Develosil C30-UG Column (3 μ m, ID 4.6 mm x L 250 mm – UG17346250W), using methanol/MTBE/water (81:15:4) and methanol/MTBE/water (7:90:3) as mobile phases. The flow rate employed was 1 mL/min and the chromatograms were recorded at 460 nm (Dugo et al., 2008). The free form pure carotenoid compounds, including β -carotene, astaxanthin, canthaxanthin, and zeaxanthin, were used as the standard in this study (Sigma-Aldrich Co. LLC. USA).

3. Results and Discussion

3.1. Gene selection and carotenoid biosynthetic pathway design

In this study, a carotenoid synthesis platform was designed, and the three algal β -carotene hydroxylase genes were individually integrated into a designed carotenoid biosynthesis pathway in the yeast host to construct three carotenoid producing strains (Fig. S1a in the Supplementary data). To increase the metabolic flux toward a higher isoprenoid precursor, the *tHMG1* gene (1500 bp) from *K. marxianus* and the *crtE* gene (1131 bp) from *X. dendrorhous* were included (Fig. S1b in the Supplementary data).

Moreover, β -carotene producing genes, *crtYB* (2022 bp) and *crtI* (1749 bp), were also cloned from *X. dendrorhous*. To move toward the production of astaxanthin, the astaxanthin synthase genes were synthesized based on the sequences of green algae, including the *bkt* gene (1335 bp) of *C. reinhardtii* and three algal β -carotene hydroxylase genes of *C. reinhardtii* (*Crchyb*, 894 bp), *Ch. zoofingiensis* (*Czchyb*, 900 bp), and *H. pluvialis* (*Hpchyb*, 822 bp) (Table 1)(Fig. S1b in the Supplementary data).

To install these designer carotenoid-biosynthetic pathways in the host genome, a Crabtree-negative yeast host *K. marxianus* was employed for achieving higher end-product productivity and for using the synthetic biological tool PGASO (Chang et al., 2012). Seven ordered gene cassettes were constructed, including KIPLac4-*crtI*-KITTLac4 (promoter-gene-terminator, No.1 gene cassette), ScPGapDH-*crtE*-ScTTGap (No.2), ScPGK-*chyb*-ScTTPGK (No.3), KIPGapDH-*kanMX*-ScTTGap (No.4), KIPGK-*bkt*-ScTTPGK (No.5), KIPADHI-*crtYB*-ScTTGap (No.6), and ScPADHI-*tHMG*-ScTTADHI (No.7) (Fig. 1a). These cassettes were co-transformed into the genome of the yeast host in a designated order as *crtI-crtE-chyb-kanMX-bkt-crtYB-tHMG*, and the three algal *chyb* genes were set within the No. 3 cassette to three kinds of gene combination, respectively. Three

engineered strains, Cr1, Cz5, and Hp9, with three different algal β -carotene hydroxylase genes, were obtained from for further characterized.

To verify that these gene cassettes were installed in the genome, seven internal primer pairs of each cassette were designed (Table 2). The PCR products showed seven specific bands, that reflected their expected sizes, 4114 bp, 2251 bp, 2200 bp, 1973 bp, 2644 bp, 3341 bp, and 3720 bp, respectively (Fig. 1b). To verify that these gene cassettes were assembled in the correct order, nine internal primer pairs spanning the gap regions of each cassette were also designed (Table 2). The PCR products showed nine specific bands, that reflected their expected sizes, 6365 bp, 4460 bp, 4180 bp, 4617 bp, 5985 bp, 6927 bp, 8577 bp, 9705 bp and 6829 bp, respectively (Fig. 1b).

