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Pathway Engineering of *E. coli* for Production of Fritschiellaxanthin and Other Carotenoids with α-Carotene Backbone and Their Singlet Oxygen-Quenching Activities

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Received: 7 November 2024; Accepted: 2 December 2024; Available online: 3 December 2024

ABSTRACT: Some photosynthetic organisms are capable of biosynthesizing carotenoids (xanthophylls) with α-carotene backbone, that is, α-carotene-derived carotenoids, such as (3*R*,3′*R*,6′*R*)-3,3′-dihydroxy α-carotene (lutein). Except for lutein, such carotenoids are minor compounds in nature. In this study, α-carotene-derived carotenoids were produced with *E. coli*. To achieve this, carotenoid biosynthesis genes from the bacterium *Pantoea ananatis* containing the 4-β-ketolase (*crtW*) gene with/without the 3-β-hydroxylase (*crtZ*) gene, in addition to *crtEBI* genes, and biosynthesis genes (*MpLCYb*, *MpLCYe*, and *MpCYP97C*) from liverwort *Marchantia polymorpha*, along with the *HpIDI* gene, were cloned into plasmids. The transformed *E. coli* cells biosynthesized (3*S*,3′*R*,6′*R*)-3,3′-dihydroxy-4-keto-α-carotene (fritschiellaxanthin (4-ketolutein)), (3′*R*,6′*R*)-3′-hydroxy-4-keto-α-carotene (4-keto-α-cryptoxanthin), and (3′*R*,6′*R*)-3′-hydroxy-α-carotene (α-cryptoxanthin), as carotenoids that have not been produced by a heterologous microbial system so far. These carotenoids show potent singlet oxygen-quenching activity.

Keywords: Singlet oxygen-quenching activity; α-carotene; α-cryptoxanthin; Fritschiellaxanthin; *Marchantia polymorpha*

1. Introduction

Carotenoids are yellow, orange, or red isoprenoid compounds with long conjugated double bonds that are produced by some species of fungi, archaea, and bacteria, as well as by all plants and algae [1–6]. Among these carotenogenic organisms, only photosynthetic organisms, including land plants, green algae, and Rhodophyta (red algae), can biosynthesize carotenoids (xanthophylls) with an α-carotene [(6′*R*)-β,ε-carotene] backbone, namely α-carotene-derived carotenoids. For example, (3*R*,3′*R*,6′*R*)-3,3′-dihydroxy-α-carotene (lutein) is a major carotenoid (45% of total) in the leaves of higher plants and a crucial component of light-harvesting complex II [7]. Other carotenoids with α-carotene backbone include (3′*R*,6′*R*)-3′-hydroxy α-carotene (α-cryptoxanthin) and (3*S*,3′*R*,6′*R*)-3,3′-dihydroxy-4-keto α-carotene (fritschiellaxanthin (4-keto-lutein)) [2]. α-Cryptoxanthin is a minor component of some species of algae (Rhodophyta) and petals of *Medicago* species. Fritschiellaxanthin is a minor carotenoid present in the green alga, *Fritschiella tuberosa.* Fritschiellaxanthin has also been found in some aqueous animals, including from the crab *Sesarma* (*Holometopus*) *haematocheir* (Akategani in Japanese), as a major carotenoid (43% of total) [8], as well as from the goldfish *Carassius auratus* (Hibuna) in specimens fed lutein [9].

Animals are not typically able to biosynthesize carotenoids and utilize them orally for health, often by metabolizing them. In humans, lutein is present in the macula lutea together with zeaxanthin. In humans, lutein protects the eyes from photooxidative damage by filtering blue light and preventing ocular diseases, such as age-related macular degeneration

https://doi.org/10.70322/sbe.2024.10018

and cataracts [10,11]. With regards to other carotenoids with α-carotene backbone, studies on their biofunction remain scarce due to their low frequency in nature and the difficulty in preparing pure compounds.

Carotenoids with α-carotene backbone are metabolically synthesized from GGPP (geranylgeranyl diphosphate) by way of α-carotene. Pathway engineering of *Escherichia coli* for the efficient production of lutein has been previously performed using a combination of carotenoid biosynthesis genes from the bacterium *Pantoea ananatis* and the liverwort *Marchantia polymorpha* via the efficient formation of α-carotene [12]. The heterologous microbial production of lutein has also been performed in *Saccharomyces cerevisiae* [13]. With the exception of lutein, the heterologous production of α-carotene-derived carotenoids has seldom been performed. In the present study, pathway engineering of *E. coli* was performed to produce fritschiellaxanthin and other α-carotene-derived carotenoids, as well as to evaluate their antioxidant activity.

2. Materials and Methods

2.1. Bacterial Strains

E. coli K12 DH5α and JM101(DE3) were used for DNA manipulation and the expression of the carotenoid biosynthesis genes, respectively.

2.2. Construction of Plasmids Containing Carotenogenesis Genes for Expression in Escherichia coli

The *Paracoccus* sp. N81106 *crtW* gene was amplified by PCR and inserted into the pRK vector-based plasmid pRK-HIEBI-MpLCYbΔTP-MpLCYe-Z [12], resulting in the construction of pRK-HIEBI-MpLCYb-MpLCYe-ZW (accession no. LC835499). This *crtW* fragment was also ligated with the *M. polymorpha LCYe* (*MpLCYe*) gene fragment and inserted into the plasmid, pRK-HIEBI-MpLCYbΔTP [12], to construct pRKHIEBI- MpLCYb-MpLCYe-W (accession no. LC835500) The structures of these plasmids are shown in Figure 1. The plasmids were introduced with CDF-MpCYP97C-MpLCYe, which carries the 3-ε-hydroxylase (*MpCYP97C*) gene that is absent from each pRK vector-based plasmid, and the lycopene ε-cyclase (*MpLCYe*) gene also included in each plasmid (accession no. LC654938) [12] in wild-type *E. coli* (JM101 (DE3)).

