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Research review paper

Progress on molecular breeding and metabolic engineering of biosynthesis pathways of C_{30}, C_{35}, C_{40}, C_{45}, C_{50} carotenoids

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Abstract

At least 700 natural carotenoids have been characterized; they can be classified into C_{30}, C_{40} and C_{50} subfamilies. The first step of C_{40} pathway is the combination of two molecules of geranylgeranyl pyrophosphate to synthesize phytoene by phytoene synthase (CrtB or PSY). Most natural carotenoids originate from different types and levels of desaturation by phytoene desaturase (CrtI or PDS+ZDS), cyclization by lycopene cyclase (CrtY or LYC) and other modifications by different modifying enzyme (CrtA, CrtU, CrtZ or BCH, CrtX, CrtO, etc.) of this C_{40} backbone. The first step of C_{30} pathway is the combination of two molecules of FDP to synthesize diapophytoene by diapophytoene synthase (CrtM). But natural C_{30} pathway only goes through a few steps of desaturation to form diaponeurosporene by diapophytoene desaturase (CrtN). Natural C_{50} carotenoid decaprenoxanthin is synthesized starting from the C_{40} carotenoid lycopene by the addition of 2 C_5 units. Concerned the importance of carotenoids, more and more attention has been concentrated on achieving novel carotenoids. The method being used successfully is to construct carotenoids biosynthesis pathways by metabolic engineering. The strategy of metabolic engineering is to engineer a small number of stringent upstream enzymes (CrtB, CrtI, CrtY, CrtM, or CrtN), then use a lot of promiscuous downstream enzymes to obtain large number of novel carotenoids. Two key enzymes phytoene desaturase ($CrtI_m$) and lycopene cyclase ($CrtY_m$) have been modified and used with a series of downstream modifying enzymes with broad substrate specificity, such as monooxygenase (CrtA), carotene desaturase (CrtU), carotene hydroxylase (CrtZ), zeaxanthin glycosylase (CrtX) and carotene ketolase (CrtO) to extend successfully natural C_{30} and C_{40} pathways in *E. coli*. Existing C_{30} synthase CrtM to synthesize carotenoids with different chain length have been engineered and a series of novel carotenoids have been achieved using downstream modifying enzymes. C_{35} carotenoid biosynthesis pathway has been constructed in *E. coli* as described. C_{45} and C_{50} carotenoid biosynthesis pathways have also been constructed in *E. coli*, but it is still necessary to extend these two pathways. Those novel acyclic or cyclic carotenoids have a potential ability to protect against photooxidation and radical-mediated peroxidation reactions which makes them interesting pharmaceutical candidates.

Keywords: Natural carotenoids; Unnatural carotenoids; Extended pathways; Constructed pathways; CrtM

Contents

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1. Introduction

At least 700 carotenoids (Hornero-Me'ndez and Britton, 2002) have been characterized from the two known C_{30} and C_{40} carotenoid biosynthetic pathways. Most of them are distributed in the C_{40} pathway, which is found in thousands of plant and microbial species. In this pathway, two molecules of geranylgeranyl pyrophosphate (GGDP) (C_{20}PP) are condensed to synthesize phytoene, phytoene is transformed into lycopene through a few steps of desaturation, and β-carotene is formed by lycopene through cyclization. The typical structure of C_{40} carotenoids is cyclic β-ionone end group, which can be substituted by oxo, hydroxy and epoxy groups at different positions (Olson and Krinsky, 1995). Our laboratory had cloned and analyzed the sequences of phytoene synthase (PSY) (Yan et al., 2005) and phytoene desaturase (PDS) (Zhu et al., 2005) from *Dunaliella salina*. The lycopene cyclase (LCY) from the algae is also being cloned and sequenced. C_{30} pathway is known in only a few bacterial species, such as those of *Staphylococcus* and *Heliobacterium* (Takaichi et al., 1997). In this pathway, two molecules of farnesyl pyrophosphate (FDP) (C_{15}PP) undergo condensation to form diapophytoene (dehydrosqualene), diapophytoene is transformed into diaponeurosporene through a few steps of desaturation. Except for C_{30} and C_{40} pathways, there are few bacteria (such as *Corynebacterium* and *Halobacterium* spp.) which are known to accumulate C_{50} carotenoids (e.g. decaprenoxanthin), but these longer-chain structures are synthesized starting from the C_{40} carotenoid lycopene by the addition of 2 C_5 (isoprene) units (Krubasik et al., 2001) (Fig. 1).

The most important biological function of carotenoids is as antioxidants owing to their potential to inactivate singlet oxygen and to quench carboxy radicals (Britton, 1995; Van den Berg et al., 2000). More and more evidences are being accumulated to show that carotenoids play an important role in human health. A number of epidemiological studies have revealed that an increased consumption of a diet rich in carotenoids is correlated with a diminished risk for various types of cancer, cardiovascular or ophthalmological diseases (Mayne, 1996). Carotenoids with unsubstituted β-ionone end groups are precursors of vitamin A. Vitamin A may inhibit tumor promotion, carotenoids are also protective because of their antioxidant effects to prevent cells and tissues from oxidative damage (Stahl and Sies, 2003). Carotenoids also influence cellular signaling and may trigger redox-sensitive regulatory pathways (Stahl et al., 2002). Their value in medical and pharmaceutics has triggered an increased interest in the synthesis of new carotenoid structures and the economic production of compounds in engineered cells.

