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REVIEW

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Structural diversity and functional novelty of new carotenoid biosynthesis genes

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Abstract Many new carotenoid synthesis genes have recently been identified through genomic sequencing or functional cloning. Some of them exhibit novel structures and/or novel functions. This review describes such examples in the families of lycopene β-cyclases, putative homologues of phytoene dehydrogenases and new carotenoid hydroxylases. Both the functionally novel lycopene β-monocyclases and structurally novel fusion-type of lycopene β-cyclases were described. Another newly discovered sequence of lycopene β-cyclase described might represent a new class of lycopene β-cyclases previously not identified in several cyanobacteria. Three examples of putative homologues of phytoene dehydrogenases were described, however, they were confirmed to encode different and/or new functions such as β-carotene ketolase, 4,4′-diapolycopene oxygenase or prolycopene isomerase. Two new carotenoid hydroxylase genes were described that encoded the new function of 2,2′-β-ionone ring hydroxylase or 3,3′-isorenieratene hydroxylase. Phylogenetic analysis of these genes shed light on their possible evolutionary origins. These new genes also provide tools for synthesis of novel and desirable carotenoids by genetic engineering.

Keywords Carotenoid biosynthesis · Lycopene β-cyclase · Phytoene dehydrogenase homologues · Carotenoid hydroxylase · Sequence homology · Phylogenetic analysis

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Introduction

Over 700 carotenoids are widely distributed in nature including bacteria, fungi, algae, and plants [9]. They carry out important functions in photosynthesis, nutrition, and protection against oxidative damage. Colors of carotenoids range from yellow, orange to red with variations of brown and purple. Several eye-catching examples include β-carotene from carrots, lycopene from tomatoes and lutein from marigold flowers. Carotenoid biosynthesis starts with the isoprenoid pathway (mevalonate-dependent or mevalonate-independent) to generate the C5 isoprene unit, isopentenyl pyrophosphate (IPP). IPP is condensed with its isomer dimethylallyl pyrophophate (DMAPP) to C10 geranyl pyrophosphate (GPP) and elongated to C15 farnesyl pyrophosphate (FPP). Most common C40 carotenoids are synthesized from FPP precursor (Fig. 1) via geranyl geranyl pyrophosphate synthase (CrtE), phytoene synthase (CrtB), phytoene dehydrogenase (CrtI), and lycopene cyclase (CrtY/L). Further functionalizations of the carotenes such as hydroxylation, oxidation, glycosylation, aromatization, sulfonation give rise to a variety of carotenoids with different colors and different properties. C30 carotenoids are relatively uncommon but have been described in *Staphylococcus* and in methylotrophic bacteria. The proposed pathways (Fig. 2) involve synthesis of C30 carotene backbone from FPP followed by functionalization of the C30 carotene backbone.

There also appears to be a great diversity of carotenoid synthesis genes. Genomic sequencing of microbes and plants uncovered many putative carotenoid synthesis genes based on their apparent homology with known carotenoid synthesis genes. Experimental data showed that some of these genes encode the predicted functions with novel characteristics, some even encode different functions from what predicted based on homology analysis. Examples of recently discovered