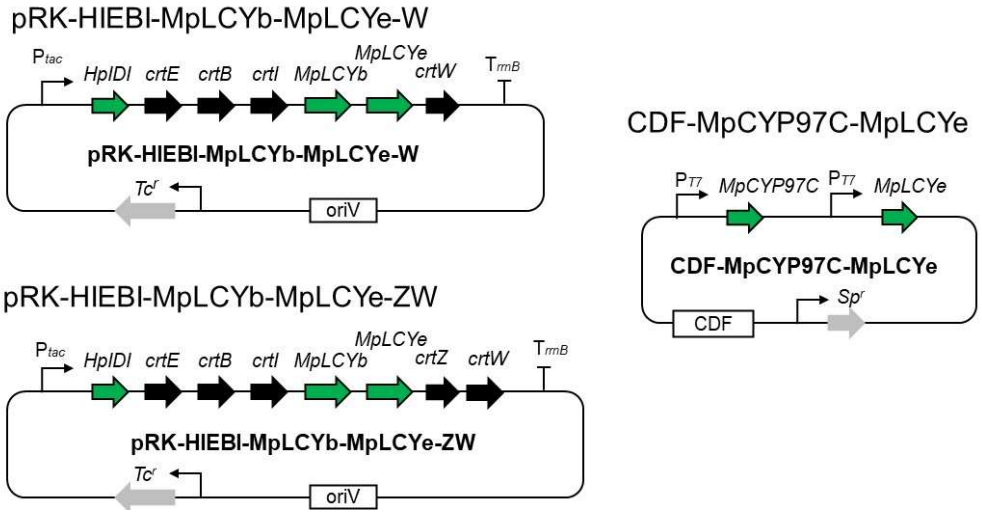

Figure 1. Plasmid constructs used in this study.

2.3. Solvents and Reagents

Analytical grade dichloromethane (CH_2Cl_2), *n*-hexane, acetone, methanol (MeOH), ethyl acetate (EtOAc), and tetrahydrofuran (THF) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.4. Spectroscopic Analysis Using NMR and MS

NMR spectra [^{1}H and ^{13}C NMR, Double Quantum Filtered Correlation Spectroscopy (DQF COSY), Heteronuclear Single Quantum Correlation (HSQC), Heteronuclear Multiple Bond Coherence (HMBC), Nuclear Overhauser Effect Spectroscopy (NOESY)] were obtained via spectrometry (Bruker AVANCE400 Spectrometer, Billerica, MA, USA)

and analyzed in TopSpin1.3. Chemical shifts were referenced to the solvent signals ($CDCl_3$: δ_H = 7.26, δ_C = 77.0). Electrospray ionization mass spectrometry (ESI-MS) was performed using a JEOL JMS-T100LP instrument.

2.5. Culture of E. coli Transformant

E. coli transformants were grown in 100 mL 2 × YT (2YT) medium (16 g/L of tryptone, 10 g/L of yeast extract, 5 g/L of NaCl) containing 10 mg/L of tetracycline and 100 mg/L of spectinomycin in a 500 mL Sakaguchi flask at 25 °C at 150 rpm for 48 h.

2.6. Extraction and HPLC Analysis of Carotenoids from E. coli Cells

The cultured *E. coli* cells (100 mL) were centrifuged (8000× *g*, 15 min), and the precipitate was extracted with methanol (MeOH)–acetone (1:1) (5 mL) with sonication. After centrifugation (8000× *g*, 5 min), the supernatant (10 mL) was analyzed using octadecylsilyl silica gel (ODS) HPLC (4.6 mm × 150 mm, PEGASIL ODS SP100; Senshu Scientific Co., Ltd., Tokyo, Japan), with a solvent of MeOH-THF (9:1) at a flow rate of 1.0 mL/min and detection at 450 nm.

2.7. Isolation of Carotenoids Produced by Cells Transfected with pRK-HIEBI-MpLCYb-MpLCYe-W

The transformed *E. coli* cells carrying pRK-HIEBI-MpLCYb-MPLCYe-W were collected from 2 L of culture by centrifugation (8000× *g*, 15 min) and extracted using 90 mL of acetone-MeOH (7:2) and 100 mL of CH_2Cl_2-MeOH (1:1) by sonication in a stepwise manner. The combined extract (190 mL) was concentrated to a small volume *in vacuo* and partitioned into *n*-hexane/90% (*v*/*v*) *n*-hexane and MeOH (each 150 mL). The *n*-hexane layer was concentrated to dryness to obtain orange oil (720.7 mg). The orange oil was subjected to preparative ODS HPLC (20 mm × 250 mm, Develosil C30-UG; Nomura Chemical, Co., Ltd., Aichi, Japan) with a solvent of MeOH:tetrahydrofuran (THF) (3:1) at a flow rate of 8.0 mL/min and detection at PDA (250–700 nm). Each peak at t_R 10.9 min, t_R 12.9 min, and t_R 18.2 min was collected and concentrated to dryness to obtain pure carotenoids **1**, **2**, and **3** [**1** (t_R 10.9 min), 17.7 mg; **2** (t_R 12.9 min), 9.0 mg; **3** (t_R 18.2 min), 2.0 mg], respectively.