Secondary metabolic pathways appear to be able to explore new chemical structures at minimal cost (Firn and Jones, 2000). Many secondary pathways have a "reverse tree" topology, where the backbone structures of metabolites are dictated by a small number of stringent, upstream enzymes (Sacchettini and Poulter, 1997). Key to promoting facile exploration is the extensive use of promiscuous enzymes that can accept

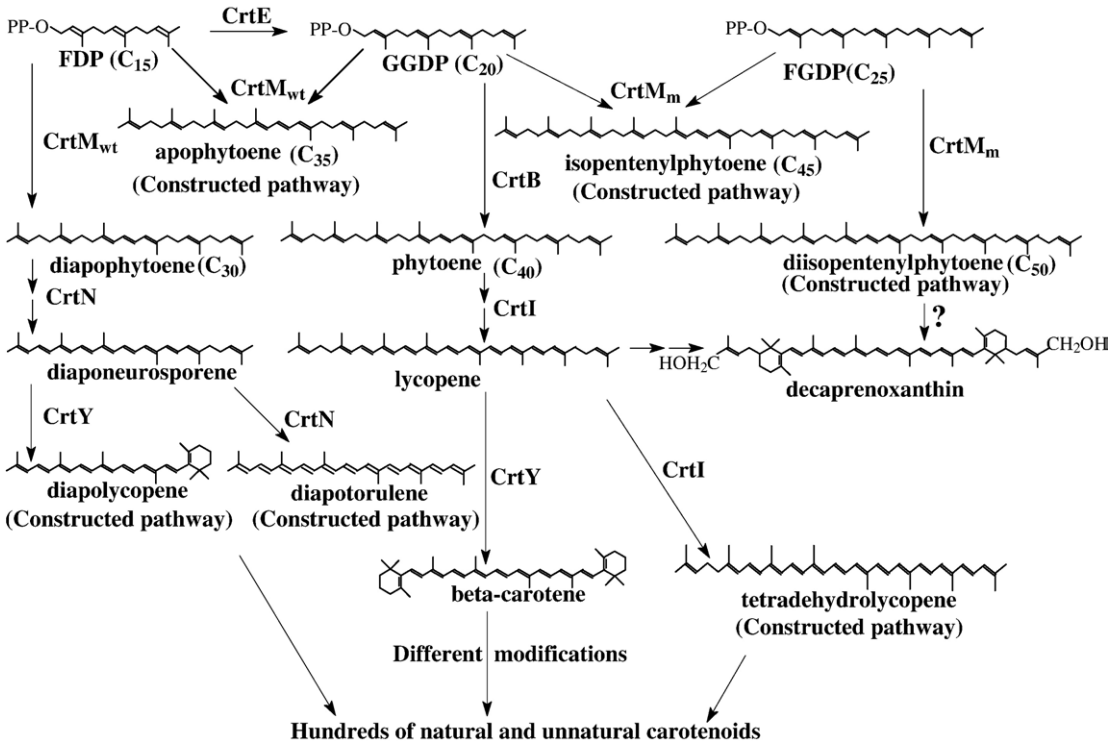

Fig. 1. Natural and unnatural pathways for carotenoid biosynthesis. In addition to natural C_{30}, C_{40} and C_{50} carotenoid pathways, C_{30} and C_{40} carotenoid pathways have been extended in *E. coli*. Unnatural C_{35}, C_{45}, and C_{50} carotenoid pathways also have been constructed in *E. coli*. CrtE, GGDP synthase; $CrtM_{wt}$, wild type diapophytoene synthase; $CrtM_m$, mutant diapophytoene synthase; CrtB, phytoene synthase; CrtN, diapophytoene desaturase; CrtI, phytoene desaturase, CrtY, lycopene cyclase.

a variety of substrates. Thus, once a new compound is produced, as a result of either enzyme recruitment or mutation in an existing enzyme, it is further metabolized by downstream modifying enzymes, thereby allowing a series of novel compounds to emerge.

In recent years, researchers have constructed several biosynthesis pathways of carotenoids in *E. coli* which don't exist in nature. Some of them have used mutant of two modifying enzymes and wild type of other downstream modifying enzymes in C_{30} and C_{40} pathways to extend those natural pathways (Lee et al., 2003). Through this kind of construction, they have achieved a series of novel carotenoids with C_{30} and C_{40} backbone (e.g. diapolycopene and diapotorulene) and proved that these modifying enzymes have broad substrate specificity. The others have engineered existing C_{30} carotenoid synthase (CrtM) and used its mutant to incorporate other carotenoids with different chain length, such as apophytoene (C_{35}) (Umeno and Arnold, 2003), isopentenylphytoene (C_{45}) and diisopentenylphytoene (C_{50}) (Umeno and Arnold, 2004). After this kind of construction, a series of novel carotenoids with C_{35}, C_{45} and C_{50} backbone can be achieved using downstream modifying enzymes which have been proved to have broad substrate specificity. But it is still a question whether the constructed carotenoid diisopentenylphytoene can be transformed into decaprenoxanthin through direct desaturations and cyclization (Fig. 1).

We have made this review to summary the progress on molecular breeding and metabolic engineering of biosynthesis pathways of C_{30}, C_{35}, C_{40}, C_{45}, C_{50} carotenoids. In the following text, we'll describe more detailed these natural and unnatural pathways constructed in *E. coli*.

2. Natural pathways of C_{30} and C_{40} carotenoids

The genes of most enzymes existing in natural C_{40} carotenoids biosynthesis pathway of both microbial resources and cyanobacterial/plant are listed in Table 1. The products of the extension of isopentenyl disphosphate (IDP) by the enzymes' farnesyl pyrophosphate synthase (FDS) and GGDP synthase (CrtE in microorganisms or GGPS in plant) are FDP and GGDP respectively. The first committed step in C_{40} carotenoid biosynthesis is the combination of two molecules of GGDP to form the colorless carotenoid