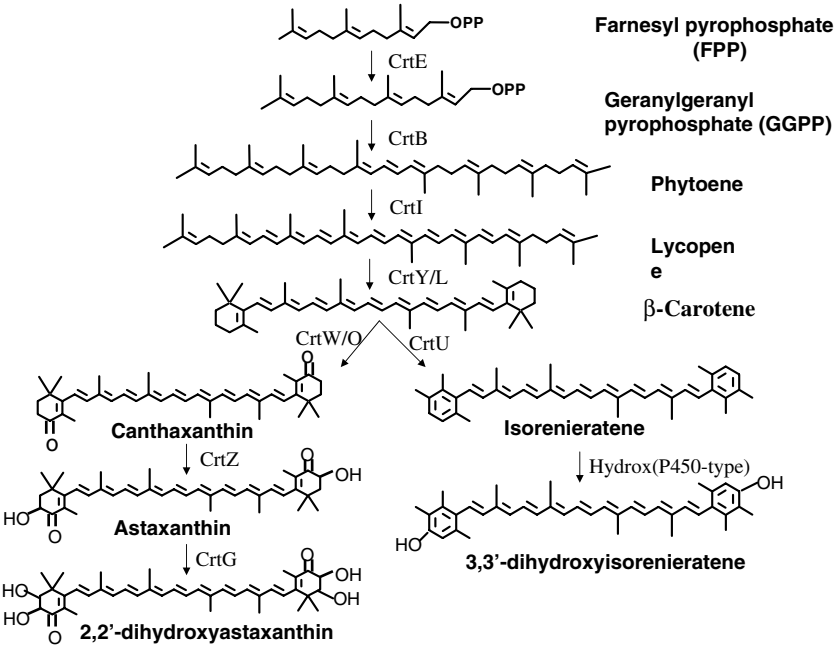

Fig. 1 Pathway for C40 carotenoids biosynthesis. CrtE geranyl geranyl pyrophosphate synthase; CrtB phytoene synthase; CrtI phytoene dehydrogenase; CrtY/L lycopene β-cyclase; CrtW/O 4,4′-β-ionone ring ketolase; CrtZ 3,3′-β-ionone ring hydroxylase; CrtG 2,2′-β-ionone ring hydroxylase; CrtU β-carotene desaturase; Hydrox cytochrome P450-type isorenieratene hydroxylase. The β-carotene portion of the pathway is commonly present in many C40 cyclic carotenoids producing strains. Extension to 2,2′-dihydroxyastaxanthin is present in *Brevundimonas* sp. SD212. Extension to 3,3′-dihydroxyisorenieratene is present in *Brevibacterium linens*

genes from genomic sequencing or functional cloning will be given in this review (Table 1) to illustrate the novelty of gene structures and gene functions.

Diversity of lycopene β-cyclases in microorganisms

Carotenoids are described as acyclic, monocyclic, or bicyclic depending on whether the ends of the hydrocarbon backbones have been cyclized [2]. Lycopene β-cyclases are a class of enzymes responsible for catalyzing the formation of β-cyclic carotenoids from acyclic lycopene (ψ,ψ-carotene). A recent review [24] surveyed different types of lycopene β-cyclases and postulated their possible evolutionary relationship. Here we would like to describe several new lycopene β-cyclases that have been identified since the review.

Lycopene β-monocyclases CrtYm and CrtLm

The common lycopene β-cyclases consist of the bacterial CrtY-type [17, 25, 31, 35, 39] and the plant CrtL-type or Lcy-b type [10, 11, 19, 37]. The CrtY-type and the

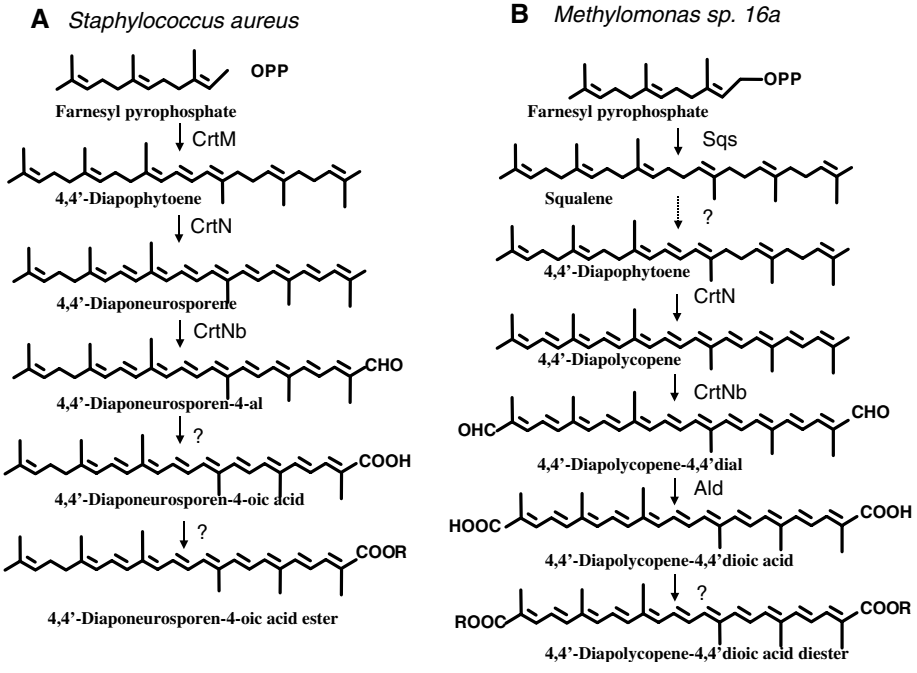

Fig. 2 Proposed pathways for biosynthesis of C30 carotenoids in *Staphylococcus aureus* (**a**) and *Methylomonas* sp. 16a (**b**). Sqs squalene synthase; CrtM 4,4′-diapophytoene synthase; CrtN 4,4′-diapophytoene desaturase; CrtNb 4,4′-diapolycopene oxygenase; Ald aldehyde dehydrogenase