In green algal biosynthesis of carotenoids, the first committed step, the head-to-head condensation of GGPP to phytoene, is mediated by phytoene synthase (PSY). The subsequent steps of the pathway leading to the synthesis of ester-form colored carotenoids are carried out by membrane-localized enzymes such as phytoene desaturase (PDS), lycopene β -cyclase (LCY), ζ -carotene desaturase (ZDS), carotenoid isomerase (CRTISO), β -carotene ketolase (BKT), and β -carotene hydroxylase (CHY). The astaxanthin with 3S, 3'S stereoisomer, which is the major form in green algae, has much higher antioxidant activity than the 3R, 3'R stereoisomer, which is the major form

of astaxanthin in the fungus *X. dendrorhous* (Visser et al., 2003). However, this long pathway might reduce its carotenoids productivity, and PSY (phytoene synthase) has been considered the rate-limiting entry reaction into the carotenoid biosynthesis pathway in photosynthetic organisms (Paniagua-Michel et al., 2012). On the other hand, the carotenoid-biosynthetic pathway in *X. dendrorhous* includes bifunctional phytoene synthase/lycopene cyclase (*crtYB*), phytoene desaturase (*crtI*), bifunctional astaxanthin synthase (*crtS*), and a cytochrome P450 (*crtR*) (Niklitschek et al., 2008). Although *X. dendrorhous* has a shorter pathway, its major form of free-form astaxanthin, 3R, 3'R stereoisomer, has a lower antioxidant activity (Visser et al., 2003). Installing these designer carotenoid-biosynthetic pathways in the yeast *K. marxianus* may lead to a better designer carotenoid producer.

3.2. Characterization of the designed strains

Three engineered strains, Cr1, Cz5, and HP9, were selected and sub-cultured with 10 generations as a stable clone (Table 1). Furthermore, an engineered yeast strain Xd7-3 (*crtI-crtE-crtR-kanMX-crtS-crtYB-tHMG*), which contained the *crtS* and *crtR* from *X. dendrorhous* (Table 1), was also constructed into *K. marxianus* as the control strain. These four carotenoid pathways in yeast could accumulate carotenoids, changing the color of the yeast cell from white (WT) to yellow (Xd7-3), orange or red (Cr1, Cz5,

and HP9) (Fig. S2a in the Supplementary data), and HP9 showed a deeper color than Cr1 and Cz5. To test the engineered strains for carotenoids production, the wild type strain (WT), the control strain (Xd7-3), and the three engineered strains (Cr1, Cz5, and Hp9) were cultured in the 5 ml YP medium at 30°C with 1% different carbon sources, including galactose, lactose, glucose, and sucrose. After 2 days culturing, all the engineered strains, especially the Hp9 strain, could show a significant orange color in 1% galactose compared to other carbon sources (Fig. S2b in the Supplementary data). Different galactose concentrations and different culturing temperatures were further tested for the Hp9 strain. The data indicated that the optimal culturing condition for the Hp9 strain was YP medium with 2% galactose at 25°C (Fig. S2c in the Supplementary data). A 50 ml batch fermentation culturing was employed to compare these engineered strains with the optimal culturing condition. After 3 days culturing, the growth curve data indicated that the Hp9 and Cz5 strains had grown slightly faster than the WT strain, the Cr1 strain, and the Xd7-3 strain (Fig. 2). At the late stationary phase, the WT strain showed white color, the Xd7-3 strain showed yellow color, while the Cr1, Cz5, and Hp9 strains showed orange-red color in each culture. The Hp9 strain still showed brighter color than others.

The carotenoids, including yellow carotenoids (β -carotene, echienone, β -cryptoxanthin, and zeaxanthin) and pinkish-red carotenoids (adonixanthin, astaxanthin and canthaxanthin), belong to a family of natural lipid-soluble pigments that are responsible for the wide variety of colors in natural hosts. The data indicated that the engineered strains with the carotenoids synthesis pathway genes from algae could show orange-red color compared with yellow color of the Xd7-3 with the genes from *X. dendrorhous*. Furthermore, the Hp9 strain, which possesses the *Hpchyb* gene from *H. pluvialis*, showed a stronger red color than Cr1 and Cz5. The color of these engineered strains might be due to the accumulation of different types or combinations of carotenoids in the cell. The galactose was the inducer for the strong KITT_{Lac4} promoter, and it drove the key enzyme gene, *crtI*, in the No.1 gene cassette (Verwaal et al., 2007; Ukibe et al., 2009). This might be the reason why galactose was the carbon source of the optimized carotenoids production condition for these engineered strains.