Table 1
Genes coding for C_{40} carotenoids biosynthesis enzymes

Carotenoids biosynthesis enzymes	Genes	Organisms	Accession number
Assembly of carotenoid backbone			
GGPP synthase	*crtE*	*Erwinia uredevora*	D90087
	crtE	*Synechocystis* PCC6803	D90899
	al-3	*Neurospora crassa*	X53979
	Ggps	*Arabidopsis thaliana*	L25813
	Ggps	*Capsicum annuum*	P80042
Phytoene synthase	*crtB*	*Agrobacterium aurantiacum*	D58420
	crtB	*Synechocystis* PCC6803	X69172
	al-2	*N. crassa*	L27652
	Psy	*A. thaliana*	L25812
	Psy	*Narcissus pseudonarcissus*	X78814
	Psy1	*Lycopersicon esculentum*	A21360
	Psy2	*L. esculentum*	L23424
Biosynthesis of acyclic carotenoids			
Phytoene desaturase	*Pds*	*A. thaliana*	L16237
	Pds	*Zea mays*	L39266
	Pds	*N. pseudonarcissus*	X78815
	Pds	*L. esculentum*	M88683
	crtP	*Synechocystis* PCC6803	X62574
	crtI	*Rhodobacter capsulatus*	Z11165
	crtI	*E. uredevora*	D90087
	al-1	*N. crassa*	M57465
ζ-carotene desaturase	*crtQ*	*Synechocystis* PCC6803	X62574
	Zds	*A. thaliana*	U38550
	Zds	*C. annuum*	X68058
Hydroxyneurosporene synthase	*crtC*	*Rhodobacter sphaeroides*	X82458
Methoxyneurosporene desaturase	*crtD*	*R. capsulatus*	Z11165
Spheroidene monooxygenase	*crtA*	*R. capsulatus*	Z11165
Biosynthesis of cyclic carotenoids			
Lycopene-β-cyclase	*crtY*	*E. uredevora*	D90087
	crtY	*Synechocystis* PCC6803	X74599
	β-Lyc	*A. thaliana*	Z29211
Lycopene-ε-cyclase	ε-Lyc	*A. thaliana*	U50738
β-carotene hydroxylase	*crtZ*	*A. aurantiacum*	D58420
	Bch	*A. thaliana*	U58919
	Bch	*C. annuum*	Y09225
Zeaxanthin glucosylase	*crtX*	*E. uredevora*	M87280
β-carotene C(4) oxygenase	*crtW*	*A. aurantiacum*	D58420
	crtW	*Alcaligenes PC1*	D58422
	crtO	*Synechocystis* PCC6803	D64004
Zeaxanthin epoxidase	*Zep1*	*A. thaliana*	T45502
	Zep1	*C. annuum*	X91491
Violaxanthin deepoxidase	*Vde1*	*A. thaliana*	N37612
Violaxanthin cleavage	*Vpl4*	*Z. mays*	U95953
Capsanthin/capsorubin synthase	*Ccs*	*C. annuum*	X77289
β-carotene desaturase	*crtU*	*Streptomyces griseus*	X95596

phytoene by the enzyme phytoene synthase (CrtB in microorganisms or PSY in cyanobacterial/plant). The introduction of additional double bonds into phytoene by the enzyme phytoene desaturase (CrtI in microorganisms or PDS + ZDS in cyanobacterial/plant) produces the colored carotenoids ζ-carotene (two desaturations), neurosporene (three desaturations) or lycopene (four desaturations). The desaturations in microorganisms are all catalyzed by CrtI, but the first two steps of desaturation are catalyzed by PDS and the last two by ZDS in cyanobacterial/plant. Lycopene cyclase (CrtY in microorganisms or LYC in cyanobacterial/plant) catalyzes the introduction of rings into either end of lycopene to synthesize β-carotene (Britton, 1998). Then carotene desaturase (CrtU in microorganisms) catalyzes the desaturation of β-carotene to form isorenariatene, carotene hydroxylase (CrtZ in microorganisms or BCH cyanobacterial/plant) and zeaxanthin glycosylase (CrtX in microorganisms) catalyzes the hydroxylation and glycosylation of β-carotene to form zeaxanthin and zeaxanthin diglucoside, carotene ketolase (CrtO in microorganisms) catalyzes the ketonization of β-carotene to form cantaxantion, etc. (Fig. 2).

Diverse acyclic C_{30} diapocarotenoids are synthesized by several nonphototrophic bacteria such as *Staphylococcus*, *Streptococcus*, and *Methylobacterium* species (Taylor, 1984). The combination of two molecules of FDP forms diapophytoene by CrtM. The introduction of additional double bonds into diapophytoene by the enzyme diapophytoene desaturase (CrtN) produces the yellow diaponeurosporene through diapophytoene and diapo-ζ-carotene (Fig. 2) (Wieland et al., 1994).

A number of genes encoding the enzymes for central carotenoid biosynthetic routes have been cloned, and genes from different species have been shown to function cooperatively when combined (Lee and Schmidt-Dannert, 2002). Enzymes catalyzing the synthesis and desaturation of acyclic C_{30} and C_{40} carotenoid backbones and initial cyclization reactions of C_{40} carotenoids appear well conserved in carotenoid-producing organisms. Less is known about the enzymes responsible for