Table 1 Novel carotenoid biosynthesis genes described in this review

Gene	Protein	Source/ reference	Homology between homologs[a]	Accession number	Novelty
crtYm	Lycopene-β-cyclase	Marine bacterium strain P99-3 [44]		AB097813	Monocyclase
crtLm	Lycopene-β-cyclase	*Rhodococcus erythropolis* [42] *Deinococcus radiodurans* [42]	31%	AY437860 AAF10377 (DR0801)	Monocyclase, plant-type
crtYcd	Lycopene-β-cyclase	*Halobacterium salinarum* [36] *Sulfolobus solfataricus* [18]	29%	AE005102 (AAG20275) AAK43013 (CT0456)[b]	Fusion-type
cruA	Lycopene-β-cyclase	*Chlorobium tepidum* [28]		(slr0088)[b]	New sequence
crtO	4,4′-β-ionone ring ketolase	*Synechocystis* sp. [15] *Anabaena* sp. [32] *Rhodococcus erythropolis* [41] *Deinococcus radiodurans* [41]	64% between the two from cyanobacteria 38% between the two from bacteria	(alr3744)[b] AY705709 AAF10377 (DR0093)	Non-homologous to CrtW-type of ketolases
crtNb	4,4′-diapolycopene oxygenase	*Methylomonas* sp. [43] *Staphylococcus aureus* [30]	51%	AY841893 NP_373088	New sequences New function
crtH/*crtISO*	Prolycopene isomerase	*Synechocystis* sp. [8, 29] *Arabidopsis thaliana* [34] *Lycopersicon esculentum* [21]	59–60% between the ones from the cyanobacterium and the plant; 85% between the two from plants	(sll0033)[b] NP_172167 AAL91366	New sequences New function
crtG	2,2′-β-ionone ring hydroxylase	*Brevundimonas* sp. SD212 [33] *Brevundimonas vesicularis* (Q. Cheng, unpublished)	79%	AB181388 DQ309446	New sequences New function
orf10	3,3′-isorenieratene hydroxylase	*Brevibacterium linens* [12, 13]		AF139916	Cytochrome P450-type, new function

[a]Amino acid identity between the listed homologs in each category
[b]Gene designation in cyanobase (http://www.kazusa.or.jp/cyanobase/)

CrtL-type of lycopene cyclases share little overall homology to each other. Despite the sequence divergence, most lycopene β-cyclases are bicyclic, which cyclize both ends of lycopene to produce bicyclic β-carotene. Recently, a novel CrtY-type lycopene β-monocyclase (CrtYm) and a novel CrtL-type lycopene β-monocyclase (CrtLm) have been identified.

The gene (*crtYm*) was isolated from a unique marine bacterium strain P99-3, belonging to the family of *Flavobacteriaceae*, that produced myxol [44]. Myxol is the aglycone of monocyclic myxoxanthophyll likely derived from γ-carotene. It was therefore expected that this bacterium possessed a gene for lycopene β-monocyclase. The gene (*crtYm*) was cloned by functional screening of a cosmid library in the lycopene-accumulating *Escherichia coli* reporter strain. One of the positive clones was shown to encode a carotenoid synthesis gene cluster. Deletion analysis of the gene cluster confirmed that one of the ORFs (*crtYm*) encode the lycopene β-monocyclase. It should be noted that myxol could not be detected from the *E. coli* cells containing the positive cosmid and the lycopene reporter plasmid. Lycopene and γ-carotene were the only pigments that could be detected. It is possible that other carotenoid synthesis genes in this cluster were not expressed well in *E. coli*. An alignment of the amino acid sequences of CrtYm and two other lycopene β-cyclases from bacteria and two from plants showed that CrtYm shared the conserved domains as the other lycopene bicyclases. Six amino acid residues were identified that were conserved in all the aligned lycopene β-cyclases except CrtYm. It was postulated that at least one of these six amino acid residues might determine the number of β-rings added to lycopene.

The gene (*crtLm*) was isolated from *Rhodococcus erythropolis* strain AN12 [42], a non-photosynthetic gram-positive bacterium that belongs to the family of *Nocardiaceae*. Strain AN12 produced monocyclic 4-keto-γ-carotene as the major carotenoid and γ-carotene as the minor carotenoid. The gene (*crtLm*) encoding the lycopene β-cyclase was identified by genome sequencing and was located separately from a gene cluster containing the rest of the carotenoid synthesis genes. The *crtLm* gene was confirmed to encode a lycopene β-monocyclase that produced almost exclusively γ-carotene from lycopene. This monocyclase also showed high activity towards neurosporene and low activity towards diaponeurosporene. No detectable activity was observed towards ζ-carotene. One interesting characteristics of CrtLm was that its lycopene cyclase activity could be inhibited by 2-(4-chlorophenylthio)-triethylamine (CPTA), an inhibitor specific for plant-type lycopene β-cyclases. A CrtLm

homologue was also identified in the genome of *Deinococcus radiodurans* strain R1 and was shown to exhibit similar properties as the *Rhodococcus* CrtLm. Phylogenetic analysis showed that the CrtLm from the non-photosynthetic *Rhodococcus* or *Deinococcus* were closely related to the CrtL from plants and cyanobacteria. It was postulated that the CrtL-type of lycopene β-cyclases from non-photosynthetic bacteria might be an evolutionary link between the common bacterial CrtY-type of lycopene β-cyclases and plant lycopene β- and ε-cyclases.