To quantify the carotenoids in the cell, acetone was employed for extracting these pigments from the yeast culture. The full-spectrum UV/V spectrophotometry was used to estimate the total amount of carotenoids, and the free form pure carotenoids compounds, including β -carotene, astaxanthin, canthaxanthin, and zeaxanthin, were used as the four standards. Based on the analysis results, all the carotenoid compounds

could show an absorption spectrum between 400 nm to 530 nm (Fig. S3a in the Supplementary data). The carotenoids extracted from all of these engineered strains (Xd7-3, Cr1, Cz5, and Hp9) also showed an absorption spectrum between 400 nm to 530 nm, except the WT strain (Fig. S3b in the Supplementary data). Based on the calculation method in Methods 2.3, these engineered strains could accumulate different amounts of carotenoids in the cell, i.e., 4.4 ug/g in the WT strain, 89.5 ug/g in the Xd7-3 strain, 101.2 ug/g in the Cr1 strain, 124.7 ug/g in the Cz5 strain, and 161.7 ug/g in the Hp9 strain. These data indicated that the engineered strains possessed *bkt* and *chyb* from algae could produce more carotenoids than the engineered strain, Xd7-3 strain, which possessed *crtS* and *crt R* from a fungus. The Hp9 strain showed the highest carotenoid productivity.

The heterologous carotenoid biosynthesis pathway of the engineered strains seemed to contribute to the carotenoids production in the yeast cell. The data indicated that the algal β -carotene ketolase gene and β -carotene hydroxylase gene have a higher efficiency than the astaxanthin synthase and cytochrome P450 from the fungus, *X. dendrorhous*, to produce the total amount of carotenoids production in the yeast system.

In the plant system, the transgenic tobacco expressing the algal β -carotene ketolase showed a 1.8-fold increase in total carotenoids (Gerjets et al., 2007; Mann et al., 2000;

Zhong et al., 2011). Moreover, the Hp9 strain, which possessed the *Hpchyb* from *H. pluvialis*, showed a 25 % higher efficiency in the total amount of carotenoid production than the strains that possessed *Crchyb* or *Czchyb*. The engineered yeast system could grow faster by a fermentation of 3 days, showing a 7 fold increase in productivity for industrial application compared with the algal systems, such as *H. pluvialis*, which required a >20 days culturing. However, the total amount of carotenoids production of the *H. pluvialis* system (1~ 4 %) was almost 250 folds higher than the engineered yeast system. It is still difficult to use the current engineered yeast system for industrial application. Further improvement of the system, such as increasing the gene copy number is needed.

3.3. Analysis of the composition of the carotenoids

To analyze the compositions of the carotenoids, the HPLC assay was carried out for the specific carotenoids analysis, and the mixture of free form carotenoid compounds could be separated with different retention times, such as astaxanthin (7.8 min), zeaxanthin (9.7 min), canthaxanthin (12.8 min), and β -carotene (32 min) (data not shown); the four standard curves were set up for accurate quantification. With the 50 ml batch fermentation yeast culturing, the β -carotene could be detected in all of the engineered strains, except the WT strain (Table 3). After estimating β -carotene

concentration with a standard, these engineered strains could accumulate 244.7 ug/g in the Xd7-3 strain, 59.6 ug/g in the Cr1 strain, 93.9 ug/g in the Cz5 strain, and 224.4 ug/g in the Hp9 strain in the cell, respectively. The data indicated that the Hp9 strain could produce 3.8 folds faster than the Cr1 strain. Furthermore, except the WT strain and the Xd7-3 strain, all the engineered strains that possessed algal *bkt* and *chyb* genes could accumulate canthaxanthin in the cell with 18.4 ug/g in the Cr1 strain, 12.8 ug/g in the Cz5 strain, and 39.8 ug/g in the Hp9 strain (Table 3). The data indicated that the Hp9 strain could produce 3.1 times faster than the Cz5 strain.