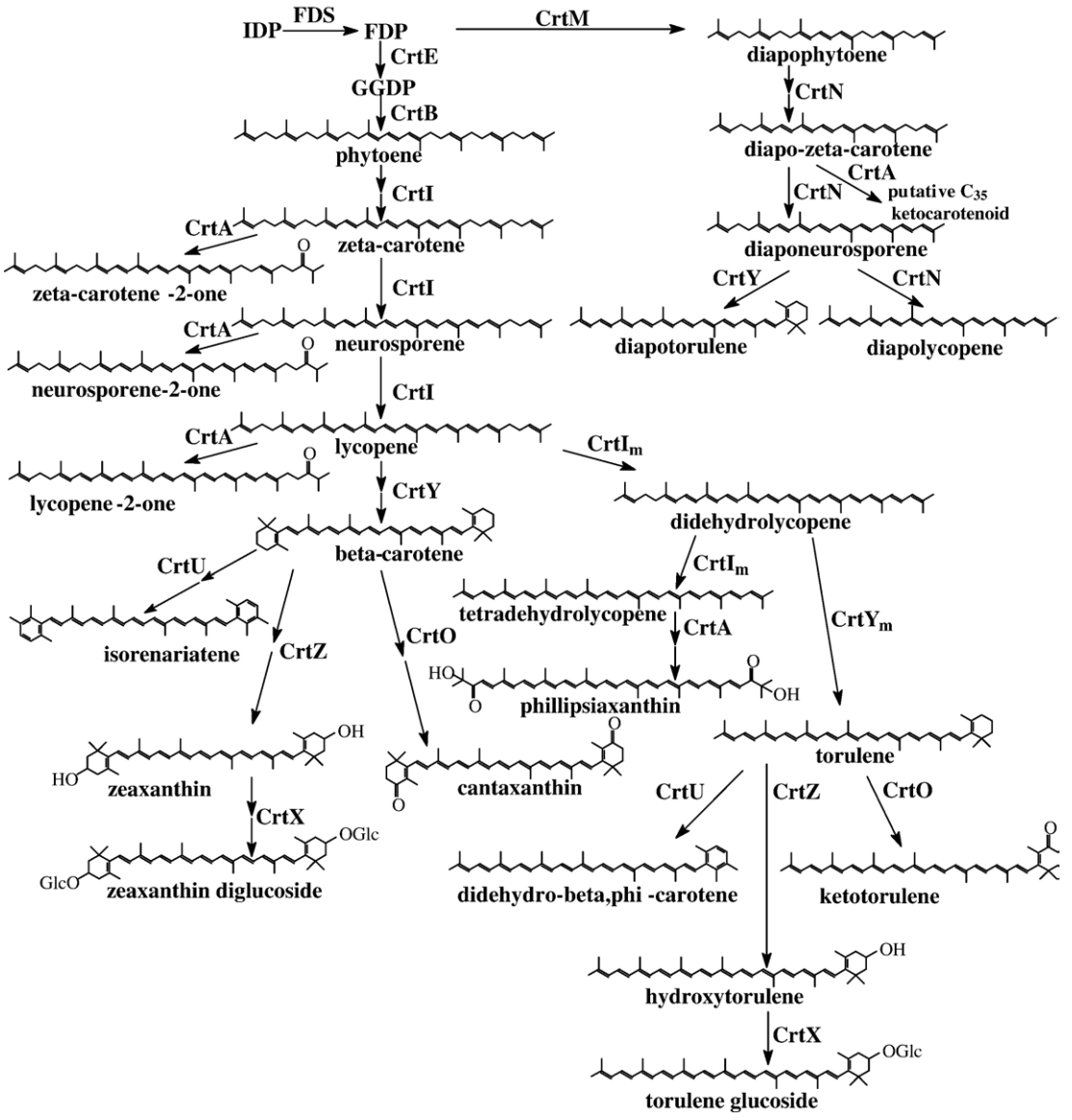

Fig. 2. Biosynthetic routes to different acyclic and cyclic C_{40} and C_{30} carotenoids in engineered *E. coli*. In addition to natural C_{30} and C_{40} carotenoid pathways, extended C_{30} and C_{40} carotenoid pathways have been constructed in *E. coli*. FDS, pyrophosphate synthase; $CrtI_m$, mutant phytoene desaturase; $CrtY_m$, mutant lycopene cyclase; CrtA, monooxygenase; CrtO, carotene ketolase; CrtU, carotene desaturase; CrtZ, carotene hydroxylase; CrtX, zeaxanthin glycosylase. (Adapted from Lee et al., 2003).

the additional structural modifications that contribute to the tremendous diversity of natural carotenoids (Straub, 1987). However, a number of these modifying enzymes have been identified and characterized from microbial sources including monooxygenase (CrtA) from *Rhodobacter capsulatus* (Armstrong et al., 1989); CrtO from *Synechocystis* sp. (Fernandez-Gonzalez et al., 1997); CrtU from *Streptomyces griseus*, *Brevibacterioum linens*, and *Mycobacterium aurum* (Krugel et al., 1999; Krubasik and Sandmann, 2000; Viveiros et al., 2000); and CrtZ and CrtX from *Erwinia herbicola* (Hundle et al., 1994).

3. Extended pathways of C_{30} and C_{40} carotenoids

Some researchers have constructed a six-step phytoene desaturase ($CrtI_m$) which is capable of synthesizing the fully conjugated tetradehydrolycopene in *E. coli* (Fig. 2) (Schmidt-Dannert et al., 2000). They also have modified CrtY by directed evolution to generate $CrtY_m$, a variant that

cyclizes didehydrolycopene, the precursor of tetradehydrolycopene, to produce the red carotenoid torulene in *E. coli* (Fig. 2).

Other researchers sought to probe the catalytic promiscuity of carotenoid-modifying enzymes toward new carotenoid substrates synthesized by in vitro evolved biosynthetic routes. They reported on the successful extension of the in vitro evolved tetradehydrolycopene and torulene pathways as well as the wild type lycopene, β-carotene, and diaponeurosporene (a C_{30} carotenoid) pathways with carotenoid-modifying genes to produce structurally novel carotenoids in *E. coli* (Fig. 2) (Lee et al., 2003).

3.1. Extended pathways of C_{40} carotenoids

3.1.1. Oxygenation

In order to generate new, acyclic, purple C_{40} xanthophylls in *E. coli* from the wild-type lycopene and in vitro evolved tetradehydrolycopene biosynthetic pathways, Lee et al. (2003) applied CrtA to introduce keto groups.

Extension of the lycopene pathway by coexpression of pUC-*crtA* with pAC-*crtE–crtB–crtI* in *E. coli* resulted in the synthesis of three novel acyclic xanthophylls: æ-carotene-2-one, neurosporene-2-one, and lycopene-2-one (Fig. 2) through the identification of absorption maxima, polarity, and mass fragmentation spectrum (Enzell et al., 1968, 1969).

Extension of the tetradehydrolycopene pathway by coexpression of pUC-*crtA* with pAC-*crtE–crtB–crtI*$_m$ in *E. coli* resulted in the synthesis of a deep purple cartotenoid, phillipsiaxanthin (Schmidt-Dannert et al., 2000). Phillipsiaxanthin shows absorption characteristics of lycopene and neurosporene but with masses corresponding to the respective diketo- and dihydroxy-diketo-derivatives (Schwieter et al., 1966). It is the first reported deep purple carotenoid produced in recombinant cells (Fig. 2).

The novel lipophilic carotenoid phillipsiaxanthin may be a powerful cellular antioxidant. Its ability to protect against photooxidation and radical-mediated peroxidation reactions is linked to the length of the conjugated double-bond system and the presence of a single hydroxy group which makes it an interesting pharmaceutical candidate. But its antioxidative activity in a liposome-membrane model system still needs to be determined. Phillipsiaxanthin also can be thought as a potential colorant for its unique purple color.

3.1.2. Ketonization

One branch of extension of the evolved torulene pathway by coexpression of pUC-*crtO* with pAC-*crtE–crtB–crtI*$_m$–*crtY*$_m$ in *E. coli* resulted in the synthesis of a new major carotenoid, 4-keto-torulene, through the identification of absorption maxima, polarity, and mass fragmentation spectrum (Fig. 2) (Fernandez-Gonzalez et al., 1997).

