

Biosynthetic routes of hydroxylated carotenoids (xanthophylls) in *Marchantia polymorpha*, and production of novel and rare xanthophylls through pathway engineering in *Escherichia coli*

Miho Takemura · Takashi Maoka ·
Norihiro Misawa

Received: 7 October 2014 / Accepted: 23 November 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract

Main conclusion *MpBHY* codes for a carotene β -ring 3,(3')-hydroxylase responsible for both zeaxanthin and lutein biosynthesis in liverwort. *MpCYP97C* functions as an ϵ -ring hydroxylase (zeinoxanthin 3'-hydroxylase) to produce lutein in liverwort.

Abstract Xanthophylls are oxygenated or hydroxylated carotenoids that are most abundant in the light-harvesting complexes of plants. The plant-type xanthophylls consist of α -xanthophyll (lutein) and β -xanthophylls (zeaxanthin, antheraxanthin, violaxanthin and neoxanthin). The α -xanthophyll and β -xanthophylls are derived from α -carotene and β -carotene by carotene hydroxylase activities, respectively. β -Ring 3,3'-hydroxylase that mediates the route of zeaxanthin from β -carotene via β -cryptoxanthin is present in higher plants and is encoded by the *BHY* (*BCH*) gene.

Gene accession numbers The nucleotide sequence reported in this paper has been submitted to DDBJ under accession numbers, AB981062 (*MpBHY*), AB981063 (*MpCYP97A*), AB981064 (*MpCYP97B*) and AB981065 (*MpCYP97C*).

Electronic supplementary material The online version of this article (doi:10.1007/s00425-014-2213-0) contains supplementary material, which is available to authorized users.

M. Takemura (✉) · T. Maoka · N. Misawa
Research Institute for Bioresources and Biotechnology, Ishikawa
Prefectural University, 1-308, Suematsu, Nonoichi,
Ishikawa 921-8836, Japan
e-mail: mtake@ishikawa-pu.ac.jp

T. Maoka
Research Institute for Production Development, Sakyo-Ku,
Kyoto 606-0805, Japan

On the other hand, *CYP97A* (or *BHY*) and *CYP97C* genes are responsible for β -ring 3-hydroxylation and ϵ -ring 3'-hydroxylation, respectively, in routes from α -carotene to lutein. To elucidate the evolution of the biosynthetic routes of such hydroxylated carotenoids from carotenoids in land plants, we identified and functionally analyzed carotenoid hydroxylase genes of liverwort *Marchantia polymorpha* L. Three genes homologous to higher plants, *BHY*, *CYP97A*, and *CYP97C*, were isolated and named *MpBHY*, *MpCYP97A*, and *MpCYP97C*, respectively. *MpBHY* was found to code for β -ring hydroxylase, which is responsible for both routes starting from β -carotene and α -carotene. *MpCYP97C* functioned as an ϵ -ring hydroxylase not for α -carotene but for zeinoxanthin, while *MpCYP97A* showed no hydroxylation activity for β -carotene or α -carotene. These findings suggest the original functions of the hydroxylation enzymes of carotenoids in land plants, which are thought to diversify in higher plants. In addition, we generated recombinant *Escherichia coli* cells, which produced rare and novel carotenoids such as α -echinenone and 4-ketozeinoxanthin, through pathway engineering using bacterial carotenogenic genes that include *crtW*, in addition to the liverwort *MpLCYb*, *MpLCYe* and *MpBHY* genes.

Keywords α -Xanthophyll · CYP97 · Liverwort · Carotene hydroxylase · *Marchantia polymorpha*

Abbreviations

BCH	β -Carotene 3,3'-hydroxylase
BHY	β -Carotene 3,3'-hydroxylase
CD	Circular dichroism
CYP	Cytochrome P450
LCYb	Lycopene β -cyclase
LCYe	Lycopene ϵ -cyclase
NMR	Nuclear magnetic resonance

Introduction

Carotenoids are the most widespread group of pigments found in bacteria, fungi, plants, and animals. Carotenoids are synthesized in all photosynthetic organisms and in some bacteria and fungi. On the other hand, since animals are unable to synthesize carotenoids *de novo*, they must obtain them by dietary means. β -Carotene, α -carotene and β -cryptoxanthin serve as dietary precursors of vitamin A (Castenmiller and West 1998). Numerous reports have shown that several carotenoids, which include β -carotene, β -cryptoxanthin, lycopene, lutein, zeaxanthin, and astaxanthin, exert various beneficial effects on human health, e.g., potential anti-cancer therapeutic properties and protective roles against cardiovascular and eye diseases as well as bone loss (Mayne 1996; Nishino et al. 2000; Iwamoto et al. 2000; Krinsky et al. 2003; Talegawkar et al. 2008; Sugiura et al. 2012). Thus, these carotenoids are used as functional food supplements or food additives and in cosmetics.

Xanthophylls are oxygenated or hydroxylated carotenes that are most abundant in the light-harvesting complexes (Niyogi et al. 2001; Tian et al. 2003; Dall'Osto et al. 2007). The xanthophyll composition of higher plants is conserved and consists of α -xanthophyll (lutein) and β -xanthophylls (zeaxanthin, antheraxanthin, violaxanthin and neoxanthin). α -Xanthophyll and β -xanthophylls are derived from α -carotene and β -carotene, respectively. Lutein is unexceptionally present in land plants, including bryophytes, but only in limited divisions of algae (Rhodophyta, Cryptophyta, Euglenophyta, Chlorarachnophyta and Chlorophyta) (Takaichi 2011), whereas it has never found in prokaryotes, including cyanobacteria. On the other hand, zeaxanthin is distributed not only in land plants and algae but also in some species of bacteria that include cyanobacteria.

Plant-type α -xanthophyll and β -xanthophylls are converted from α -carotene [(6'R)- β , ϵ -carotene] and β -carotene (β , β -carotene) by carotene hydroxylase activities, respectively. Lutein [(3R,3'R,6'R)- β , ϵ -carotene-3,3'-diol] is biosynthesized from α -carotene by the action of both β -ring and ϵ -ring hydroxylases, while zeaxanthin [(3R,3'R)- β , β -carotene-3,3'-diol] is synthesized from β -carotene by only β -ring hydroxylase. Two different types of enzymes that catalyze these hydroxylation reactions have been found in higher plants, i.e., cytochromes P450 that belong to the CYP97 family, which catalyze the hydroxylations of β - and ϵ -rings, and the non-heme di-iron enzyme BH_Y (also called BCH, HYD, or CrtR-b) as an ortholog of bacterial CrtZ, which catalyzes the hydroxylation of β -rings (Kim et al. 2009). BH_Y enzymes function mainly as 3,3'-hydroxylase for the β -rings in the route of β -carotene to zeaxanthin via β -cryptoxanthin. In many plant species,

the redundancy of the BH_Y (BCH) genes has been reported (Kim et al. 2009; Vallabhaneni et al. 2009; Li et al. 2010; Qin et al. 2012; Kim et al. 2001). Phylogenetic analyses suggest that the duplication of BH_Y occurred in higher plants after the split of monocot and dicot (Kim et al. 2009). The BH_Y genes of higher plants show gene-specific expression patterns, which provide different carotenoid levels in a tissue-specific manner. On the other hand, BH_Y in green algae investigated thus far is not redundant (Cui et al. 2013). In contrast to BH_Y type enzymes, P450s belonging to the CYP97 family can hydroxylate both β - and α -carotenes, while their preferred substrate is α -carotene. *Arabidopsis* CYP97A3 acts predominantly on the β -ring of α -carotene, whereas *Arabidopsis* CYP97C1 can efficiently hydroxylate both the β - and ϵ -rings of α -carotene (Kim et al. 2009). Moreover, *Arabidopsis* CYP97B3 is reported to be able to hydroxylate the β -ring of α -carotene (Kim et al. 2010). The CYP97 gene subfamilies (CYP97A, B and C) are thought to have originated before the separation of higher plants and green algae lineage (Bak et al. 2011; Cui et al. 2013). However, the molecular evolution of these genes is yet understood.

As described above, the physiology of several carotenoids, including β -carotene, β -cryptoxanthin, lycopene, lutein, zeaxanthin, and astaxanthin, has been well studied; however, others have not been investigated thus far because of their scarceness in nature. To produce these carotenoids in abundance, the pathway engineering (metabolic engineering) approach with *Escherichia coli* is one of the most powerful tools (Lee and Schmidt-Danner 2002; Das et al. 2007; Ye and Bhatia 2012; Misawa 2011). Since more than two decades ago, various carotenoids such as lycopene, β -carotene, zeaxanthin, and astaxanthin have been synthesized in *E. coli* (Cunningham et al. 1993; Misawa et al. 1990; Ruther et al. 1997). However, there are a number of rare and novel carotenoids that have not been synthesized in *E. coli*. In particular, there are few reports on the production of carotenoids that belong to α -xanthophylls in *E. coli* (Quinlan et al. 2007). Numerous kinds of carotenoids are synthesized among plants, algae, bacteria, and fungi (Britton et al. 2004). Therefore, the combination of carotenoid biosynthesis genes from different organisms makes it feasible to produce rare or novel carotenoids (Albrecht et al. 1997; Yokoyama et al. 1998; Shindo et al. 2008).