2.8. Isolation of Carotenoids Produced by Cells Transfected with pRK-HIEBI-MpLCYb-MpLCYe-ZW

The transformed *E. coli* cells carrying pRK-HIEBI-MpLCYb-MpLCYe-ZW were collected using centrifugation (8000× *g*, 15 min) from 2 L of culture and extracted using 90 mL of acetone-MeOH (7:2) and 100 mL of CH_2Cl_2-MeOH (1:1) by sonication in a stepwise manner. The combined extract (190 mL) was concentrated to a small volume *in vacuo* and partitioned into *n*-hexane/90% (*v*/*v*) *n*-hexane and MeOH (each 150 mL). The 90% MeOH layer was concentrated to dryness *in vacuo*, and further partitioned using EtOAc/H_2O (each 150 mL). The EtOAc layer containing the produced carotenoids was concentrated to dryness to obtain orange oil (56.4 mg). Orange oil was subjected to preparative ODS HPLC (20 mm × 250 mm, Develosil C30-UG) with a solvent of MeOH:THF (9:1) at a flow rate of 8.0 mL/min and detection at PDA (250–700 nm). In this preparative HPLC, three peaks were observed at t_R 15.3 min, t_R 16.9 min, and t_R 18.7 min. The eluate of the t_R 15.3 min and t_R 18.7 min peaks were concentrated to dryness to obtain pure carotenoids **4** and **5** [**4** (t_R 15.3 min), 20.4 mg; **5** (t_R 18.7 min), 10.8 mg], respectively. Since the peak at t_R 16.9 min was a mixture of two carotenoids, the eluate was concentrated to dryness [orange powder (16.2 mg)] and further purified by preparative silica gel HPLC (10 mm × 250 mm, COSMOSIL 5SL-II; Nacalai Tesque Inc., Kyoto, Japan) with a solvent of *n*-hexane-acetone (4:1) at a flow rate of 3.0 mL/min and detection at PDA (250–700 nm). In this chromatography, two peaks were observed at t_R 16.0 min and t_R 19.2 min, and each eluate was concentrated to dryness to obtain pure carotenoids **6** and **7** [**6** (t_R 16.0 min), 6.0 mg; **7** (t_R 19.2 min), 6.5 mg], respectively.

2.9. Singlet Oxygen-Quenching Activity

For the measurement of singlet oxygen-quenching activity, 160 μL of 12.5 μM methylene blue and 200 μL of 0.12 M linoleic acid, with or without 40 μL of carotenoid (final concentration, 1–25 μM; each dissolved in ethanol), were added to 5-mL glass test tubes. The tubes were thoroughly mixed and illuminated at 7000 lx and 22 °C for 3 h in a Styrofoam box (exposure to methylene blue plus light mediates the formation of singlet oxygen from oxygen, and the resulting singlet oxygen oxidizes linoleic acid to absorb UV light at 235 nm). Subsequently, 120 μL of the reaction mixture was removed and diluted to 3.48 mL with ethanol. OD_{235} was measured to estimate the formation of conjugated dienes. OD_{235}, in the absence of carotenoids, was used as the negative control [no singlet oxygen ($^1O_2$)-quenching

activity]. The $^1O_2$-quenching activity of the carotenoids was calculated from the OD_{235} in the presence of carotenoids relative to this reference value. Activity was represented as the half-maximal inhibitory concentration (IC_{50}) value.

*2.10. Assigned ^1H and ^{13}C NMR Data of (3'R,6'R)-3'-Hydroxy-4-keto-α-carotene (4-keto-α-cryptoxanthin) (**1**), (3'R,6'R)-3'-Hydroxy-α-carotene (α-cryptoxanthin) (**3**), and (3S,3'R,6'R)-3,3'-Dihydroxy-4-keto-α-carotene (fritschiellaxanthin (4-keto-lutein)) (**4**)*

2.10.1. (3′*R*,6′*R*)-3′-Hydroxy-4-keto-α-carotene (4-keto-α-cryptoxanthin) (**1**)

^1H NMR ($CDCl_3$) δ: 0.85 (3H, s, H-16′), 0.99 (3H, s, H-17′), 1.19 (3H, s, H-16), 1.19 (3H, s, H-17), 1.36 (1H, dd, *J* = 6.8, 13.2 Hz, H-6′b), 1.62 (3H, s, H-18′), 1.83 (1H, m, H-6′a), 1.85 (2H, H-2a and H-2b), 1.87 (3H, s, H-18), 1.91 (3H, s, H-19′), 1.97 (3H, s, H-20′), 1.98 (3H, s, H-20), 2.00 (3H, s, H-19), 2.40 (1H, d, *J* = 9.7 Hz, H-2′), 2.50 (2H, H-3a and H-3b), 4.25 (1H, m, H-5′), 5.43 (1H, dd, *J* = 9.7, 15.3 Hz, H-7′), 5.54 (1H, s, H-4′), 6.14 (1H, d, *J* = 15.3 Hz, H-8′), 6.23 (1H, d, *J* = 16.0 Hz, H-7), 6.24 (1H, d, *J* = 10.3 Hz, H-14)a, 6.26 (1H, d, *J* = 10.5 Hz, H-14′)a, 6.27 (1H, d, J = 10.5 Hz, H-10), 6.37 (1H, d, *J* = 16.0 Hz, H-8), 6.43 (1H, d, *J* = 15.0 Hz, H-12), 6.43 (1H, d, *J* = 15.2 Hz, H-12′), 6.63 (1H, dd, *J* = 10.5, 15.0 Hz, H-11), 6.63 (1H, dd, *J* = 10.2, 15.0 Hz, H-11′), 6.65 (1H, m, H-15), 6.65 (1H, m, H-15′) (Figure S1). ^{13}C NMR ($CDCl_3$) δ: 12.6 (C-19), 12.8 (C-20), 12.8 (C-20′), 13.1 (C-19′), 13.8 (C-18), 22.9 (C-18′), 24.3 (C-16′), 27.7 (C-16), 27.7 (C-17), 29.5 (C-17′), 34.0 (C-1′), 35.7 (C-1), 37.4 (C-2), 34.3 (C-3), 44.6 (C-6′), 55.0 (C-2′), 65.9 (C-5′), 124.0 (C-7), 124.4 (C-11′), 124.5 (C-4′), 125.1 (C-11), 128.9 (C-7′), 129.6 (C-15), 129.9 (C-5), 130.7 (C-10′), 130.8 (C-15′), 132.4 (C-14′), 133.7 (C-14), 134.5 (C-10), 134.6 (C-9), 135.3 (C-9′), 136.2 (C-13), 136.9 (C-12′), 137.5 (C-13′), 137.7 (C-8′), 137.9 (C-5′), 139.4 (C-12), 141.3 (C-8), 161.3 (C-6), 199.3 (C-4) (Figure S2). [a]: Interchangeable.