3.1.3. Desaturation

The symmetrical aromatization of β-carotene to isoreneriatene (φ,φ-carotene) by CrtU involves the introduction of two double bonds and a concurrent methyl group shift for each β-ring (Fig. 2) (Krugel et al., 1999).

Another branch of extension of the evolved torulene pathway by coexpression of pUC-*crtU* with pAC-*crtE–crtB–crtI*$_m$–*crtY*$_m$ in *E. coli* resulted in the synthesis of a new major asymmetrical aromatic carotenoid, didehydro-β,φ-carotene, through the identification of absorption maxima, polarity, and mass fragmentation spectrum (Fig. 2) (Lee et al., 2003).

3.1.4. Hydroxylation and glucosylation

CrtZ and CrtX also exhibit the same broad substrate specificities as CrtO and CrtU (Hundle et al., 1994).

Another branch of extension of the evolved torulene pathway by coexpression of pUC-*crtZ* with pAC-*crtE–crtB–crtI*$_m$–*crtY*$_m$ in *E. coli* resulted in the synthesis of a new polar carotenoid, hydroxytorulene, with an absorption spectrum similar to torulene but with a mass spectrum different from torulene (Fig. 2) (Lee et al., 2003).

Subsequent combination in *E. coli* of pAC-*crtE–crtB–crtI14–crtY*$_m$–*crtZ*, together with the terminal enzyme CrtX of the glucosylation pathway expressed on pUC-*crtX*, produces a number of very polar carotenoid structures in *E. coli*.

Neither hydroxytorulene nor its precursor torulene accumulated in *E. coli* cells carrying the assembled torulene glycosylation pathway, but a new carotenoid identified as torulene glucoside was synthesized in addition to different hydroxylated and glycosylated β-carotene derivatives (Fig. 2). The formation of carotenoids where only one-ring is hydroxylated or glycosylated indicates that CrtZ and CrtX catalyze β-ring modification irrespective of the other end structure present in a carotenoid molecule.

3.2. Extended pathways of C_{30} carotenoids

3.2.1. Desaturation

To extend the isoprenoid pathway for synthesis of C_{30} carotenoids, *E. coli* cells were transformed with pAC-*crtM–crtN* (Lee et al., 2003). They developed a

deep yellow-orange color, suggesting the production of diapocarotenoids. Analysis of the cell extracts by HPLC-mass spectrometry showed that CrtN efficiently introduced four double bonds into dehydrosqualene to synthesize the fully conjugated diapolycopene in recombinant *E. coli* instead of the three-step desaturation to form diaponeurosporene in recombinant *E. coli* which was reported earlier (Wieland et al., 1994) (Fig. 2).

E.coli cells harboring pAC-*crtM–crtN* also accumulated significant amounts of polar carotenoids. Molecular masses and absorption spectra showed them to be various diapolycopene and diaponeurosporene derivatives carrying methoxy- and/or hydroxy-functional groups at one or both of their ends. Acyclic end groups of bacterial C_{30} diapocarotenoids are frequently oxidized to hydroxy, aldehyde, or carboxy groups, which can be further acylated and/or glycosylated (Kleinig et al., 1978; Marshall and Willmoth, 1981). Other researchers also reported formation of modified diapocarotenoids in recombinant *E.coli* (Raisig and Sandmann, 2001).

3.2.2. Cyclization

Cyclization of C_{30} diapocarotenoids, which is a common modification of C_{40} carotenoids, is so far unknown. Lycopene cyclase CrtY acts on ψ-end groups (Takaichi et al., 1996), which are the same in acyclic C_{40} carotenoids (e.g., lycopene) and C_{30} carotenoids (e.g., diaponeurosporene or diapocarotene). Extension of the diapolycopene pathway by coexpression of pUC-*crtY* with pAC-*crtM–crtN* in *E. coli* resulted in the synthesis of a novel cyclic carotenoid, diapotorulene, along with diaponeurosporene through the identification of absorption maxima, polarity, and mass fragmentation spectrum (Lee et al., 2003) (Fig. 2). Other possible monocyclic and dicyclic diapocarotenoids derived from diapocarotene were not detected.

3.2.3. Oxygenation

Although many bacteria produce a large number of different acyclic xanthophylls (oxygenated carotenoids), only four genes encoding a hydratase (*crtC*), desaturase (*crtD*), methyl transferase (*crtF*), and *crtA* have been cloned from *Rhodobacter* strains (Armstrong et al., 1989). CrtA was chosen as a possible enzyme for the introduction of keto groups into diapolycopene to obtain acyclic carotenoids with expanded chromophores.

To produce acyclic C_{30} xanthophylls in engineered *E. coli* cells, the diapolycopene pathway was extended in *E. coli* by coexpression of pUC-*crtA* with pAC-*crtM–crtN* (Lee et al., 2003). The cotransformed cells appeared more yellow than *E. coli* pAC-*crtM–crtN*. HPLC analysis of the cell extract showed three new very polar peaks. The absorption maxima and spectral fine structure of the major carotenoid corresponds to an acyclic carotenoid without conjugated carbonyl functions and with eight conjugated double bonds as opposed to the nine conjugated double bonds in diaponeurosporene. The two minor peaks showed spectral properties similar to diapocarotene and diapophytoene. Further structural analysis of the yellow carotenoid by HPLC-mass spectrometry showed that it was a putative C_{35} backbone structure rather than C_{30} (Fig. 2).

As described above, researchers have used the extension of an in vitro evolved metabolic pathway with a functionally diverse array of modifying enzymes to achieve the recombinant production of nine new C_{30} and C_{40} carotenoid structures and a putative C_{35} carotenoid structure in *E. coli*. More importantly, several of the novel carotenoids have never before been isolated in nature (e.g., diapotorulene) or synthesized in engineered cells (e.g., the first deep purple carotenoid phillipsiaxanthin). Furthermore, modifying genes located later in a biosynthetic pathway exhibit a higher catalytic promiscuity than those earlier in the pathway, allowing them to accept unnatural substrates. Therefore, the combination of directed evolution to diverge natural pathways toward new possible metabolic routes and extension of these pathways with additional genes is a powerful approach to discover novel natural and unnatural compounds and produce these compounds in microbial hosts.