In this study, to gain insight into the evolution of carotenoid hydroxylases in plants, we isolated and functionally analyzed genes for carotenoid hydroxylases from liverwort *Marchantia polymorpha* L., which is thought to be one of the first land plants. We also report that we were able to effectively produce lutein in *E. coli* using the liverwort *MpBH_Y* and *MpCYP97C* genes. In addition, we

successfully produced novel and rare carotenoids belonging to α -xanthophylls through pathway engineering approach of *E. coli*.

Materials and methods

Cloning of the carotenoid hydroxylase genes from *M. polymorpha*

Homology search to the *M. polymorpha* genome, cDNA and EST sequences of the liverwort (Kohchi et al. personal communication) was performed to find the homologous sequences to the known *BHY* (*BCH*), *CYP97A* and *CYP97C* genes. Based on the homologous sequences obtained, the primers were designed and the coding regions without the expected transit peptides of each gene were amplified by PCR of the liverwort cDNAs. The following primers were used:

MpBHYF: 5'-CATATGACAGAAATATTCGGAACA-3'.

MpBHYR: 5'-GGATCCTACTTGGAGGATGCAGAG-3'.

MpCYP97AF: 5'-CATATGCGAACTACAGTGGCAGTAA-3'.

MpCYP97AR: 5'-GGATCCCTAAGATTGCTCGAGTGTG-3'.

MpCYP97CF: 5'-CATATGTCTGGATATGGAGAAAGAG-3'.

MpCYP97CR: 5'-GGATCCTTATATGCTTGCGAGCTC-3'.

(The underlined sequences were added for the cloning.)

Then, PCR products were cloned into the plasmid vector and sequenced.

Sequence analysis

DNA sequences of the genes were analyzed using DNASIS DNA analysis software (Hitachi Solutions, Tokyo, Japan). Homology search was performed by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Amino acid sequences were aligned by CLUSTAL W (<http://www.clustal.org/>), and a neighbor-joining tree was constructed with a 500 bootstrap replication support using MEGA6 software (Tamura et al. 2013). Transit peptides of the gene products were predicted by ChloroP (<http://www.cbs.dtu.dk/services/ChloroP/>). Transmembrane helical regions were predicted by TMHMM2.0 (<http://www.cbs.dtu.dk/services/TMHMM>).

Expression of the liverwort carotenoid biosynthesis genes in *E. coli*

The coding regions of the liverwort *BHY* (*MpBHY*), *CYP97A* (*MpCYP97A*) and *CYP97C* (*MpCYP97C*) were amplified with the *M. polymorpha* cDNA as the template by PCR and cloned into the pET21a or pRSFDuet (Merck Millipore, Darmstadt, Germany) independently. These plasmids were named pET21-MpBHY, pET21-MpCYP97A and pET21-MpCYP97C, or pRSFDuet-MpBHY, pRSFDuet-MpCYP97A and pRSFDuet-MpCYP97C. We also simultaneously inserted two of the genes *MpBHY* and *MpCYP97C*, and *MpCYP97A* and *MpCYP97C* into the pRSFDuet (Merck Millipore) and called the constructed vectors pRSFDuet-MpBHY/MpCYP97C and pRSFDuet-MpCYP97A/MpCYP97C, respectively. The *crtW* gene from *Brevundimonas* sp. strain SD212 (Nishida et al. 2005; accession no. AB181388) was also introduced with *MpBHY* into pRSFDuet. This plasmid was named pRSFDuet-MpBHY/*crtW*. They were transformed into the *E. coli* (BL21 (DE3)) that produced lycopene (with pACCRT-EIB) (Misawa et al. 1995), β -carotene (with pACHP-Beta), or α -carotene (with pACCRT-EIB and pETDuet-MpLCYb/MpLCYe) (Takemura et al. 2014). To construct pACHP-Beta, the *Haemotococcus pluvialis* *idi* gene was isolated from pHP11 (Kajiwara et al. 1997; accession no. AB019034) with *Xho*I and *Not*I, and inserted into the *Ava*I-*Sal*I site of pACCAR16 Δ crtX (Misawa et al. 1995) with an amplified *Not*I-*Sal*I fragment including the *Pantoea ananatis* *crtE* gene. The transformed *E. coli* was grown in 2YT medium at 37 °C until an optical density of 0.8–1.0, induced with 0.05 mM of IPTG, and further cultured at 21 °C for 2 days.

An N-terminally truncated form of the *Arabidopsis* NADPH-P450 reductase 2 gene (Hull and Celenza 2000; Urban et al. 1997) was amplified by PCR and cloned into the CDF vector (Merck Millipore). This plasmid was named CDF-AtATR2 and co-transformed with the plasmids described above into *E. coli*.

Extraction and analysis of carotenoids from *E. coli* cells

Extraction of carotenoids from *E. coli* was performed by the method as previously described (Fraser et al. 2000). *E. coli* cultures were centrifuged and cell pellets were extracted in methanol using mixer for 5 min. Tris-HCl (50 mM, pH 7.5) (containing 1 M NaCl) was added and mixed. Then, chloroform was added to the mixture and incubated for 5 min. After centrifugation, the chloroform phase was removed and dried by centrifugal evaporation. Dried residues were re-suspended with ethyl acetate and applied to the HPLC-PDA.

Chromatography was carried out on a Waters Alliance 2695–2996 system (Waters, Milford, MA, USA) with a column, TSKgel ODS-80Ts (4.6 × 150 mm, 5 μm; Tosoh, Tokyo, Japan), according to the method described previously (Yokoyama and Miki 1995). Briefly, the extract was eluted at a flow rate of 1.0 ml/min at 25 °C with solvent A (water–methanol, 5:95, v/v) for 5 min, followed by a linear gradient from solvent A to solvent B (tetrahydrofuran–methanol, 3:7, v/v) for 5 min, solvent B alone for 8 min, and then back to solvent A. Carotenoids were identified by comparing both their retention times and absorption spectra monitored using PDA relative to those of the authentic standards.

Spectroscopic data of lutein, zeaxanthin, β-cryptoxanthin, rubixanthin, astaxanthin, canthaxanthin, echinenone, and the other ketocarotenoids in addition to β-carotene, α-carotene, δ-carotene and lycopene were described (Britton et al. 2004). α-Echinenone, zeinoxanthin, and 4-ketozeinoxanthin were identified by NMR, HR-MS and CD. Since 4-ketozeinoxanthin [(3*R*,6'*R*)-3-hydroxy-β,ε-caroten-4-one] was a novel compound, its detailed data are reported (Maoka et al. 2014).

Spectroscopic data

α-Echinenone [(6'*R*)-β,ε-caroten-4-one]: UV–vis λ max (Ether) 445–470 nm; HR-ESI MS; *m/z* 550.4195 (*M*⁺, calcd for C₄₀H₅₄O, 550.4175); ¹H NMR (CDCl₃, 500 MHz) δ 0.83 (H₃-17', s), 0.91 (H₃-16', s), 1.20 (H₃-16, 17, s), 1.18 (H-2'β, m), 1.43 (H-2'α, m), 1.59 (H₃-18', s), 1.85 (H₂-2, t, 7), 1.88 (H₃-18, s), 1.91 (H₃-19', s), 1.95 (H₃-20', s), 1.98 (H₃-20, s), 2.00 (H₃-19, s), 2.00 (H₂-3' m), 2.51 (H₂-4, t, 7), 5.42 (H-4', br s), 5.53 (H-7', dd, 15, 9.5), 6.11 (H-8', d, 15), 6.12 (H-10', d, 11), 6.24 (H-7, d, 16), 6.24 (H-14', d, 11), 6.28 (H-10, d, 11), 6.30 (H-14, d, 11), 6.34 (H-12', d, 15), 6.37 (H-8, d, 16), 6.45 (H-12, d, 15), 6.61 (H-11', dd, 15, 11), 6.62 (H-15', dd, 15, 11) and 6.67 (H-15, dd, 15, 11), 6.65 (H-11, dd, 15, 11); CD (Ether) λ (Δε) 210 (0), 245 (+4.3), 270 (0), 285 (-0.7), 298 (0), 344 (+3.7), 380 (0).