2.10.2. (3′*R*,6′*R*)-3′-Hydroxy α-carotene (α-cryptoxanthin) (**3**)

^1H NMR ($CDCl_3$) δ: 0.85 (3H, s, H-16′), 1.00 (3H, s, H-17′), 1.03 (6H, s, H-16 and H-17), 1.36 (1H, dd, *J* = 6.8, 13.2 Hz, H-2′b), 1.47 (2H, m, H-2a and H-2b), 1.62 (3H, s, H-18′), 1.62 (2H, m, H-3), 1.72 (3H, s, H-18), 1.83 (1H, dd, *J* = 5.9, 13.2 Hz, H-2′), 1.91 (3H, s, H-19′), 1.96 (3H, s, H-20), 1.97 (3H, s, H-19), 1.97 (3H, s. H-20′), 2.02 (2H, dd, *J* = 6.4, 6.4 Hz, H-4), 2.40 (1H, d, 9.9 Hz, H-6′), 4.25 (1H, m, H-3′), 5.43 (1H, dd, J=9.9, 15.4 Hz, H-7′), 5.54 (1H, s, H-4′), 6.14 (1H, d, *J* = 15.2 Hz, H-8), 6.14 (1H, d, *J* = 15.4 Hz, H-8′), 6.16 (1H, d, *J* = 15.2 Hz, H-7), 6.16 (1H, d, *J* = 10.8 Hz, H-10), 6.16 (1H, d, *J* = 10.8 Hz, H-10′), 6.25 (2H, H-14 and H-14′), 6.35 (1H, d, *J* = 14.9 Hz, H-12), 6.35 (1H, d, *J* = 14.9 Hz, H-12′), 6.62 (1H, dd, *J* = 10.2 Hz, 14.9 Hz, H-11′), 6.63 (2H, H-15 and H-15′), 6.65 (1H, dd, *J* = 10.5, 14.9 Hz, H-11) (Figure S3). ^{13}C NMR ($CDCl_3$) δ: 12.8 (C-19), 12.8 (C-20), 12.8 (C-20′), 13.1 (C-19′), 19.3 (C-3), 21.8 (C-18), 22.9 (C-18′), 24.3 (C-16′), 29.0 (C-16), 29.0 (C-17), 29.5 (C-17′), 33.1 (C-4), 34.0 (C-1′), 34.3 (C-1), 39.7 (C-2), 44.6 (C-2′), 55.0 (C-6′), 65.9 (C-3′), 124.5 (C-4′), 124.7 (C-11′), 125.1 (C-11), 126.7 (C-7), 128.7 (C-7′), 129.4 (C-5), 129.9 (C-15)c, 130.1 (H-15′)c, 130.8 (C-10), 130.8 (C-10′), 132.3 (C-14′)b, 132. 6 (C-14)b, 135.0 (C-9′), 136.1 (C-9), 136.3 (C-13)a, 136.6 (C-13′)a, 137.2 (C-12), 137.2 (C-12′), 137.6 (C-8), 137.7 (C-8′), 137.9 (C-6), 138.0 (C-18′) (Figure S4). [a, b]: Interchangeable.

2.10.3. (3*S*,3′*R*,6′*R*)-3,3′-Dihydroxy-4-keto-α-carotene (fritschiellaxanthin (4-keto-lutein)) (**4**)

^1H NMR ($CDCl_3$) δ: 0.85 (3H, s, H-16′), 0.99 (3H, s, H-17′), 1.21 (3H, s, H-16), 1.32 (3H, s, H-17), 1.36 (1H, dd, *J* = 6.8, 13.2 Hz, H-2′b), 1.82 (1H, m, H-2b), 1.62 (3H, s, H-18′), 1.83 (1H, m, H-2′a), 1.91 (3H, s, H-19′), 1.94 (3H, s, H-18), 1.97 (3H, s, H-20′), 1.98 (3H, s, H-20), 1.99 (3H, s, H-19), 2.15 (1H, m, H-2a), 2.40 (1H, d, *J* = 9.7 Hz, H-6′), 4.24 (1H, m, H-3′), 4.32 (1H, dd, *J* = 5.5, 13.8 Hz, H-3), 5.43 (1H, dd, *J* = 9.7, 15.3 Hz, H-7′), 5.54 (1H, s, H-4′), 6.14 (1H, d, *J* = 10.0 Hz, H-10′), 6.14 (1H, d, *J* = 15.3 Hz, H-8′), 6.21 (1H, d, *J* = 15.0 Hz, H-7), 6.24 (2H, H-14 and H-14′), 6.27 (1H, d, *J* = 10.0 Hz, H-10), 6.43 (1H, d, *J* = 15.0 Hz, H-12), 6.43 (1H, d, *J* = 15.0 Hz, H-12′), 6.44 (1H, d, *J* = 15.0 Hz, H-8), 6.63 (1H, dd, *J* = 10.0, 15.0 Hz, H-11), 6.63 (1H, dd, *J* = 10.0, 15.0 Hz, H-11′), 6.65 (2H, H-15 and H-15′) (Figure S5). ^{13}C NMR ($CDCl_3$) δ: 12.5 (C-19), 12.8 (C-20′), 12.8 (C-20), 13.1 (C-19′), 14.0 (C-18), 22.8 (C-18′), 24.3 (C-16′), 26. 1 (C-17), 29.5 (C-17′), 30.7 (C-16), 34.0 (C-1′), 36.8 (C-1), 44.6 (C-2′), 45.4 (C-2), 55.0 (C-6′), 65.9 (C-3′), 69.2 (C-3), 123.1 (C-7), 124.3 (C-11′), 124.5 (C-4′), 125.2 (C-11), 126.8 (C-5), 128.9 (C-15)b, 128.9 (C-7′), 130.7 (C-10′), 130.9 (C-15′)b, 132.4 (C-14′)a, 134.0 (C-14)a, 134.3 (C-9), 135.3 (C-10), 135.3 (C-9′), 136.1 (C-12), 137.1 (C-12′), 137.4 (C-13′), 137.7 (C-8′), 137.9 (C-5′), 139.9 (C-12), 142.4 (C-8), 162.3 (C-6), 200.4 (C-4) (Figure S6). [a, b]: Interchangeable.