4. Natural pathways of C_{50} carotenoids

Researchers used rescued DNA from transposon color mutants of Gram-positive bacterium *Corynebacterium glutamicum* to clone the carotenoid biosynthetic gene cluster (Krubasik et al., 2001). By sequence comparison and functional complementation, the genes involved in the synthesis of C_{50} carotenoids were identified. The genes *crtE*, *crtB*, and *crtI* are responsible for the formation of lycopene. The products of three novel genes *crtYe* and *crtYf*, with sequence similarities to heterodimeric lycopene cyclase *crtYc* and *crtYd*, together with *crtEb* which exhibits a prenyltransferase motif, were involved in the conversion of C_{40} acyclic lycopene to cyclic C_{50} carotenoids.

The formation of cyclic C_{50} carotenoids from lycopene involves two individual steps, *crtEb* and *crtYe*, *crtYf* play different roles in these two steps (Krubasik et al., 2001) (Fig. 3). It was shown that *crtEb* converts lycopene via the

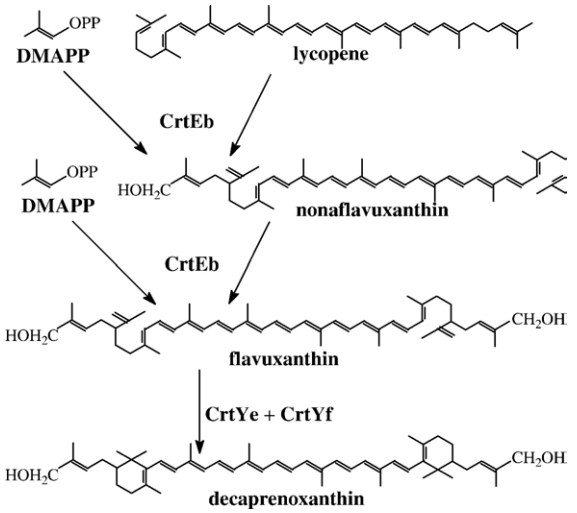

Fig. 3. Biosynthesis pathway and proposed mechanism for the individual steps in the formation of decaprenoxanthin from lycopene (adapted from Krubasik et al., 2001). DMAPP, dimethylallyal pyrophosphate.

C_{45} intermediate nonaflavuxanthin into the acyclic C_{50} carotenoid flavuxanthin by the addition of two C_5 isoprenoid groups. The gene products of *crtYe* and *crtYf* have sequence similarities to a new type of the heterodimeric lycopene cyclase CrtYc and CrtYd found in *B. linens* and *Mycobacteriumaurum*. In contrast with *crtYc* and *crtYd*, the related genes from *C. glutamicum* are not involved in lycopene cyclization but encode the proteins of a carotene cyclase that accepts C_{45} and C_{50} carotenoids as substrates. The reactions catalyzed by CrtYe, CrtYf, and CrtEb lead to the formation of decaprenoxanthin, a C_{50} carotenoid with two ε-ionone rings and its glucosides.

5. Constructed pathways of C_{35}, C_{45} and C_{50} carotenoids

The first committed step in carotenoid biosynthesis is the head-to-head condensation of two prenylpyrophosphates catalyzed by a synthase. For carotenoids, the highly specific synthase reaction appears to be the major point of control over product diversity. As we know, many enzymes in carotenoid pathways have been reported to have broad substrate specificity, the various downstream modification enzymes therefore represent potential targets for generating biosynthetic routes to novel carotenoids (Garcia-Asua et al., 2002).

To create new pathways for the biosynthesis of carotenoids with backbones other than C_{30} and C_{40}, some researchers focused on engineering the carotenoid synthase. Although very little is known of the structure or basis for the specificity of these membrane-associated enzymes, it has been elucidated that the C_{30} synthase CrtM generates diapophytoene in two distinct steps: (i) abstraction of a diphosphate group from a prenyl donor, followed by head-to-head condensation of the donor and acceptor molecules, and (ii) rearrangement of the cyclic intermediate, followed by removal of a second diphosphate and a final carbocation quenching process (Umeno and Arnold, 2004) (Fig. 4). It was reasoned that wild-type CrtM is able to perform the first half-reaction of phytoene (C_{40}) synthesis (condensation of 2 GGPP molecules to form prephytoenediphosphate) but that the reaction is prevented from going to completion by bulky residues which sterically inhibit the second, rearrangement step. When F26, W38 or E180 is replaced with smaller or more flexible amino acids, the reaction can proceed, and phytoene is produced (Umeno and Arnold, 2004).

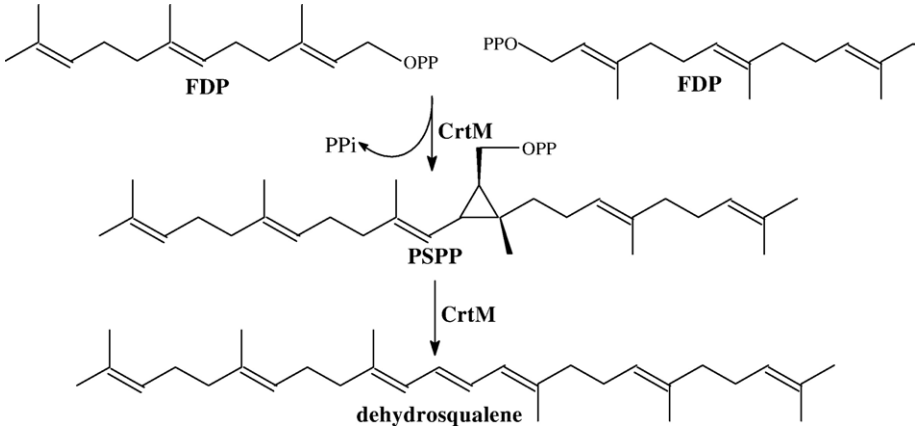

Fig. 4. Reaction schemes for CrtM (adapted from Umeno and Arnold, 2004). PSPP, presqualene pyrophosphate.