Zeinoxanthin [(3*R*,6'*R*)-β,ε-caroten-3-ol]: UV–vis λ max (Ether) 421, 444, and 471 nm; HR-ESI MS; *m/z* 552.4319 (*M*⁺, calcd for C₄₀H₅₆O₂, 552.4331); ¹H NMR (CDCl₃, 500 MHz) δ 0.83 (H₃-17', s), 0.91 (H₃-16', s), 1.07 (H₃-16, 17, s), 1.18 (H-2'β, m), 1.43 (H-2'α, m), 1.48 (H-2, β, dd, 12, 12), 1.59 (H₃-18', s), 1.74 (H₃-18, s), 1.78 (H-2α, ddd, 12, 5, 1.5), 1.91 (H₃-19', s), 1.97 (H₃-19, 20, 20', s), 2.00 (H₂-3' m), 2.04 (H-4β dd, 16, 10), 2.39 (H-4α, ddd 16, 6, 1.5), 2.18 (H-6', d, 9.5), 4.00 (H-3, m), 5.42 (H-4', br s), 5.53 (H-7', dd, 15, 9.5), 6.10 (H-7, d, 16), 6.11 (H-8', d,

15), 6.12 (H-10', d, 11), 6.15 (H-8, d, 16), 6.15 (H-10, d, 11), 6.24 (H-14, d, 11), 6.24 (H-14', d, 11), 6.34 (H-12', d, 15), 6.35 (H-12, d, 15), 6.61 (H-11', dd, 15, 11), 6.62 (H-15 and H-15', m), 6.64 (H-11, dd, 15, 11); ¹³C NMR (CDCl₃, 125 MHz) δ 12.8 (C-19, 20, 20'), 13.1 (C-19'), 21.6 (C-18), 23.1 (C-18'), 27.0 (C-3', 16'), 27.7 (C-17'), 28.7 (C-16), 30.2 (C-17), 31.7 (C-2'), 32.5 (C-1'), 37.1 (C-1), 42.5 (C-4), 48.4 (C-2), 54.9 (C-6'), 65.1 (C-3), 120.8 (C-4'), 125.0 (C-11, 11'), 125.5 (C-7), 126.1 (C-5), 129.9 (C-15'), 130.1 (C-15, 10'), 131.1 (C-7'), 131.3 (C-7'), 131.3 (C-10), 132.3 (C-14'), 132.6 (C-14), 134.5 (C-5'), 135.6 (C-9, 9'), 136.2 (C-13, 8', 13'), 137.1 (C-12'), 137.6 (C-6, 12), 138.5 (c-8), CD (Ether) λ (Δε) 245 (+3.4), 280 (0), 290 (-1.5), 340 (0), 360 (+1.0).

Expression analysis of the *MpBHY*, *MpCYP97A*, *MpCYP97B*, and *MpCYP97C* genes

Expression analysis of the four genes was performed by real-time PCR. Total RNAs were extracted from liverwort thalli using an RNeasy Plant mini kit (Qiagen, Hilden, Germany) and treated with DNaseI. One μg each of total RNA was reverse-transcribed with oligo-dT primer using PrimeScript RT Master Mix (Takara, Shiga, Japan). Amplification of real-time PCR was performed using SYBR Premix DimerEraser (Takara) and data analysis was carried out using the ABI 7300 Real-Time PCR System (Life Technologies).

For real-time PCR, the following primers were used:

MpBHYRT1F: 5'-CCGTCTCTCTGATGCTCTACGG-3',
MpBHYRT1R: 5'-CATCGTGAACGAACATGTAG-3',
MpCYP97ART1F: 5'-GCATCTGGAGAGTTTACAGT-3',
MpCYP97ART1R: 5'-CAACCTTCTCTCGTAATTGG-3',
MpCYP97BRT1F: 5'-AAGGTTAGAAGGCGAGCTAT-3',
MpCYP97BRT1R: 5'-GCTCTATGCAGACGAGTTTC-3',
MpCYP97CRT1F: 5'-AGTATCTGGCGACTATGGTG-3',
MpCYP97CRT1R: 5'-GGTCAGCTGAGAGAATCTTG-3',
MpACTF: 5'-TGGCCGACTCTGAGGATGTT-3',
MpACTR: 5'-TTCCAGATCCATTGTGCGAG-3'.

The expression levels of the various genes were normalized by *MpACT* as reference genes for internal control. The value relative to the expression level for female thalli was calculated.

Results

Isolation of the carotenoid hydroxylase genes from *M. polymorpha*

Figure 1 shows the xanthophyll biosynthetic pathway in higher plants, whose biosynthesis genes have been identified. Recently, we carried out detailed analysis of carotenoids in liverwort, *M. polymorpha* (Takemura et al. 2014). Consequently, no significant differences were found in the carotenoid content and composition between its male and female. In the male thalli, lutein (56.3 %) and β -carotene (30.9 %) were major carotenoids, and α -carotene (1.6 %), zeaxanthin (1.0 %), antheraxanthin (2.3 %), violaxanthin (1.6 %), and 9'-*cis*-neoxanthin (2.7 %) were also found. These results suggest that the liverwort has the same carotenoid biosynthetic pathway to that of higher plants and the same set of carotenoid biosynthesis genes. To find homologous genes with *Arabidopsis* *BHY* (*BCH*), *CYP97A3*, and *CYP97C1* genes, homology searches for the EST and genome sequences were performed (Kohchi et al. personal communication). As a result, each gene was found and named *MpBHY*, *MpCYP97A*, and *MpCYP97C*. From in silico analysis of current genome sequencing and transcriptome data from different tissues and conditions, all were expected to be single copy genes (Fig. 2).

Four transmembrane domains were predicted in *MpBHY* peptides as in other plant *BCHs* (*BHYs*; Supplementary Fig. S1). *MpBHY* also had the Motif1 (Sun et al. 1996). *MpBHY* showed approximately 50 and 21 % amino

acid identities to *AtBCH* and the *P. ananatis* *CrtZ*, respectively. The 137 amino acids of N-terminus were predicted to be a transit peptide to chloroplast.

Phylogenetic analysis of CYP97 proteins indicated that three gene subfamily members existed in the liverwort as in higher plants (Fig. 2b) (Kim et al. 2009). The *CYP97A* and *CYP97C* have been reported to be involved in carotenoid biosynthesis, while the function of *CYP97B* has not well been elucidated. The liverwort CYP97 s included consensus sequences for both oxygen-binding pocket and heme-binding domain (Chapple 1998) (Supplementary Fig. S2). The 60 and 79 amino acids of N-terminus of *MpCYP97A* and *MpCYP97C*, respectively, were predicted to be signal sequences to chloroplast.

MpBHY acts as the β -carotene hydroxylase

To identify the β -carotene hydroxylase of the liverwort, we used *E. coli* expression system (Misawa et al. 1995). The *E. coli* having plasmid pACHP-Beta that carries the *Pantoea ananatis* *crtE*, *crtB*, *crtI*, *crtY* genes in addition to the *Haematococcus pluvialis* *idi* [isopentenyl diphosphate (IPP) isomerase] gene produces β -carotene (β , β -carotene) (Figs. 3, 4a). We introduced *MpBHY*, *MpCYP97A* and *MpCYP97C* genes independently into this *E. coli*. Although 138 amino acids of the *MpBHY* N-terminal were predicted to be the transit signal to chloroplast, this region contained the first predicted TM-helix (Supplementary Fig. S1). Therefore, we constructed the pET-*MpBHY* without the first 120 amino acids. On the other hand, *MpCYP97A* and *MpCYP97C*, whose individual predicted transit peptides were eliminated, were fused with pET vector. When the empty vector pET21a was expressed in this *E. coli*, β -carotene was detected as expected (Fig. 4a). When pET-*MpBHY* was introduced, zeaxanthin [(3*R*, 3'*R*)- β , β -carotene-3,3'-diol] was detected (Fig. 4b). A small amount of the monohydroxylated carotenoid β -cryptoxanthin [(3*R*)- β , β -caroten-3-ol] and rubixanthin [(3*R*)- β , ψ -caroten-3-ol] were also detected. Rubixanthin is a 3-hydroxylated product of γ -carotene that is the monocyclic intermediate of β -carotene synthesis from lycopene. These results demonstrated that *MpBHY* exerts β -ring hydroxylation activity for β -carotene and γ -carotene to produce zeaxanthin (via β -cryptoxanthin) and rubixanthin, respectively. In contrast to *MpBHY*, neither *MpCYP97A* nor *MpCYP97C* influenced the composition of carotenoids when introduced into the β -carotene-producing *E. coli* (Fig. 4c, d). These results suggested that neither *MpCYP97A* nor *MpCYP97C* exerted β -carotene hydroxylase activity.