3. Results and Discussion

Pigments produced by recombinant *E. coli* cells carrying the plasmids pRK-HIEBI-MpLCYb-MpLCYe-W or pRK-HIEBI-MpLCYb-MpLCYe-ZW, in addition to the plasmid CDF-MpCYP97C-MpLCYe, were analyzed using ODS HPLC (Figure 2). Both *E. coli* transformants produced several carotenoids, as shown in Figure 2. Subsequently, carotenoid compounds **1–7** (Figure 2) were purified by extraction from the cells with organic solvents, two-layer partitioning, preparative ODS, and silica gel HPLCs. Purified carotenoids **1**–**7** were analyzed using ESI-MS (+), 1D (^{1}H and ^{13}C), and 2D (^{1}H-^{1}H DQF COSY, HSQC, HMBC, and NOESY) NMR spectra. The ^{1}H and ^{13}C NMR spectra of carotenoids **1–7** in $CDCl_3$ are shown in Figures S1–S14.

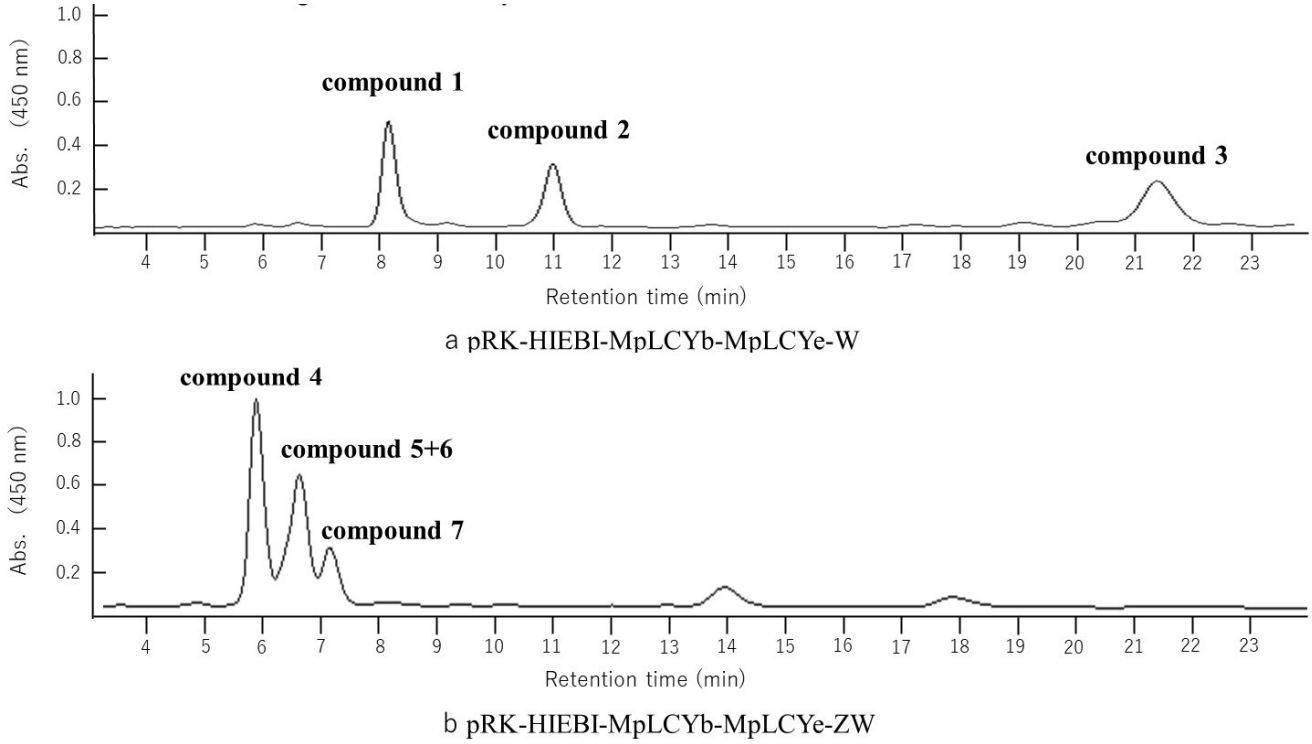

Figure 2. HPLC analysis of the extracts of *E. coli* transformants.