All the engineered strains with heterologous β -carotene synthesis genes, including *tHMG1* from *K. marxianus*, and *crtE*, *crtYB*, and *crtI* from *X. dendrorhous*, can produce β -carotene. However, the *crtS* and *crtR* genes from *X. dendrorhous* in the Xd7-3 strain might not be efficient in the downstream carotenoids conversion from β -carotene, showing a higher β -carotene accumulation than the other engineered strains. On the other hand, the Cr1, Cz5, and Hp9 strains may convert the β -carotene by the algal *bkt* and *chyb* genes to downstream carotenoids and may therefore improve their total carotenoids productivity. The data showed that there were many un-confirmed peaks that could be detected under the 470 nm (data not shown). The *K. marxianus* host has been reported to be able to convert many kinds of fatty acids to their ester-forms, such

as the 2-phenylethyl acetate, which can be converted from 2-phenylethylethanol during the fermentation process (Chang et al., 2014). These un-confirmed carotenoids-like derivatives might be the intermediates converted from β -carotene by the algal β -carotene hydroxylase, and then esterified by the esterase in the host. It will be further confirmed by HPLC-MS analysis.

To reduce the cost of carotenoid production, many biosynthesis approaches have been studied. A zeaxanthin producing *E. coli* (820 ug/g dry weight) was observed in the transformant with a plasmid in which the gene order corresponds to the order of the bacterial zeaxanthin metabolic pathway (*crtE-crtB-crtI-crtY-crtZ*) (Nishizaki et al., 2006). Furthermore, an engineered strain was expressed with the bacterial β -carotene ketolase (*crtW*) and the bacterial β -carotene hydroxylase (*crtZ*) from *Pantoea ananatis* (*Erwinia uredovora*), which directed the pathway exclusively towards the desired product that produced β -carotene (6.2 mg/g dry weight), and astaxanthin (1.4 mg/g dry weight) (Lemuth et al., 2011). In the engineered yeast with carotenoid biosynthesis genes on the episome plasmid, it was capable of producing high levels of β -carotene (5.9 mg/g dry weight dry weight), but it resulted in the accumulation of only a small amount of astaxanthin and intermediates, such as echinenone, canthaxanthin, and zeaxanthin (Verwaal et al., 2007; Ukibe et al., 2009).

Currently, the green alga, *Haematococcus pluvialis*, which possesses the most concentrated natural source of astaxanthin, is the only host for industry production.

However, cultivation of green algae requires many factors, such as a large area of land, strong sunlight, long culturing time, and a large amount of fresh water. Therefore, either a more cost-effective process or a high-productivity host is needed. To reduce the cost of carotenoid production, many biosynthesis approaches have been proposed. However, it is important to deal with some practical issues, such as to find out the rate limiting steps and to find efficient enzymes to improve the carotenoid biosynthesis pathway.

Although yeast has no endogenous carotenoid ketolase and hydroxylase, it has been considered as a host for producing astaxanthin. The advantages of the yeast expression system are easy culturing, easy harvesting, and easy genetic engineering. Also, it has a high growth rate. In this study, several key enzymes, β -carotene ketolases and β -carotene hydroxylases, were expressed and their efficiencies were compared in the yeast system. The data suggested that the algal enzymes showed higher carotenoids biosynthesis efficiencies than the enzymes from *X. dendrorhous*, and the chyb from *H. pluvialis* showed a higher efficiency than the enzymes from other green algae.

The total carotenoid productivity in this study was still low compared with previous studies. It might be because a low copy number of gene cassettes were

integrated into the genome in this study. Increasing the gene copy numbers may increase the productivity.

Astaxanthin has three isomers: 3S, 3'S; 3S, 3'R; and 3R, 3'R. The 3S, 3'S stereoisomer, which is the major form in green algae (Visser et al., 2003), has much higher antioxidant activity than the 3R, 3'R stereoisomer, which is the major form of astaxanthin in the fungus *X. dendrorhous*. Therefore, the algal astaxanthin synthase enzyme system has been employed and tested in the study. Although it resulted in the accumulation of β -carotene and only a small amount of canthaxanthin, there were many un-known intermediates, such as adonixanthin, echienone, β -cryptoxanthin, and zeaxanthin that should be confirmed. The algal β -carotene ketolase gene, which effects the last ketolation that converts β -carotene to canthaxanthin or zeaxanthin to astaxanthin, was functionally characterized using an *E. coli* system, and three *bkt* genes, from *C. reinhardtii*, *Ch. Zofingiensis*, and *H. pluvialis*, were identified with high, moderate, and low conversion efficiency, respectively. These data indicated that *bkt* from *H. pluvialis* was less efficient than the other two, although it provided the most concentrated natural source of astaxanthin than all green algae. Based on these results, the algal-carotene hydroxylase, which effects the last hydroxylation that converts β -carotene to zeaxanthin or canthaxanthin to astaxanthin, was functionally characterized

using a yeast system, and the *Hpchyb* from *H. pluvialis* showed a higher efficiency than those from other green algae. It was suggested that β -carotene hydroxylase plays a crucial role in the biosynthesis of astaxanthin in *H. pluvialis*.