Most of the plant P450s involved in the secondary metabolism require a FAD and FMN-containing NADPH-P450 reductase to receive the electrons from NADPH and reduce P450 itself (Hannemann et al. 2007). For the

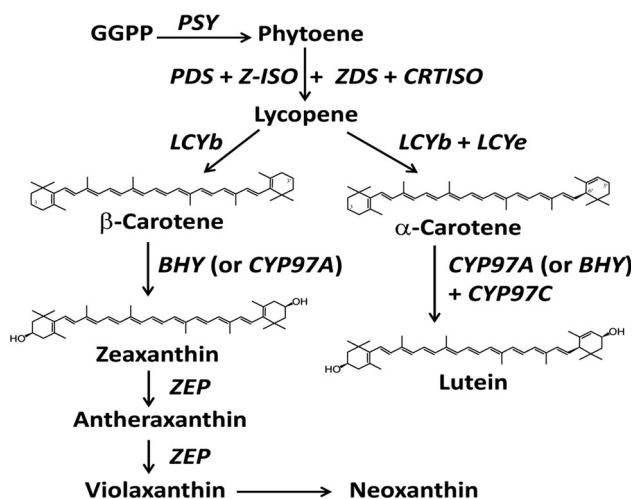


Fig. 1 Xanthophyll biosynthesis pathways in higher plants. *GGPP* geranylgeranyl pyrophosphate, *PSY* phytoene synthase, *PDS* phytoene desaturase, *Z-ISO* 15-*cis*- ζ -carotene isomerase, *ZDS* ζ -carotene desaturase, *CRTISO* carotenoid isomerase, *LCYb* lycopene β -cyclase, *LCYe* lycopene ϵ -cyclase, *BHY* β -carotene hydroxylase, *CYP97A* cytochrome P450 97A, *CYP97C* cytochrome P450 97C, *ZEP* zeaxanthin epoxidase

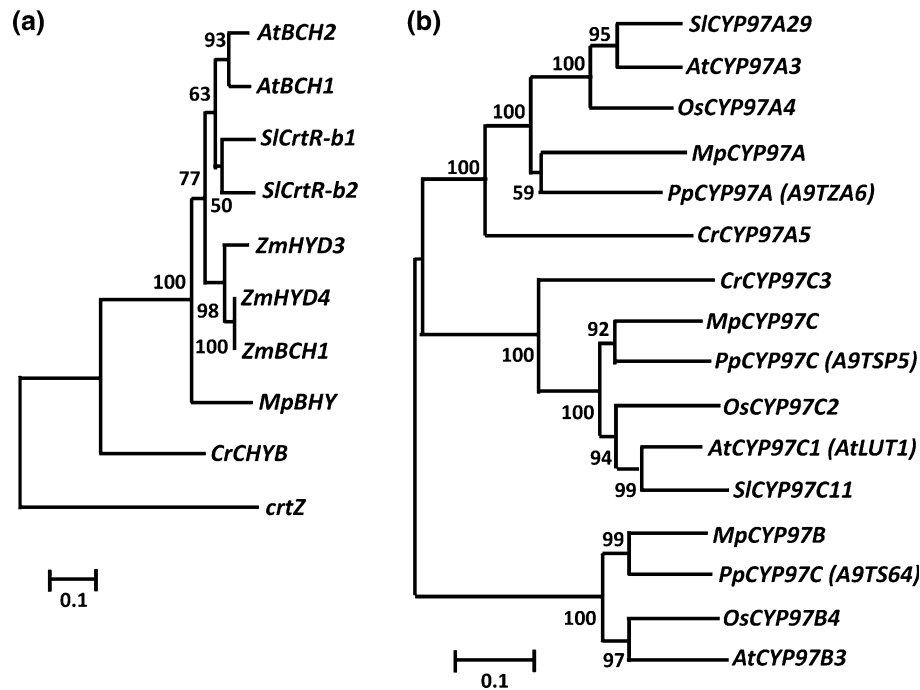


Fig. 2 Phylogenetic relationship between carotene hydroxylases. A rooted neighbor-joining tree was constructed. Numbers adjacent to branches are bootstrap values supporting the presented final tree. **a** Phylogenetic tree of β -carotene hydroxylases. Accession numbers are: Maize BCH1 (ZmBCH1), GQ131287; Maize HYD3 (ZmHYD3), AY844958; Maize HYD4 (ZmHYD4), AY844956; Arabidopsis BCH1 (AtBCH1), AY113923; Arabidopsis BCH2 (AtBCH2), AY117225; Tomato CrtR-b1 (SlCrtR-b1), Y14809; Tomato CrtR-b2 (SlCrtR-b2), Y14810; Liverwort BHY (MpBHY), AB981062; Chlamydomonas CHYB (CrCHYB), AY860819; Pantoea CrtZ (crtZ), D90087. **b** Phylogenetic tree of CYP97A, CYP97B and CYP97C proteins. Accession numbers are: Arabidopsis CYP97A3

(AtCYP97A3), NM_102914; Tomato CYP97A29 (SlCYP97A29), EU849605; Rice CYP97A4 (OsCYP97A4), AK068163; Chlamydomonas CYP97A5 (CrCYP97A5), EF587911; Liverwort CYP97A (MpCYP97A), AB981063; Physcomitrella CYP97A (PpCYP97A), A9TZA6; Arabidopsis CYP97B3 (AtCYP97B3), NM_117600; Rice CYP97B4 (OsCYP97B4), TC299269; Liverwort CYP97B (MpCYP97B), AB981064; Physcomitrella CYP97B (PpCYP97B), A9ST64; Arabidopsis CYP97C1 (AtCYP97C1), AY424805; Tomato CYP97C11 (SlCYP97C11), EU849604; Rice CYP97C2 (OsCYP97C2), AK065689; Chlamydomonas CYP97C3 (CrCYP97C3), EF587910; Liverwort CYP97C (MpCYP97C), AB981065 Physcomitrella CYP97C (PpCYP97C), A9TSP5

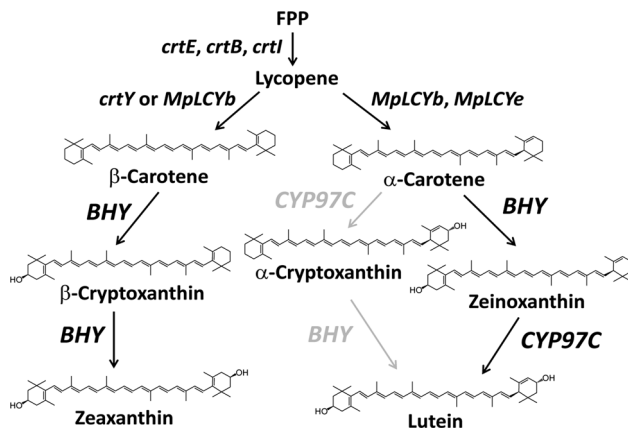


Fig. 3 Pathway of carotenoid biosynthesis in transformed *E. coli* used in this study. The reactions indicated by gray arrows have not been detected

functional expression of such plant P450 genes in *E. coli*, a gene for plant NADPH-P450 reductase, is necessary to be co-expressed (Harada et al. 2011; Schückel et al. 2012),

while a redox partner flavoprotein endogenous in *E. coli* is sometimes likely to complementarily function with foreign P450s such as higher plant CYP97A and CYP97C (Tian et al. 2003, Quinlan et al. 2012, Christopher and Waterman 1998). However, we do not know whether the *M. polymorpha* CYP97s need a foreign redox partner such as a plant NADPH-P450 reductase. For this reason, we tested the requirement of a higher plant NADPH-P450 reductase for MpCYP97A or MpCYP97C by co-expressing the *Arabidopsis* NADPH-P450 reductase 2 (*AtATR2*) gene (Hull and Celeza 2000; Urban et al. 1997). As a result, no differences were found in the products between the presence and absence of *AtATR2* (Supplementary Fig. S3).

MpBHY and MpCYP97C act to produce lutein

To further investigate the enzyme activities of these three genes, we used the *E. coli* producing α -carotene [(6'R)- β , ϵ -carotene] due to the presence of the plasmids pACCRT-EIB and pETDuet-MpLCYb/MpLCYe (Fig. 5a; Takemura