Based on the results of these analyses and the catalysis property of the gene products (MpLCYe, MpCYP97C and CrtZ), compounds **2**, **5**, **6** were unambiguously identified as canthaxanthin [14], zeaxanthin [15], and adonixanthin [16], which possess β-carotene backbone, and identified **7** as (3*R*,3′*R*,6′*R*)-3,3′-dihydroxy-4-keto-α-carotene (lutein) [17], which possess α-carotene backbone, by comparison with the previously reported their ^{1}H and ^{13}C NMR spectral data. The chemical structures of these compounds are shown in Figure 3.

All remaining compounds (**1**, **3**, and **4**) were confirmed to possess a 3-hydroxy α-ionone ring structure as one end structure and all *trans* olefin structures (=α-carotene backbone), as determined by 1D and 2D NMR analyses. Thus, the other end structures were further analyzed using NMR spectral data.

The other end structure of **1** was determined to be a 4-keto β-ionone ring by the observation of a ketone carbon (C-4, δ 199.3) in the ^{13}C NMR spectrum, the ^{1}H-^{13}C long-range coupling from H-18 (δ 1.87), H-2 (δ 1.85), and H-3 (δ 2.50) to C-4 in the HMBC spectrum (Figure 3). Thus, **1** was identified as (3′*R*,6′*R*)-3′-hydroxy-4-keto-α-carotene (4-keto-α-cryptoxanthin (**1**)). Compound **1** in the flesh and carapace of *Aristaeomorpha foliacea* and *Heterocarpus dorsalis* has been reported in the previous study [18].

The other end structure of **3** was determined to be a β-ionone ring based on the vicinal spin networks H-2 (δ 1.47), –H-3 (δ 1.62)–H-4 (δ 2.02) in the DQF COSY spectrum. Thus, **3** was identified as (3′*R*,6′*R*)-3′-hydroxy-α-carotene (α-cryptoxanthin (**3**)).

The other end structure of **4** was determined to be a 3-hydroxy-4-keto β-ionone ring by the observation of a ketone carbon (C-4, δ 200.4) in the ^{13}C NMR spectrum, vicinal spin couplings of H-2 (δ 1.82, δ 2.15)—H-3 (δ 4.32) in the DQF COSY spectrum, and the ^{1}H-^{13}C long-range coupling from H-2 and H-18 (δ 1.94) to the ketone carbon in the HMBC spectrum (Figure 3). Thus, **4** was identified as (3*S*,3′*R*,6′*R*)-3,3′-dihydroxy-4-keto-α-carotene (fritschiellaxanthin (4-keto-lutein) (**4**)).

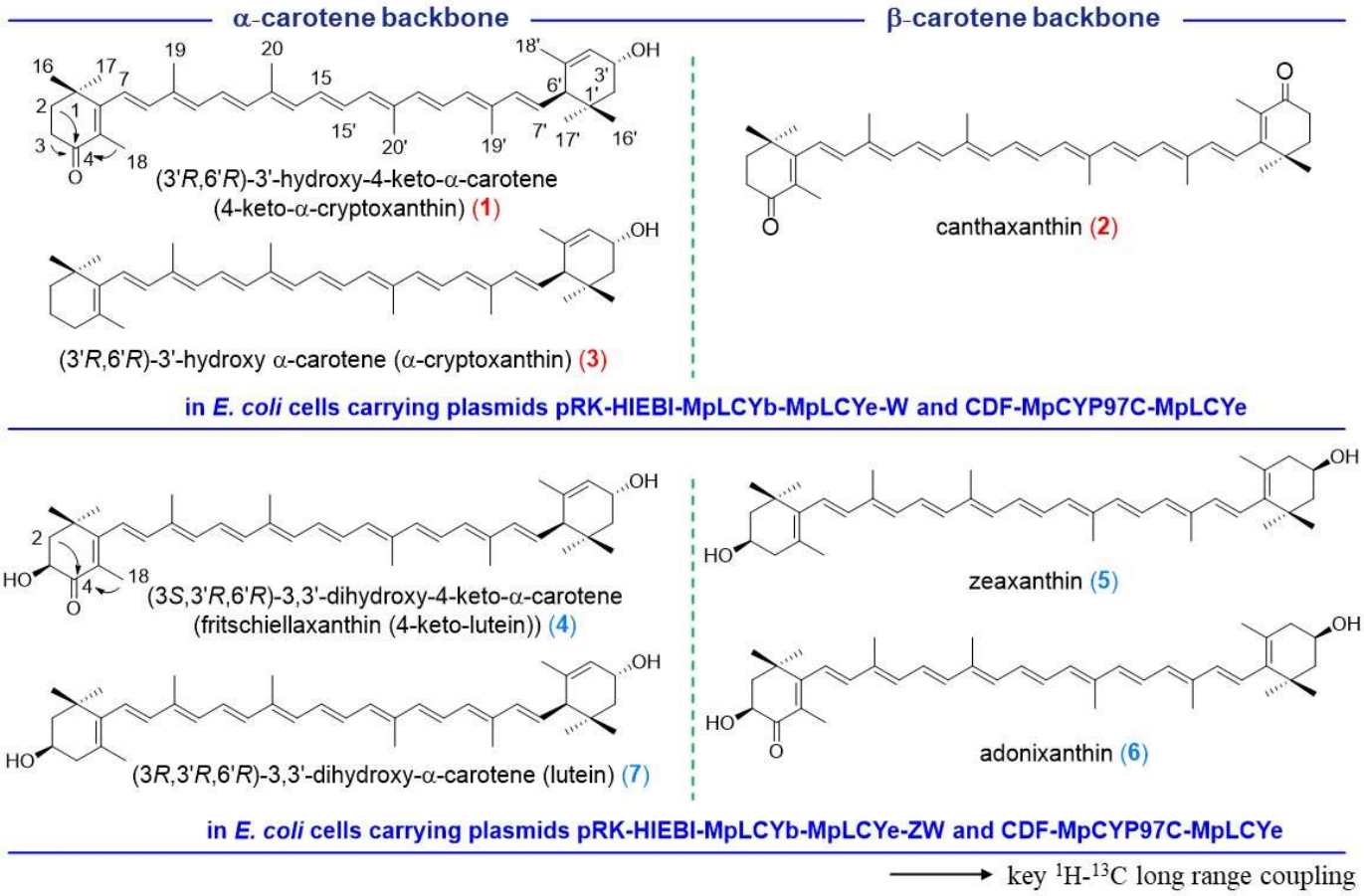

Figure 3. Carotenoids produced by recombinant *E. coli*.