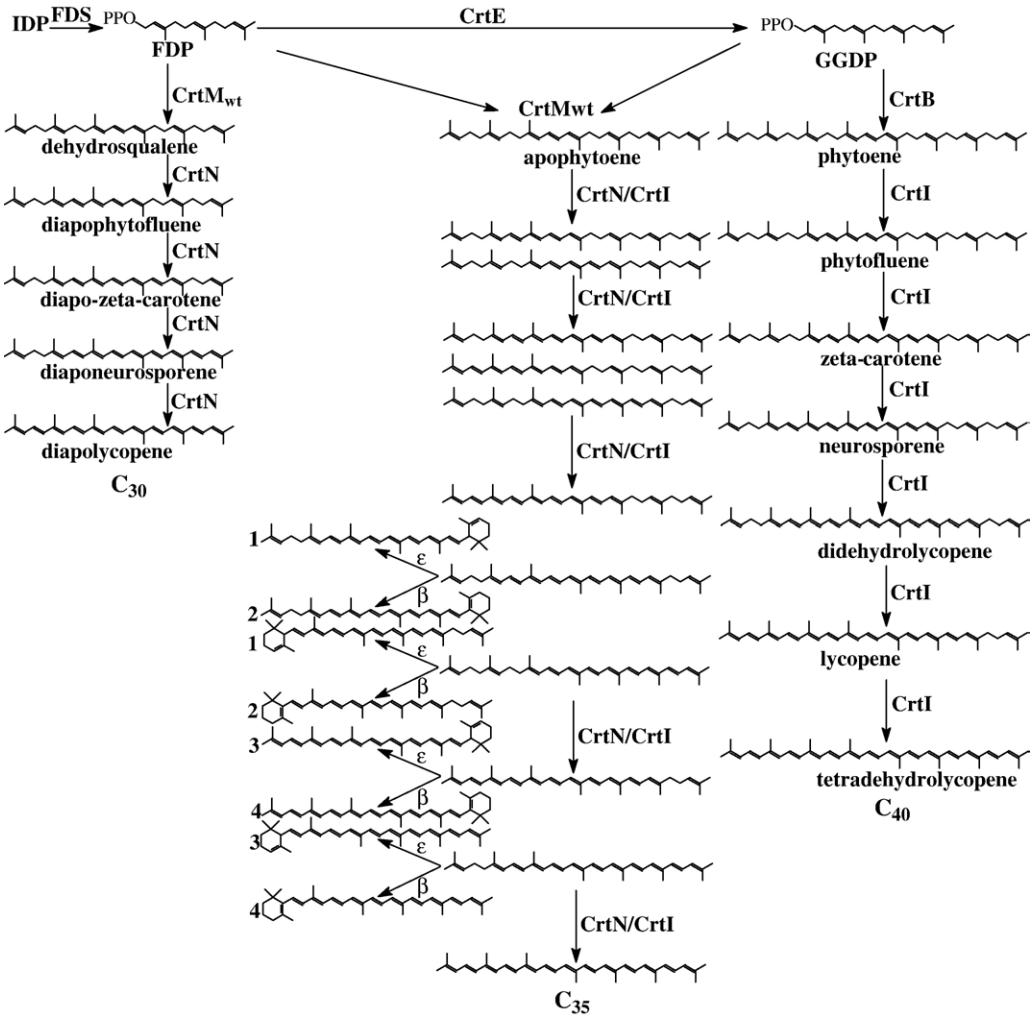

Fig. 5. Three pathways for carotenoid biosynthesis (adapted from Umeno and Arnold, 2003). In addition to natural C_{30} and C_{40} carotenoid pathways, a C_{35} carotenoid pathway has been constructed in *E. coli*. 1, 3 are the constructed novel monocyclic ε-end C_{35} carotenoids. 2, 4 are the constructed novel monocyclic β-end C_{35} carotenoids.

Using random mutagenesis and a functional complementation screen for C_{40} synthase activity, they identified single-amino-acid substitutions in CrtM (F26L or F26S) that confer the C_{40} function (Umeno et al., 2002). By repeating this experiment with a random mutant library that was free from mutation at F26, they found two more amino acid substitutions, W38G and E180G, that confer the same phenotype (Umeno et al., 2003). Upon further mutagenesis at these three residues, they showed that the specificity of the carotenoid synthase CrtM is controlled at the second (rearrangement) step of its two-step reaction. Furthermore, they have engineered synthase variants that can make previously unknown C_{45} and C_{50} carotenoid backbones (mono- and di-isopentenylphytoenes) from the appropriate C_{20} and C_{25} isoprenyldiphosphate precursors (Umeno and Arnold, 2004). With this ability to produce the new backbones in *E. coli* comes the potential to generate whole series of novel carotenoids upon addition of carotenoid-modifying enzymes to the engineered pathway.

5.1. Constructed pathways of C_{35} carotenoids

5.1.1. Synthesization

Researchers subcloned crtM and crtN, both from *Staphylococcus aureus* (Wieland et al., 1994), into a pUC19 derivative (Schmidt-Dannert et al., 2000) to generate plasmid pUC-crtM–crtN. Then transformed XL1 cells with pUC-*crtM–crtN–crtE*, where *crtE* from *Erwinia*