4. Conclusion

Biosynthesis of valuable carotenoids by yeast can be used to study the astaxanthin synthesis genes and can reduce the cost of bioprocessing. In this study, a designer carotenoid biosynthesis pathway was provided for heterologous production via synthetic biology. The algal β -carotene hydroxylases have a higher production efficiency than the enzymes from *X. dendrorhous*. Moreover, *Hpchyb* from *H. pluvialis* can produce more carotenoids than *Crchyb* and *Czchyb* from other green algae.

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Figure Captions

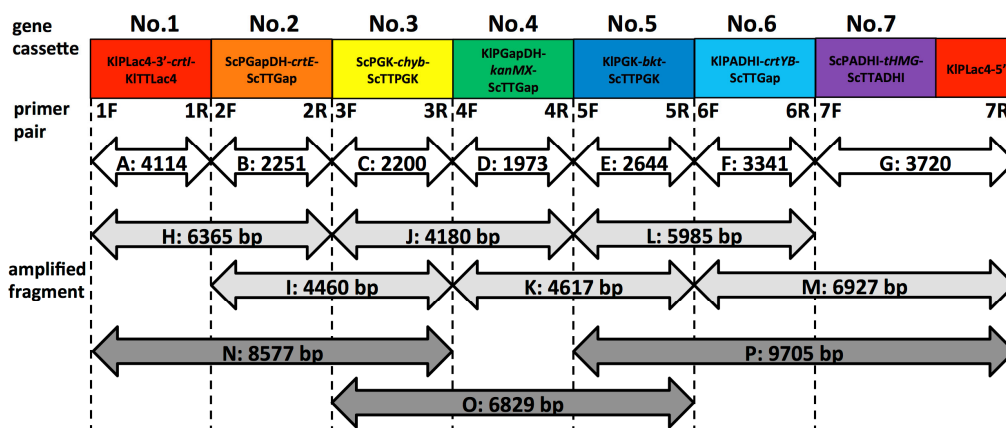
Figure 1. Designer strains construction. (a) The designer blue map. (b) Confirmation of these designer strains. The gene cassettes were labeled with colorful bricks and the amplification fragments were labeled with double arrowhead. The fragments were amplified by the specific primer pairs, including A(1F-1R), B(2F-2R), C(3F-3R), D(4F-4R), E(5F-5R), F(6F-6R), G(7F-7R), H(1F-2R), I(2F-3R), J(3F-4R), K(4F-5R), L(5F-6R), M(6F-7R), N(1F-3R), O(3F-5R), and P(5F-7R).