To the best of our knowledge, the assigned ^{1}H and ^{13}C NMR data for compounds **1**, **3**, and **4** have not been reported in previous studies. The biological activities, including antioxidant activities of compounds **1**–**4**, have also not been previously reported.

Singlet oxygen quenching activity is generally expected in carotenoids [19,20]. Thus, we evaluated the activities of **1**–**7** using α-carotene, β-carotene, and astaxanthin as comparative controls. Table 1 presents the results of this comparison. The singlet oxygen-quenching activity of β-carotene (IC_{50} 12 ± 0.29 μM) was superior to that of α-carotene (IC_{50} 15 ± 1.1 μM). This is consistent with a previous study that found singlet oxygen-quenching activity to increase in proportion to the number of conjugated double bonds [20]. The OH function in the α and β ionone ring structure did not affect this activity because α-cryptoxanthin (**3**) (IC_{50} 17 ± 0.37 μM) and lutein (**7**) (IC_{50} 9.2 ± 0.45 μM) showed almost equivalent activities to α-carotene, and zeaxanthin (**5**) (IC_{50} 12 ± 0.29 mM) also showed equivalent activity to β-carotene. On the other hand, 4-keto function in the β-ionone ring, which increase the number of conjugated double bonds, was considered to be important for this activity because the activities of 4-keto-α-cryptoxanthin (**1**) (IC_{50} 4.6 ± 0.37 μM) and fritschiellaxanthin (4-keto-lutein) (**4**) (IC_{50} 4.6 ± 0.30 μM) were superior to α-carotene. The activities of canthaxanthin (**2**) (IC_{50} 2.3 ± 0.21 μM) and adonixanthin (**6**) (IC_{50} 2.3 ± 0.21 μM) were superior to β-carotene. The singlet oxygen-quenching activities of **1**, **2**, **4**, and **6** were almost equivalent to that of astaxanthin, a representative carotenoid with potent singlet oxygen-quenching activity.

In our previous studies, while MpCYP97C did not work on α-carotene, it worked on (3*R*,6′*R*)-3-hydroxy-α-carotene (zeinoxanthin) and produced lutein [21]. However, the results presented in this study indicate that MpCYP97C worked on α-carotene to make α-cryptoxanthin (Figure 4). In this study, we used a CDF vector instead of the pRSF vector used in the previous study. Therefore, the difference in vectors may have affected the activity. In a previous study, we found that CrtW worked on α-carotene and zeinoxanthin to form (6′*R*)-4-keto-α-carotene (α-echinenone) and (3*R*,6′*R*)-3-hydroxy-4-keto-α-carotene (4-keto-zeinoxanthin), respectively [21]. However, these compounds were not detected in the present study. This may be due to the fact that MpCYP97C in the CDF vector performed better than CrtW against α-carotene and zeinoxanthin.

Table 1. Singlet oxygen-quenching activities of carotenoids.

Carotenoid	IC_{50} (μM)
(3′*R*,6′*R*)-3′-hydroxy-4-keto-α-carotene (4-keto-α-cryptoxanthin) (**1**)	4.6 ± 0.37
canthaxanthin (**2**)	2.3 ± 0.21
(3′*R*,6′*R*)-3′-hydroxy-α-carotene (α-cryptoxanthin) (**3**)	17 ± 0.37
(3S,3′*R*,6′*R*)-3,3′-hydroxy-4-keto-α-carotene (fritschiellaxanthin (4-keto-lutein)) (**4**)	4.6 ± 0.30
zeaxanthin (**5**)	12 ± 0.29
adonixanthin (**6**)	2.3 ± 0.21
(3*R*,3′*R*,6′*R*)-3,3′-dihydroxy-α-carotene (lutein) (**7**)	9.2 ± 0.45
α-carotene	15 ± 1.1
β-carotene	6.3 ± 0.08
astaxanthin	4.3 ± 0.25