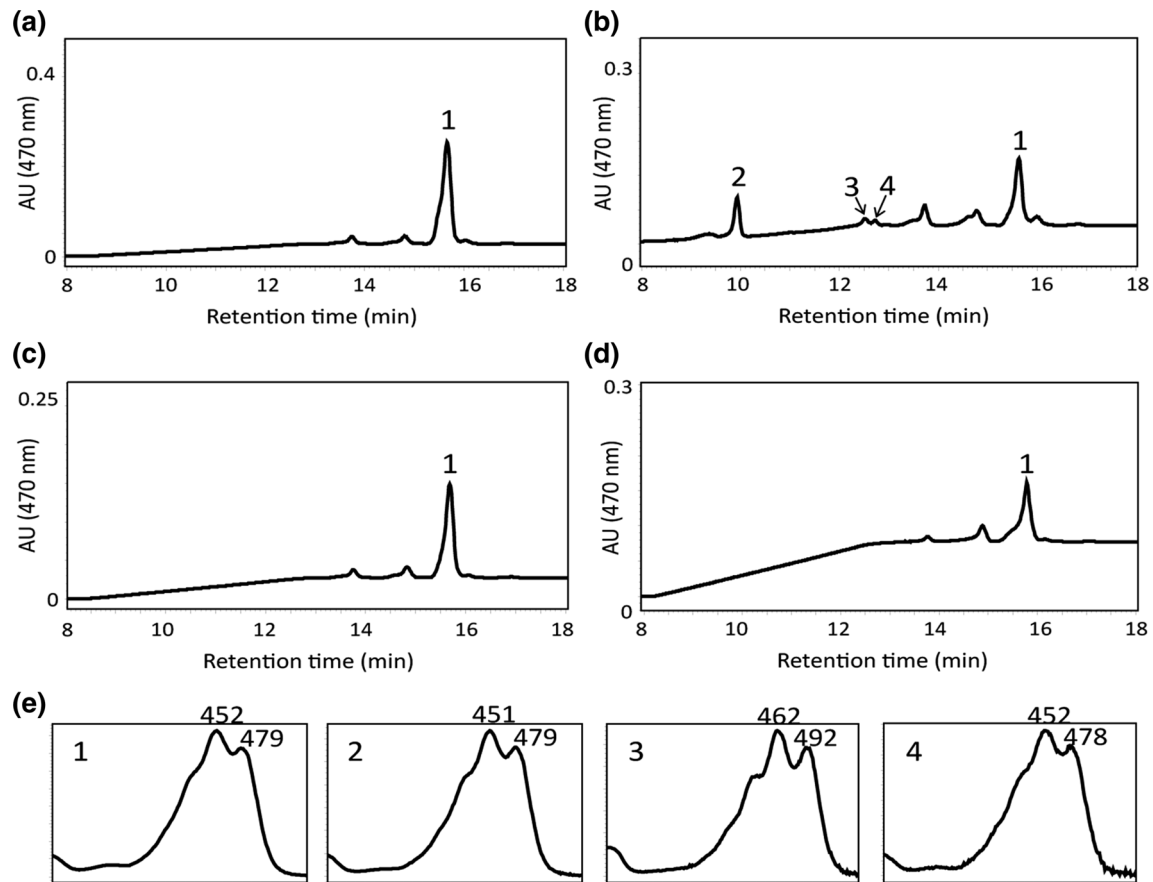


Fig. 4 HPLC analysis of products formed from β -carotene in transformed *E. coli*. **a–d** HPLC chromatograms of the extracts from *E. coli* that carried **a** pACHP-Beta plus pET21a vector; **b** pACHP-Beta plus pET-MpBHY; **c** pACHP-Beta plus pET-MpCYP97A;

d pACHP-Beta plus pET-MpCYP97C. **e** Absorption spectra of individual peaks. **1** β -carotene; **2** zeaxanthin; **3** rubixanthin; **4** β -cryptoxanthin

et al. 2014). When either the *MpCYP97A* or *MpCYP97C* gene was introduced into this recombinant *E. coli*, α -carotene was not converted to any other carotenoids (Fig. 5b, c). In addition, the *AtATR2* gene did not affect their enzyme activities (Supplementary Fig. S3). These results indicated that neither *MpCYP97A* nor *MpCYP97C* possesses ring hydroxylase activity for α -carotene. On the other hand, when the *MpBHY* gene was introduced into the α -carotene-producing *E. coli*, the monohydroxylated intermediate, zeinoxanthin, was detected, indicating that *MpBHY* has an activity to hydroxylate β -ring of α -carotene (Fig. 5d).

We further investigated the combination of *MpCYP97A*/*MpCYP97C* and *MpBHY*/*MpCYP97C*. This is because *MpBHY* and *MpCYP97A* were expected as β -ring hydroxylase, while *MpCYP97C* was expected as ϵ -ring hydroxylase. When pRSF-*MpCYP97A*/*MpCYP97C* was introduced into the *E. coli* producing α -carotene, no significant modification of the carotenoid profile was found (Fig. 5e). In contrast, the introduction of pRSF-*MpBHY*/*MpCYP97C* resulted in the dominant production of lutein

[(3*R*,3'*R*,6'*R*)- β,ϵ -carotene-3,3'-diol; Fig. 5f]. These results indicated that *MpBHY* and *MpCYP97C* function as β -ring and ϵ -ring hydroxylase of α -carotene, respectively, to produce lutein.

Expression of the *MpBHY*, *MpCYP97A*, *MpCYP97B* and *MpCYP97C* genes

To examine the expression of *MpBHY*, *MpCYP97A*, *MpCYP97B* and *MpCYP97C*, we performed a real-time PCR. The expression level of the *MpCYP97C* gene was similar in the female and male thalli (Fig. 6). On the other hand, the expressions of *MpBHY*, *MpCYP97A* and *MpCYP97B* in the male thalli were slightly higher than those in the female thalli.

Production of rare or novel carotenoids

Previous reports have indicated that astaxanthin (4,4'-ketozeaxanthin) is produced from zeaxanthin by the β -carotene ketolase (*CrtW*) in genetically engineered *E. coli*

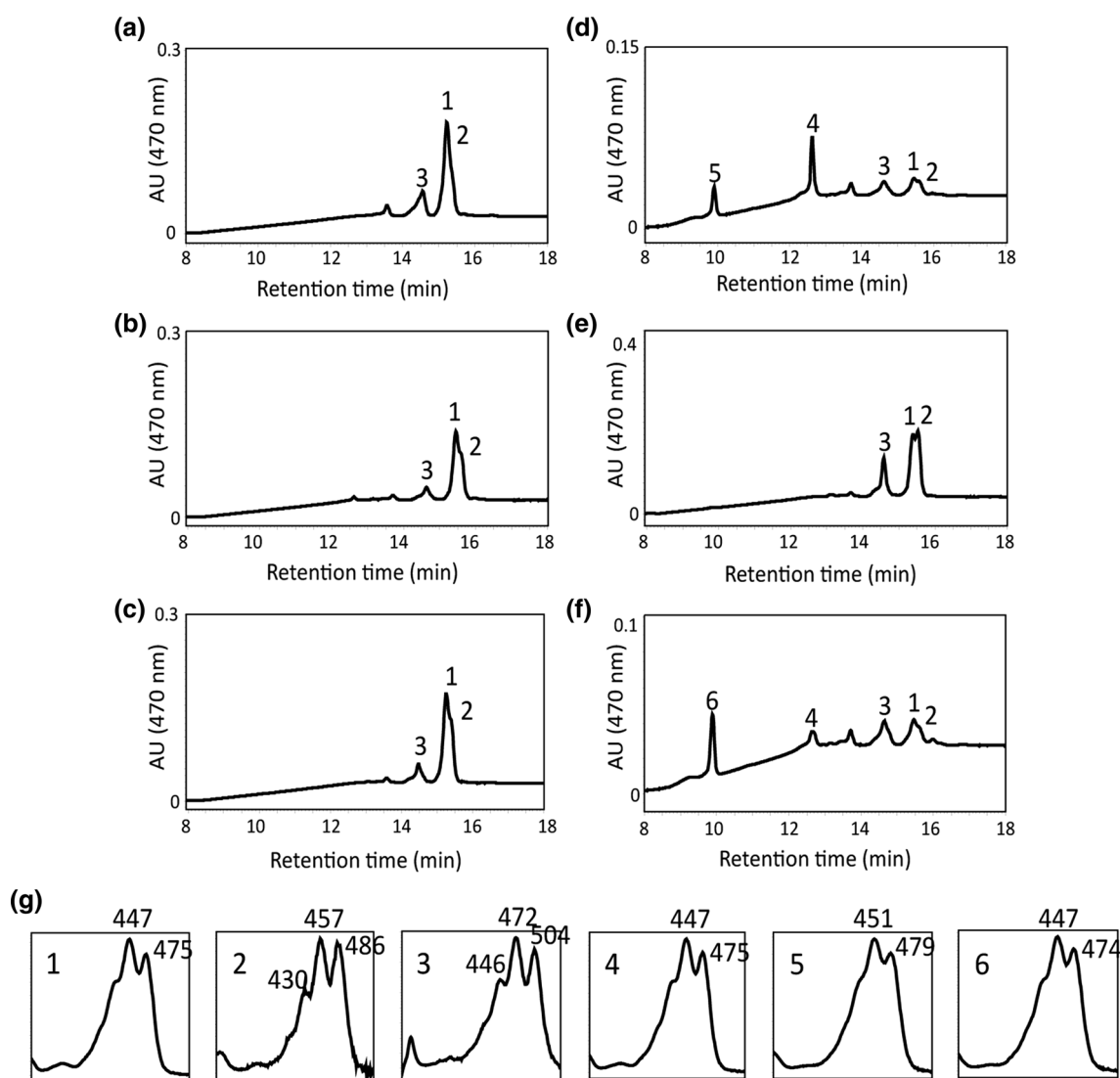


Fig. 5 HPLC analysis of products formed from α -carotene in transformed *E. coli*. **a–f** HPLC chromatograms of the extracts from α -carotene-producing *E. coli* (pACCRT-EIB and pETDuet-MpLCYb/MpLCYe) transformed by **a** pRSFDuet vector; **b** pRSFDuet -