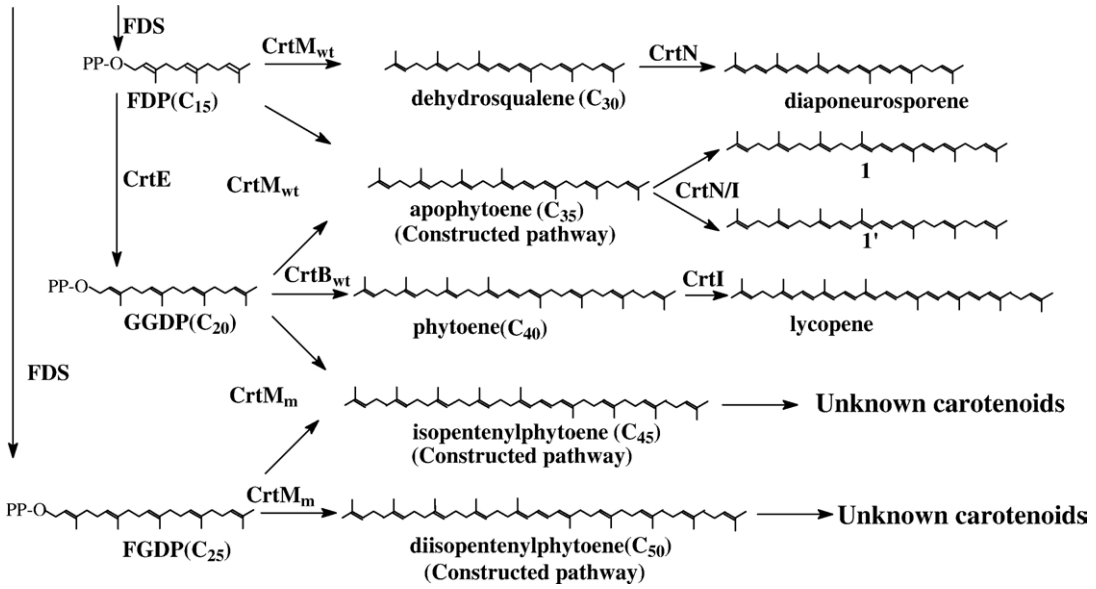

Fig. 6. Five pathways for carotenoid biosynthesis (adapted from Umeno and Arnold, 2004). In addition to natural C_{30} and C_{40} carotenoid pathways, C_{35} carotenoid pathway has been constructed in *E. coli*, C_{45} and C_{50} carotenoid pathways have also been constructed in *E. coli*, but it is still necessary to extend these two pathways. 1, 1′ are the first-step desaturation products of apophytoene.

uredovora was additionally expressed. The spectrum of the acetone extract indicated the presence of a polyene containing 13 conjugated double bonds. This carotenoid is synthesized via heterocondensation of FPP (C_{15}) and GGPP (C_{20}) ($C_{15}+C_{20}=C_{35}$) catalyzed by CrtM. CrtE readily consumes FPP for GGPP production and that FPP for C_{30} synthesis is severely limited. Based on this, it is assumed that C_{35} production is the result of uptake of GGPP by CrtM when FPP is depleted (Fig. 5).

5.1.2. Desaturation

The *S. aureus* desaturase CrtN (from a C_{30} pathway) and *E. uredovora* desaturase CrtI (from a C_{40} pathway) were functional in the C_{35} pathway (Raisig and Sandmann, 2001). C_{30} desaturase CrtN showed a higher apparent step number (four or five steps) in the C_{35} pathway than in its native C_{30} pathway (three or four steps). Thus, cells with carotenoid biosynthetic enzymes CrtM and CrtN develop intense red color in the presence of GGDP, due to the production of highly desaturated C_{35} carotenoids (Fig. 5).

5.1.3. Cyclization

Most carotenoids in plants and microorganisms are in cyclic forms, and various carotene cyclases have been isolated. However, cyclization is known only for C_{40} pathways. Several lycopene cyclases were reported to convert the 7,8-dihydro ψ end along the C_{40} backbone in addition to their natural substrate, lycopene (ψ end) (Takaichi et al., 1996). Researchers tested the function of two carotene cyclases, the β-end cyclase (CrtY) from *E. uredovora* and the ε-end cyclase from *lettuce* (Dy4), in the C_{35} pathway (Umeno and Arnold, 2003). When pUC-*crtY* was transformed into XL1 together with pAC-*crtM–crtN–crtE*, two new carotenoids were observed in small amounts. Their characteristic absorption spectra and molecular masses confirmed that the two compounds 2 and 4 are the monocyclic β-end C_{35} carotenoids (Fig. 5). Similarly, expression of pUC-*dy4* with pAC-*crtM–crtN–crtE* resulted in the production of two other carotenoids. Absorption spectra and molecular masses suggested that the two compounds 1 and 3 are the monocyclic ε-end C_{35} carotenoids (Fig. 5).

5.2. Constructed pathways of C_{45} and C_{50} carotenoids

Synthese variants have been engineered that can make previously unknown C_{45} and C_{50} carotenoid backbones (mono- and di-isopentenylphytoenes) from the appropriate C_{20} and C_{25} isoprenyldiphosphate precursors (Umeno and Arnold, 2004). When coexpressed with FDS, several CrtM variants with substitutions at positions 26 and 38 generated C_{35} (apophytoene) and C_{40} (phytoene) carotenoids along with two novel carotenoids. Based on their mass analysis, elution time, and characteristic absorption spectra, it was concluded that the two novel carotenoids are isopentenylphytoene

(the C_{45} carotenoid backbone, C_{20} plus C_{25}) and diisopentenylphytoene (C_{50} backbone, C_{25} plus C_{25}) (Fig. 6).

Based on the engineered CrtM, C_{45} and C_{50} carotenoids were achieved. C_{45} and C_{50} carotenoid biosynthesis pathways have been constructed, but it is still necessary to use downstream modification enzymes with broad substrate specificity to extend these pathways.

6. Conclusion and prospects

Although impressive in number, the known products of secondary metabolic pathways account for only a tiny fraction of the structures that could be produced. Engineering the upstream biosynthetic enzymes to accept new substrates allows us to generate whole new pathways and access very large numbers of secondary metabolites that are not known in nature but should be chemically, and biologically, possible. From above, the extension of natural carotenoid biosynthesis pathways and the research of engineering carotenoids synthases to construct different chain length carotenoids have been reviewed.

Tetradehydrolycopene and torulene as well as the wild type lycopene, β-carotene in C_{40} pathway and diaponeurosporene in C_{30} pathway were successfully extended with carotenoid-modifying enzymes to produce structurally novel carotenoids in *E. coli*. Various downstream enzymes (desaturases and cyclases) from the C_{30} and C_{40} carotenoid pathways were also functional on unnatural substrate C_{35} carotenoid which led to the production of a series of novel C_{35} carotenoids.

That the C_{30} carotenoid synthase CrtM, a key biosynthetic enzyme that determines the size of the carotenoids in the downstream pathway, is a mere single base substitution from becoming a C_{40} synthase, three or more bases substitution from becoming a C_{45}, C_{50} synthase, is a finding of evolutionary and technological significance. It appears that once a carotenoid backbone structure, for example C_{45}, is created, downstream enzymes, either natural or engineered, can accept the new substrate, and whole series of novel carotenoids can be produced. With the action of carotenoid-modifying enzymes, including desaturases, cyclases and hydroxylases, on these new extended backbones, it should be possible to double or even triple the diversity of the carotenoid kingdom. If some new carotenoid structures are proved to be superior as antioxidants or food colorants to existing natural carotenoids, there will be more space for researchers to concentrate on finding more new carotenoids and testing their biological function.

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