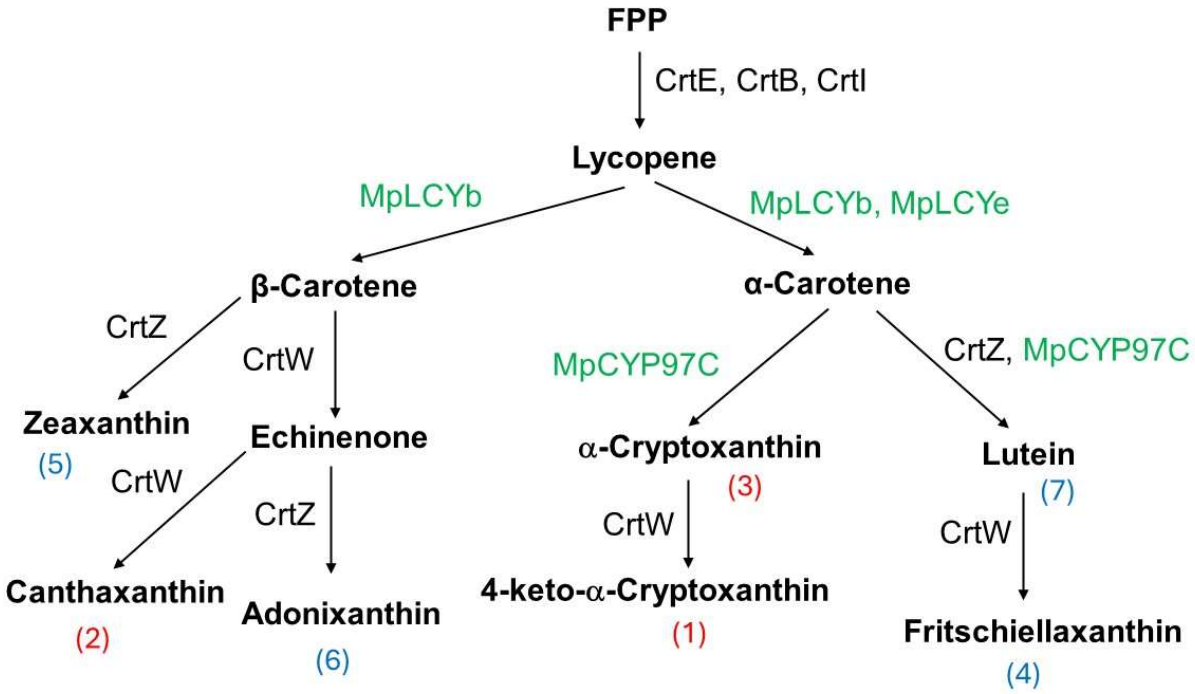

Figure 4. Biosynthetic pathway of carotenoids produced by the recombinant *E. coli.*

Among carotenoids (xanthophylls) that possess the α-carotene backbone in the molecules, 4-keto-α-cryptoxanthin (**1**), α-cryptoxanthin (**3**), and fritschiellaxanthin (4-keto-lutein) (**4**) are relatively rare in nature [2]. Fritschiellaxanthin is a 4-ketolated lutein that retains one more extended conjugated double bond than lutein and possesses the same half-molecule as astaxanthin (another notable carotenoid). Transplastomic tobacco and lettuce, in which the *crtW* gene has been introduced, have been reported to biosynthesize small amounts of fritschiellaxanthin [22,23]. Lutein and astaxanthin are commercialized carotenoids as functional food materials, since they possess distinctive beneficial functions for human health [6,10,11]. Thus, fritschiellaxanthin is likely to exert an interesting, beneficial effect on humans. It is now feasible to examine this using the specimen produced by the pathway-engineered *E. coli*.

This work represents the first successful attempt to produce three rare carotenoids with the α-carotene backbone (compounds **1**, **3**, and **4**) via pathway engineering using *E. coli*. In addition, this study is also the first to evaluate the singlet-oxygen quenching activities of these carotenoids, providing a basis for future research to examine the biological activities of these compounds and explore their usefulness.

Supplementary Materials

The following supporting information can be found at: https://www.sciepublish.com/article/pii/355, Figure S1. ^{1}H NMRspectrum of (3′*R*,6′*R*)-3′-hydroxy-4-keto α-carotene (3′-hydroxy-β,ε-echinenone) (**1**) in $CDCl_3$. Figure S2. ^{13}C NMRspectrum of (3′*R*,6′*R*)-3′-hydroxy-4-keto α-carotene (3′-hydroxy-β,ε-echinenone) (**1**) in $CDCl_3$. Figure S3. ^{1}H NMRspectrum of canthaxanthin (**2**) in $CDCl_3$. Figure S4. ^{13}C NMRspectrum of canthaxanthin (**2**) in $CDCl_3$. Figure S5. ^{1}H NMRspectrum of (3′*R*,6′*R*)-3′-hydroxy α-carotene (α-cryptoxanthin) (**3**) in $CDCl_3$. Figure S6. ^{13}C NMRspectrum of (3′*R*,6′*R*)-3′-hydroxy α-carotene (α-cryptoxanthin) (**3**) in $CDCl_3$. Figure S7. ^{1}H NMRspectrum of (3*R*,3′*R*,6′*R*)-3,3′-

dihydroxy-4-keto α-carotene (fritschiellaxanthin (4-ketolutein)) (**4**) in $CDCl_3$. Figure S8. ^{13}C NMRspectrum of (3*R*,3′*R*,6′*R*)-3,3′-dihydroxy-4-keto α-carotene (fritschiellaxanthin (4-ketolutein)) (**4**) in $CDCl_3$. Figure S9. ^{1}H NMRspectrum of zeaxanthin (**5**) in $CDCl_3$. Figure S10. ^{13}C NMRspectrum of zeaxanthin (**5**) in $CDCl_3$. Figure S11. ^{1}H NMRspectrum of adonixanthin (**6**) in $CDCl_3$. Figure S12. ^{13}C NMRspectrum of adonixanthin (**6**) in $CDCl_3$. Figure S13. ^{1}H NMRspectrum of (3*R*,3′*R*,6′*R*)-3,3′-dihydroxy α-carotene (lutein) (**7**) in $CDCl_3$. Figure S14. ^{13}C NMRspectrum of (3*R*,3′*R*,6′*R*)-3,3′-dihydroxy α-carotene (lutein) (**7**) in $CDCl_3$.

Author Contributions

K.S. and N.M.: Conceptualization; R.K. (Rinka Kanki), M.H., C.M., M.I., R.K. (Rio Kanehara), M.T. and K.S.: Investigation; K.S., M.T. and N.M.: Writing—original draft.

Ethics Statement

Not applicable.

Informed Consent Statement

Not applicable.

Funding

This research received no external funding.

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that may have influenced the work reported in this study.

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