MpCYP97A; **c** pRSFDuet -MpCYP97C; **d** pRSFDuet -MpBHY; **e** pRSFDuet-MpCYP97A/MpCYP97C; **f** pRSFDuet-MpBHY/MpCYP97C. **g** Absorption spectra of individual peaks. **1** α -carotene; **2** δ -carotene; **3** lycopene; **4** zeinoxanthin; **5** zeaxanthin; **6** lutein

(Misawa et al. 1995; Fraser et al. 1997). Recently, a rare carotenoid, α -echinenone (4-keto- α -carotene) was reported as an unexpected by-product in transgenic rice callus expressing the *Brevundimonas crtW* (Breitenbach et al. 2014). However, it is unclear whether it is possible to produce ketocarotenoids from α -carotene and/or α -xanthophyll in transgenic *E. coli*. In this study, we introduced the *crtW* gene into the α -xanthophylls-producing *E. coli*. This recombinant *E. coli* was orange (Supplementary Fig. S4). Pigment analysis showed that α -echinenone and 4-ketozeinoxanthin were produced from α -carotene and zeinoxanthin, respectively (Table 1). This result showed that CrtW catalyzed the ketolation reaction of β -ring of α -carotene and zeinoxanthin in *E. coli*. α -Echinenone is a rare

carotenoid reported to be present in sea urchin. 4-Ketozeinoxanthin is a novel carotenoid, which has never been isolated from any organism.

Discussion

In this study, we isolated and functionally analyzed carotenoid hydroxylase genes, named *MpBHY*, *MpCYP97A* and *MpCYP97C*, from a liverwort for the first time. Sequence analysis suggested that *MpBHY* and *MpCYP97A* coded for carotenoid β -ring hydroxylase and that *MpCYP97C* coded for carotenoid ϵ -ring hydroxylase. It has been reported that higher plants have more than two *BHY*

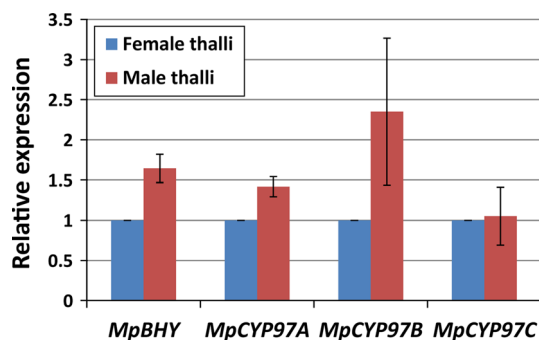


Fig. 6 Expression analysis of *MpBHY*, *MpCYP97A*, *MpCYP97B* and *MpCYP97C*. Total RNA was extracted from male and female thalli and subjected to real-time PCR analysis. Expression levels were normalized by *MpACT* as internal control and values relative to that of female thalli. Data are average $\pm$ SD of three independent measurements

Table 1 Carotenoid content in the recombinant *E. coli* cells

Carotenoid	Composition (%)	Content (μ g/gFW)
α -Carotene	10.8	6.9
β -Carotene	5.2	3.3
Lycopene	3.1	2.0
α -Echinenone	13.0	8.4
Echinenone	3.5	2.2
4-Ketozeinoxanthin	16.6	10.7
Canthaxanthin	15.7	10.1
Adonirubin	3.4	2.2
Astaxanthin	22.5	14.5
9Z-Astaxanthin	2.1	1.3
13Z-Astaxanthin	3.7	2.4

(*BCH*) genes. For example, six different *BHY* paralogs were recently identified in maize (Vallabhaneni et al. 2009). Two of these clearly encoded β -carotene hydroxylase, two were pseudogenes, and the remaining two were functionally unclear (Vallabhaneni et al. 2009). *Arabidopsis* (Sun et al. 1996; Tian and DellaPenna 2001), citrus (Kim et al. 2001), and tomato (Galpaz et al. 2006) were also reported to have two *BHY* genes. In contrast, only one *BHY* gene was found in the liverwort *M. polymorpha* genome. In flowering plants, the carotenoid compositions among flowers, fruits, and leaves are significantly different, because the genes of carotenoid biosynthesis are regulated in a tissue- and/or development-specific manner (Galpaz et al. 2006; Li et al. 2010). It is likely that duplication and subsequent functional divergence of *BHY* (*BCH*) genes occurred after the split of the bryophytes and higher plants (Moore and Purugganan 2005; Kim et al. 2009). The *BHY* is presumed to be located in the thylakoid membranes of chloroplasts in plants. *MpBHY* contains an N-terminal extension of 170 amino acids more than the corresponding

bacterial enzyme *CrtZ*. Sun et al. (1996) reported that truncation of the first 69 amino acids of the *Arabidopsis* *BCH1* (*AtBCH1*), which was predicted as the signal peptide to chloroplast, did not impair enzyme activity in *E. coli*. They also found that removing the first 129 amino acids, including the first transmembrane helix, resulted in a high ratio of β -cryptoxanthin to zeaxanthin. Therefore, they speculated that amino acid residues 70–129 of *AtBCH1* may play a role in formation of a functional homodimer (Sun et al. 1996). In this study, we produced a polypeptide lacking the first 170 amino acids, which was the extended region compared to *CrtZ*, and contained the first transmembrane region. The activity of this truncated enzyme was not impaired (Supplementary Fig. S5), suggesting that this extension was not required for the activity of *MpBHY*. Considering that the first transmembrane domain of *AtBCH1* is important for this activity, this domain may have acquired new roles during plant evolution.

MpBHY catalyzed the reaction of β -ring hydroxylation of both β - and α -carotenes in the same manner as higher plant *BCH* enzymes. On the other hand, *MpCYP97A* did not show any carotenoid hydroxylation activity, which was different from the corresponding P450s of higher plants encoded by the *CYP97A* genes such as *Arabidopsis LUT5* (*CYP97A3*) (Kim and DellaPenna 2006), rice *CYP97A4* (Lv et al. 2012) and tomato *CYP97A29* (Stigliani et al. 2011). *MpCYP97C* hydroxylated the ϵ -ring of α -carotene, which is the same as in the higher plant *CYP97C*. However, it is emphasized that this P450 functions as ϵ -ring hydroxylase not for α -carotene but for zeinoxanthin (Fig. 3). We detected zeinoxanthin but no α -cryptoxanthin in the lutein-producing *E. coli* cells, which included the *MpBHY* and *MpCYP97C* genes. Moreover, no monohydroxylated carotene (α -cryptoxanthin) was produced from α -carotene only with *MpCYP97C*. These results demonstrated that β -ring hydroxylation precedes ϵ -ring hydroxylation during lutein synthesis from α -carotene. This finding is supported by the analysis of *Arabidopsis lut1* mutant, which produced zeinoxanthin but not α -cryptoxanthin (Tian et al. 2003, 2004). Quinlan et al. (2012) have proposed the protein–protein interaction between *CYP97A* and *CYP97C* in rice. Analysis of the interaction between *MpBHY* and *MpCYP97C* proteins is also required.

We also showed that *MpBHY* and *MpCYP97C* function together to produce lutein in the liverwort, whereas in higher plants *CYP97C* functions mainly along with *CYP97A*, not with *BHY* (Kim and DellaPenna 2006; Tian et al. 2003; Quinlan et al. 2012). In the liverwort, *CYP97A* might function in the different pathway. It is possible that *MpCYP97A* needs some factor which is absent in *E. coli* to exert its activity. Since we showed that *AtATR2* conferred

no effect on MpCYP97A activity, liverwort MpCYP97A is unlikely to be involved in carotenoid biosynthesis. It needs to be determined what function MpCYP97A has in the liverwort.

Among the CYP97 family, CYP97A and CYP97C are involved in carotenoid biosynthesis in higher plants, whereas the function of CYP97B has not yet been well clarified except for two reports. One report has shown that *Arabidopsis* CYP97B3 has the activity of β -carotenoid hydroxylase (Kim et al. 2010). Other has shown that *Porphyra* PuCHY₁ has not only a β -carotenoid hydroxylation activity but also a possible ϵ -carotenoid hydroxylation activity (Yang et al. 2014). We also investigated whether liverwort CYP97B (MpCYP97B) was able to hydroxylate β -carotene or α -carotene, and it was found to have no activity as carotene hydroxylase (Supplementary Fig. S6).

In this study, we determined the carotenoid hydroxylase activities of each gene product using *E. coli* cells. Since it is important to know the physiological functions of them in the liverwort, we plan on analyzing the functions by constructing a transgenic liverwort.