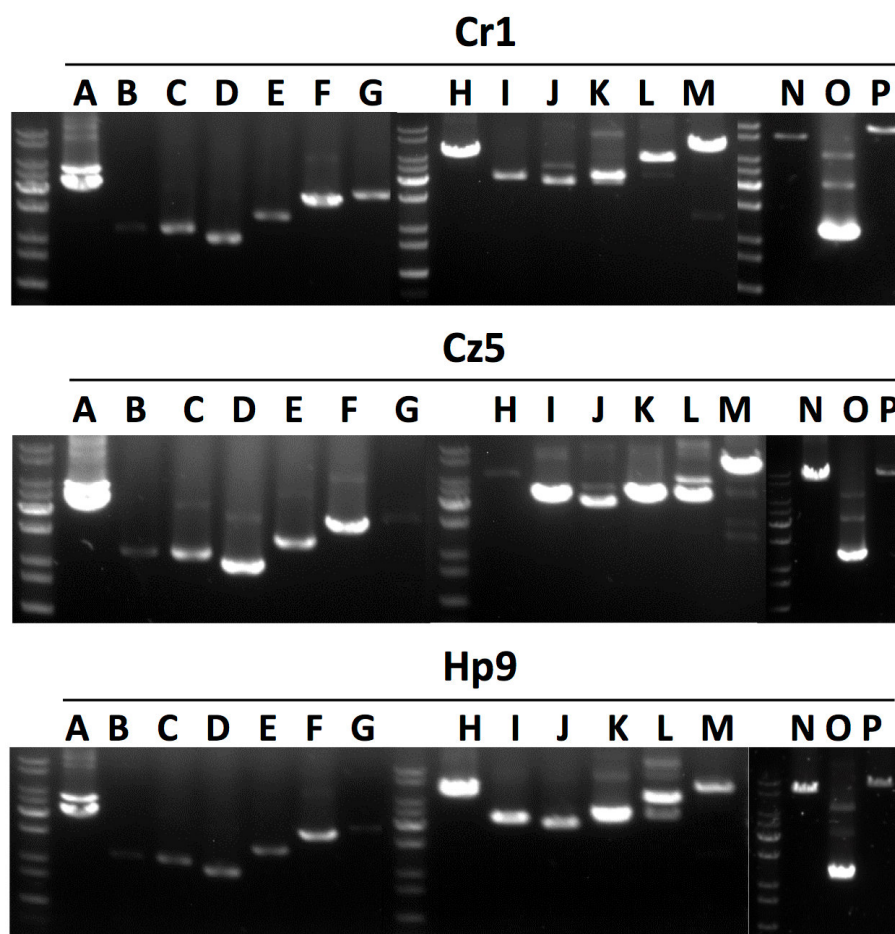
Figure 2. The growth curves of the engineered strains and the wild type. The Hp9 and Cz5 strains grew slightly faster than the WT strain, the Cr1 strain and the Xd7-3 strain

Table 1. The genes of the designer carotenoid biosynthesis pathway

Table 2. The primer pairs were used in this study.

Table 3. The HPLC analysis of the carotenoid extractions from the engineered strains.