From an evolutionary point of view, in the early plants, *BHY* (*BCH*) genes were mainly involved in β -ring hydroxylation of both β - and α -carotenes to synthesize zeaxanthin and zeinoxanthin, respectively. These plants also possessed *CYP97C* genes, which hydroxylate the ϵ -ring of zeinoxanthin to produce lutein. On the other hand, there were *CYP97A* genes in the early land plants; however, they did not function for carotenoid biosynthesis. It is interesting to know when CYP97A acquired activity as a β -carotene hydroxylase, and was replaced partially with *BHY*. The functional analysis of carotenoid hydroxylase genes from divergent plants, including algae, bryophyte, spermatophytes and so on, should reply to this question.

By the combination of carotenoid biosynthesis genes derived from different organisms, the production of novel and rare carotenoids has been successful, as noted in the Introduction. Transplastomic tobacco and lettuce plants, in which *Brevundimonas* sp. SD212 *crtZ* and *crtW* genes were introduced into their plastids, have been constructed (Hasunuma et al. 2008; Harada et al. 2014). In these plants, astaxanthin and friteschiellaxanthin (4-keto-lutein) were produced from zeaxanthin and lutein, respectively. However, α -echinenone and 4-ketozeinoxanthin that should be derived from α -carotene and zeinoxanthin, respectively, were not found in the transplastomic plants. This may be because α -carotene and zeinoxanthin were converted rapidly to lutein in the plastids, and/or because the activity of *CrtZ* (and *BHY*) is stronger than that of *CrtW*. In contrast, α -echinenone (4-keto- α -carotene) was produced in transgenic rice callus expressing the *Zmpsy*, *crtI* (*PacrtI*), and *crtW* (*BrcrtW*) (Breitenbach et al. 2014). In this case, *CrtW*

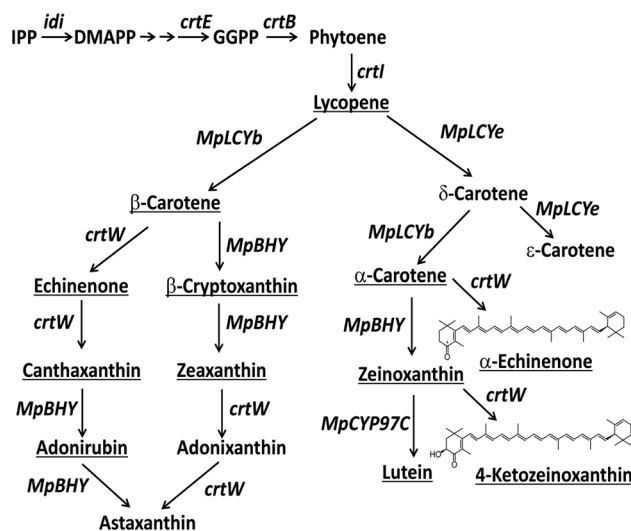


Fig. 7 Pathway of carotenoid biosynthesis in transformed *E. coli*

may show a higher activity than the native *BHY*, so that the majority of α -carotene could be converted to α -echinenone but not to lutein. We generated recombinant *E. coli* cells, which produced rare and novel carotenoids such as α -echinenone and 4-ketozeinoxanthin, through pathway engineering using bacterial carotenogenic genes that include *Brevundimonas* sp. SD212 *crtW*, in addition to the liverwort *MpLCYb*, *MpLCYe* and *MpBHY* genes (Fig. 7).

Our results indicate the feasibility of producing novel or rare carotenoids, which have not yet or hardly been produced in any hosts, in *E. coli* by appropriate combinations of carotenogenic genes derived from various organisms.

Acknowledgments The authors thank Dr. Hiroshi Shimada, Hiroshima University, for the construction of plasmid pACHP-Beta. We also thank Dr. Takayuki Kohchi, Kyoto University; Dr. Katsuyuki T. Yamato, Kinki University and Dr. Kimitsune Ishizaki, Kobe University for their assistance in searching liverwort sequences.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Albrecht M, Takaichi S, Misawa N, Schnurr G, Böger P, Sandmann G (1997) Synthesis of atypical cyclic and acyclic hydroxy carotenoids in *Escherichia coli* transformants. *J Biotechnol* 58:177–185
- Bak S, Beisson F, Bishop G, Hamberger B, Höfer R, Paquette S, Werck-Reichhart D (2011) Cytochromes P450. *Arabidopsis Book*. doi:10.1199/tab.0144
- Breitenbach J, Bai C, Rivera SM, Canela R, Capell T, Christou P, Zhu C, Sandmann G (2014) A novel carotenoid, 4-keto- α -carotene, as an unexpected by-product during genetic engineering of carotenogenesis in rice callus. *Phytochemistry* 98:85–91

- Britton G, Liaaen-Jensen S, Pfander H (2004) Carotenoids handbook. Birkhäuser Verlag, Basel
- Castenmiller JJ, West CE (1998) Bioavailability and bioconversion of carotenoids. *Annu Rev Nutr* 18:19–38
- Chapple C (1998) Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. *Annu Rev Plant Physiol Plant Mol Biol* 49:311–343
- Christopher MJ, Waterman MR (1998) NADPH-flavodoxin reductase and flavodoxin from *Escherichia coli*: characteristics as a soluble microsomal P450 reductase. *Biochemistry* 37:6106–6113
- Cui H, Yu X, Wang Y, Cui Y, Li X, Liu Z, Qin S (2013) Evolutionary origins, molecular cloning and expression of carotenoid hydroxylases in eukaryotic photosynthetic algae. *BMC Genom* 14:457–476
- Cunningham FX Jr, Chamovitz D, Misawa N, Gantt E, Hirschberg J (1993) Cloning and functional expression in *Escherichia coli* of a cyanobacterial gene for lycopene cyclase, the enzyme that catalyzes the biosynthesis of β -carotene. *FEBS Lett* 328:130–138
- Dall'Osto L, Fiore A, Cazzaniga S, Giuliano G, Bassi R (2007) Different roles of α - and β -branch xanthophylls in photosystem assembly and photoprotection. *J Biol Chem* 282:35056–35068
- Das A, Yoon S-H, Lee S-H, Kim J-Y, Oh D-K, Kim S-W (2007) An update on microbial carotenoid production: application of recent metabolic engineering tools. *Appl Microbiol Biotechnol* 77:505–512
- Fraser PD, Miura Y, Misawa N (1997) *In vitro* characterization of astaxanthin biosynthetic enzymes. *J Biol Chem* 272:6128–6135
- Fraser PD, Elisabete M, Pinto S, Holloway DE, Bramley PM (2000) Application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. *Plant J* 24:551–558
- Galpaz N, Ronen G, Khalfa Z, Zamir D, Hirschberg J (2006) A chromoplast-specific carotenoid biosynthesis pathway is revealed by cloning of the tomato *white-flower* locus. *Plant Cell* 18:1947–1960
- Hannemann F, Bichet A, Ewen KM, Bernhardt R (2007) Cytochrome P450 systems-biological variations of electron transport chains. *Biochim Biophys Acta* 1770:330–344
- Harada H, Shindo K, Iki K, Teraoka A, Okamoto S, Yu F, Hattori J, Utsumi R, Misawa N (2011) Efficient functional analysis system for cyanobacterial or plant cytochromes P450 involved in sesquiterpene biosynthesis. *Appl Microbiol Biotechnol* 90:467–476
- Harada H, Maoka T, Osawa A, Hattori J, Kanamoto H, Shindo K, Otomatsu T, Misawa N (2014) Construction of transplastomic lettuce (*Lactuca sativa*) dominantly producing astaxanthin fatty acid esters and detailed chemical analysis of generated carotenoids. *Transgenic Res* 23:303–315
- Hasunuma T, Miyazawa S, Yoshimura S, Shinzaki Y, Tomizawa K, Shindo K, Choi SK, Misawa N, Miyake C (2008) Biosynthesis of astaxanthin in tobacco leaves by transplastomic engineering. *Plant J* 55:857–868
- Hull AK, Celenza JL (2000) Bacterial expression and purification of the Arabidopsis NADPH-cytochrome P450 reductase ATR2. *Protein Expr Purif* 18:310–315
- Iwamoto T, Hosoda K, Hirano R, Kurata H, Matsumoto A, Miki W, Kamiyama M, Itakura H, Yamamoto S, Kondo K (2000) Inhibition of low-density lipoprotein oxidation by astaxanthin. *J Atheroscler Thromb* 7:216–222
- Kajiwara S, Fraser PD, Kondo K, Misawa N (1997) Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. *Biochem J* 324:421–426
- Kim J, DellaPenna D (2006) Defining the primary route for lutein synthesis in plants: the role of *Arabidopsis* carotenoid β -ring hydroxylase CYP97A3. *Proc Natl Acad Sci* 103:3474–3479
- Kim I-J, Ko K-C, Kim C-S, Chung W-I (2001) Isolation and characterization of cDNAs encoding β -carotene hydroxylase in *Citrus*. *Plant Sci* 161:1005–1010
- Kim J, Smith JJ, Tian L, DellaPenna D (2009) The evolution and function of carotenoid hydroxylases in Arabidopsis. *Plant Cell Physiol* 50:463–479
- Kim J-E, Cheng KM, Craft NE, Hamberger B, Douglas CJ (2010) Over-expression of *Arabidopsis thaliana* carotenoid hydroxylases individually and in combination with a β -carotene ketolase provides insight into in vivo function. *Phytochemistry* 71:168–178
- Krinsky NI, Landrum JT, Bone RA (2003) Biological mechanisms of the protective role of lutein and zeaxanthin in the eye. *Annu Rev Nutr* 23:171–201
- Lee PC, Schmidt-Danner C (2002) Metabolic engineering towards biotechnological production of carotenoids in microorganisms. *Appl Microbiol Biotechnol* 60:1–11
- Li Q, Farre G, Naqvi S, Breitenbach J, Sanahuja G, Bai C, Sandmann G, Capell T, Christou P, Zhu C (2010) Cloning and functional characterization of the maize carotenoid isomerase and β -carotene hydroxylase genes and their regulation during endosperm maturation. *Transgenic Res* 19:1053–1068
- Lv M-Z, Chao D-Y, Shan J-X, Zhu M-Z, Shi M, Gao J-P, Lin H-X (2012) Rice carotenoid β -ring hydroxylase CYP97A4 is involved in lutein biosynthesis. *Plant Cell Physiol* 53:987–1002
- Maoka T, Takemura M, Tokuda H, Suzuki N, Misawa N (2014) 4-Ketozeinoxanthin, a novel carotenoid produced in *Escherichia coli* through metabolic engineering using carotenogenic genes of bacterium and liverwort. *Tetrahedron Lett* 55:6708–6710
- Mayne ST (1996) Beta-carotene, carotenoids, and disease prevention in humans. *FASEB J* 10:690–701
- Misawa N (2011) Pathway engineering for functional isoprenoids. *Curr Opin Biotechnol* 22:627–633
- Misawa N, Nakagawa M, Kobayashi K, Yamano S, Izawa Y, Nakamura K, Harashima K (1990) Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. *J Bacteriol* 172:6704–6712
- Misawa N, Satomi Y, Kondo K, Yokoyama A, Kajiwarra S, Saito T, Ohtani T, Miki W (1995) Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. *J Bacteriol* 177:6575–6584
- Moore RC, Purugganan MD (2005) The evolutionary dynamics of plant duplicate genes. *Curr Opin Plant Biol* 8:122–128
- Nishida Y, Adachi K, Kasai H, Shizuri Y, Shindo K, Sawabe A, Komemushi S, Miki W, Misawa N (2005) Elucidation of a carotenoid biosynthesis gene cluster encoding a novel enzyme, 2,2'- β -hydroxylase, from *Brevundimonas* sp. strain SD212 and combinatorial biosynthesis of new or rare xanthophylls. *Appl Environ Microbiol* 71:4286–4296
- Nishino H, Tokuda H, Murakoshi M, Satomi Y, Masuda M, Onozuka M, Yamaguchi S, Takayasu J, Tsuruta J, Okuda M, Khachik F, Narisawa T, Takasuka N, Yano M (2000) Cancer prevention by natural carotenoids. *BioFactors* 13:89–94
- Niyogi KK, Shih C, Soon Chow W, Pogson BJ, DellaPenna D, Björkman O (2001) Photoprotection in a zeaxanthin- and lutein- deficient double mutant of *Arabidopsis*. *Photosynth Res* 67:139–145
- Qin X, Zhang W, Dubcovsky J, Tian L (2012) Cloning and comparative analysis of carotenoid β -hydroxylase genes provides new insights into carotenoid metabolism in tetraploid

- (*Triticum turgidum* ssp. *durum*) and hexaploid (*Triticum aestivum*) wheat grains. *Plant Mol Biol* 80:631–646
- Quinlan RF, Jaradat TT, Wurtzel ET (2007) *Escherichia coli* as a platform for functional expression of plant P450 carotene hydroxylases. *Arch Biochem Biophys* 458:146–157
- Quinlan RF, Shumskaya M, Bradbury LMT, Beltrán J, Ma C, Kennelly EJ, Wurtzel ET (2012) Synergistic interactions between carotene ring hydroxylases drive lutein formation in plant carotenoid biosynthesis. *Plant Physiol* 160:204–214
- Ruther A, Misawa N, Böger P, Sandman G (1997) Production of zeaxanthin in *Escherichia coli* transformed with different carotenogenic plasmids. *Appl Microbiol Biotechnol* 48:162–167
- Schückel J, Rylott EL, Grogan G, Bruce NC (2012) A gene-fusion approach to enabling plant cytochromes p450 for biocatalysis. *Chem BioChem* 13:2758–2763
- Shindo K, Hasunuma T, Asagi E, Sano A, Hotta E, Minemura N, Miyake C, Maoka T, Misawa N (2008) 4-Ketoantheraxanthin, a novel carotenoid produced by the combination of the bacterial enzyme β -carotene ketolase CrtW and endogenous carotenoid biosynthetic enzymes in higher plants. *Tetrahedron Lett* 49:3294–3296
- Stigliani AL, Giorio G, D'Ambrosio C (2011) Characterization of P450 carotenoid β - and ϵ -hydroxylases of tomato and transcriptional regulation of xanthophyll biosynthesis in root, leaf, petal and fruit. *Plant Cell Physiol* 52:851–865
- Sugiura M, Nakamura M, Ogawa K, Ikoma M, Yano N (2012) High serum carotenoids associated with lower risk for bone loss and osteoporosis in post-menopausal Japanese female subjects: prospective cohort study. *PLoS One* 7:e52643
- Sun Z, Gantt E, Cunningham FX Jr (1996) Cloning and functional analysis of the β -carotene hydroxylase of *Arabidopsis thaliana*. *J Biol Chem* 271:24349–24352
- Takaichi S (2011) Carotenoids in algae: distributions, biosyntheses and functions. *Mar Drugs* 9:1101–1118
- Takemura M, Maoka T, Misawa N (2014) Carotenoid analysis of a liverwort *Marchantia polymorpha* and functional identification of its lycopene β - and ϵ -cyclase genes. *Plant Cell Physiol* 55:194–200
- Talegawkar SA, Johnson EJ, Carithers TC, Taylor HA Jr, Bogle ML, Tucker KL (2008) Carotenoid intakes, assessed by food-frequency questionnaires (FFQs), are associated with serum carotenoid concentrations in the Jackson Heart Study: validation of the Jackson Heart Study Delta NIRI Adult FFQs. *Public Health Nutr* 11:989–997
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30:2725–2729
- Tian L, DellaPenna D (2001) Characterization of a second carotenoid β -hydroxylase gene from *Arabidopsis* and its relationship to the *LUT1* locus. *Plant Mol Biol* 47:379–388
- Tian L, Magallanes-Lundback M, Musetti V, DellaPenna D (2003) Functional analysis of β - and ϵ -ring carotenoid hydroxylases in *Arabidopsis*. *Plant Cell* 15:1320–1332
- Tian L, Musetti V, Kim J, Magallanes-Lundback M, DellaPenna D (2004) The *Arabidopsis* *LUT1* locus encodes a member of the cytochrome P450 family that is required for carotenoid ϵ -ring hydroxylation activity. *Proc Natl Acad Sci* 101:402–407
- Urban P, Mignotte C, Kazmaier M, Delorme F, Pompon D (1997) Cloning, yeast expression, and characterization of the coupling of two distantly related *Arabidopsis thaliana* NADPH-cytochrome P450 reductases with P450 CYP73A5. *J Biol Chem* 272:19176–19186
- Vallabhaneni R, Gallagher CE, Licciardello N, Cuttriss AJ, Quinlan RF, Wurtzel ET (2009) Metabolite sorting of a germplasm collection reveals the *Hydroxylase3* locus as a new target for maize provitamin A biofortification. *Plant Physiol* 151:1635–1645
- Yang L-E, Huang X-Q, Hang Y, Deng Y-Y, Lu Q-Q, Lu S (2014) The P₄₅₀-type carotene hydroxylase PuCHY₁ from *Porphyra* suggests the evolution of carotenoid metabolism in red algae. *J Integr Plant Biol* 56:902–915
- Ye VM, Bhatia SK (2012) Pathway engineering strategies for production of beneficial carotenoids in microbial hosts. *Biotechnol Lett* 34:1405–1414
- Yokoyama A, Miki W (1995) Composition and presumed biosynthetic pathway of carotenoids in the astaxanthin-producing bacterium *Agrobacterium aurantiacum*. *FEMS Microbiol Lett* 128:139–144
- Yokoyama A, Shizuri Y, Misawa N (1998) Production of new carotenoids, astaxanthin glucosides, by *Escherichia coli* transformants carrying carotenoid biosynthetic genes. *Tetrahedron Lett* 39:3709–3712