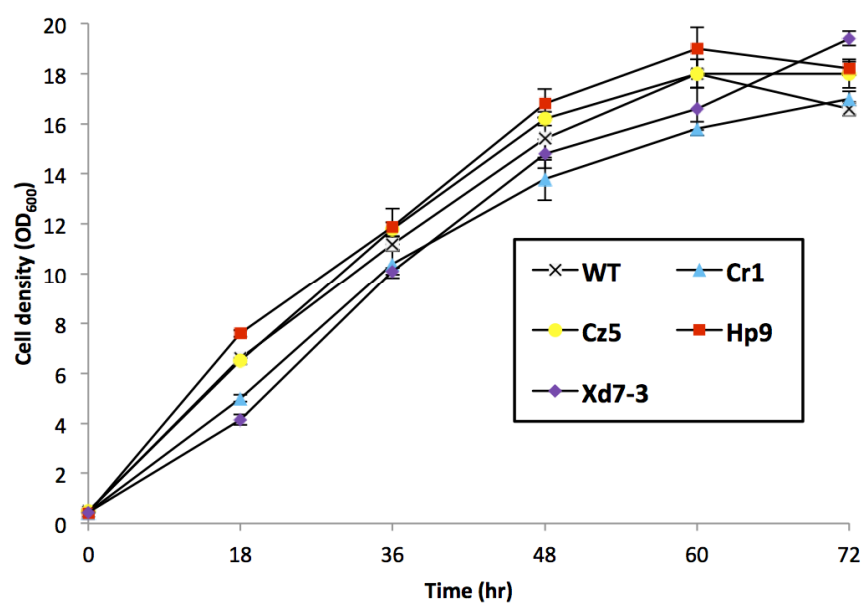


Table 1

Gene	Species	Funtion	Length (bp)	Sample
<i>tHMG1</i>	<i>Kluyveromyces marxianus</i> KY3	truncated 3-hydroxy-3-methylglutaryl-coenzyme A reductase	1500	by cloning
<i>crtE</i>	<i>Xanthophyllomyces dendrorhous</i>	geranylgeranyl pyrophosphate synthase	1131	by cloning
<i>crtYB</i>	<i>Xanthophyllomyces dendrorhous</i>	phytoene synthase/lycopene cyclase	2022	by cloning
<i>crtI</i>	<i>Xanthophyllomyces dendrorhous</i>	phytoene desaturase	1749	by cloning
<i>crtS</i>	<i>Xanthophyllomyces dendrorhous</i>	astaxanthin synthase	1674	by cloning
<i>crtR</i>	<i>Xanthophyllomyces dendrorhous</i>	cytochrome P450 reductase	2241	by cloning
<i>Crbkt</i>	<i>Chlamydomonas reinhardtii</i>	β -carotene ketolase	1335	by synthesis
<i>Crchyb</i>	<i>Chlamydomonas reinhardtii</i>	β -carotene hydroxylase	894	by synthesis
<i>Czchyb</i>	<i>Chlorella zofingiensis</i>	β -carotene hydroxylase	900	by synthesis
<i>Hpchyb</i>	<i>Haematococcus pluvialis</i>	β -carotene hydroxylase	882	by synthesis

No.	Primer name	Sequence
1F	KIPLac4-3'	5'-ccgcggggatcgactcataaaatag-3'
1R	KITTLac4_ScPGapDH	5'-CTACTATTAATTATTTACGTATTCTTTGAAATGGCAGTA TTGATAATGATAAACTTATACAACATCGAAGAAGAGTCT-3'
2F	ScPGapDH	5'-AGTTTATCATTATCAATACTGCCAT-3'
2R	ScTTGap_ScPGK	5'-GGACTCCAGCTTTTCCATTTCGCTTCGCGCTTGCCTGTA CGGTCGTTACCATACTTGGCGGAAAAAATTCATTTGTAA-3'
3F	ScPGK	5'- ACTGTAATTGCTTTTAGTTGTGTAT-3'
3R	ScTTPGK_KIPGapDH	5'-GGACTCCAGCTTTTCCATTTCGCTTCGCGCTTGCCTGTACG GTCGTTACCATACTAAGCTTTTTTCGAAACGCAGAATTTTCG-3'
4F	KIPGapDH	5'-AGTATGGTAACGACCGTACAGGCAA-3'
4R	ScTTGap_KIPGK	5'-CATATACCTTTGATACCATAAAAAACAAGCAAATATTCTTAC TTCAAACACACCCGTGGCGGAAAAAATTCATTTGTAAACT-3'
5F	KIPGK	5'-CGGGTGTGTTTGAAGTAAGAATATT -3'
5R	ScTTPGK_KIPADHI	5'-AGGTAAGTATGGTAACGACCGTACAGGCAAGCGCGAAGGC AAATGGAAAAGCTGGAAGCTTTTTTCGAAACGCAGAATTTT-3'
6F	KIPADHI	5'-CCAGCTTTTCCATTTCGCTTCGCGCTTGCC-3'
6R	ScTTGap_ScPADHI	5'-GGAATCCCGATGTATGGGTTTGGTTGCCAGAAAAGAGG AAGTCCATATTGTACACTGGCGGAAAAAATTCATTTGTAA-3'
7F	ScPADHI	5'-GTGTACAATATGGACTTCCTCTTTTC-3'
7R	ScTTADHI_KIPLac4	5'-gaaatttaggaattttaacttg-3'

-5'End

Carotenoid	Specific amount ($\mu\text{g/g}$ [dw]) of carotenoids ^a				
	Cr1	Cz5	Hp9	AST7-3	WT
β -carotene	59.6 ± 2.3 (1)	93.9 ± 3 (1.6)	224.4 ± 9.9 (3.8)	244.7 ± 5.1 (4.1)	— ^b
Canthaxanthin	18.4 ± 3.6 (1.4)	12.8 ± 1.9 (1)	39.8 ± 2.7 (3.1)	—	—

^a The data are averages of three independent cultures. The *n*-fold increases in carotenoid production levels compared to that of strain which is lowest (the value for strain is set at 1).

^b —, not detected.

Highlight

1. Carotenoid biosynthesis genes from green algae
2. A designed carotenoid biosynthesis pathway in yeast
3. Bioengineering approaches to reducing manufacturing cost