Fusion-type of lycopene β-cyclases CrtYcd

Besides the common CrtY-type and CrtL-type of lycopene β-cyclases, two other types of lycopene β-cyclases described in the review [24] were the heterodimeric CrtYc and CrtYd type [23, 47]; and the bifunctional fungal CrtYB type [4, 5, 45, 46]. The lycopene cyclase domain of the CrtYB shares homology with the heterodimeric CrtYc and CrtYd. The heterodimeric CrtYc and CrtYd from gram-positive bacteria encode two proteins, which have to interact as heterodimers for lycopene cyclization. Recently, a novel fusion-type of lycopene β-cyclases has been described in archaeon [18, 36], whose N- and C-terminal halves are homologous to the CrtYc and CrtYd subunits of the bacterial heterodimeric enzymes. We suggest designating this type of lycopene β-cyclases as CrtYcd.

The first fusion-type of lycopene β-cyclases was identified in the halophilic archaeon *Halobacterium salinarum* as a step towards the study of bacteriorhodopsin biogenesis [36]. A single gene (*crtYcd*) was identified in the genome of a closely related *Halobacterium* sp. NRC-1 that shared homology with heterodimeric Yc and Yd of lycopene β-cyclases in bacteria and the bifunctional lycopene cyclase-phytoene synthases (CrtYB) in fungi. An in-frame deletion of *crtYcd* was created in *Halobacterium salinarum*, which no longer produced β-carotene or bacteriorhodopsin, whereas lycopene was accumulated to high levels. Heterologous expression of *crtYcd* in lycopene-accumulating *E. coli* resulted in β-carotene production. Comparative sequence analysis and topology predictions provided a model for lycopene cyclase evolution. The bacterial *crtYc* and *crtYd* genes might have arisen from duplication of a gene encoding a homodimeric lycopene cyclase. The archaeal CrtYcd found in *H. salinarum* contains the fusion of CrtYc and CrtYd domain linked by an additional transmembrane segment. The fungal *crtYB* gene appears to have arisen from further fusion of an archaeal-like *crtYcd* gene with a phytoene synthase (*crtB*) gene.

Another fusion-type of lycopene β-cyclase gene was identified in a thermoacidophilic archaeon *Sulfolobus solfataricus* [18]. Its function was also verified by heterologous expression in *E. coli*. However, Peck's view [36] of the archaeal CrtYcd as the evolutionary intermediate between that of bacteria and fungi was questioned. Phylogenetic analysis of the CrtYc and CrtYd domains from bacteria, archea, and fungi showed the existence of two subgroups. Subgroup A of CrtYc or CrtYcd contains *S. solfataricus*, *Brevibacterium linens* and *Mycobacterium* sp., and subgroup B contains those of *H. salinarum*, *Myxococcus xanthus* and fungi. Hemmi [18] argued for an evolutionary independence between the two subgroups, in which the fusion-type archaeal CrtYcd are divided. At this time, we cannot exclude the possibility that the archaeal fusion-type of lycopene β-cyclases is also ancestral to some bacterial enzymes.

Novel lycopene β-cyclases CruA

Chlorobium tepidum is a photosynthetic green sulfur bacteria that was shown to produce monocyclic aryl carotenoids such as chlorobactene and its derivatives [26, 40]. The genome sequence of *Chlorobium tepidum* was completed and no homologues to any of the known lycopene β-cyclases could be detected in its genome [14]. This suggested the existence of a new type of lycopene β-cyclase. The gene (*cruA*) encoding this new type of lycopene β-cyclase was recently identified in *C. tepidum* through functional screening [28]. Expression of *cruA* in lycopene-accumulating *E. coli* produced predominantly γ-carotene, small amount of β-carotene, and some torulene. Identification of the *cruA* gene provided a molecular basis for synthesis of γ-carotene derived monocyclic carotenoids in *C. tepidum*. The only proteins similar to CruA in the databases are two groups of conserved hypothetical proteins found only in cyanobacteria and plants. They all contain a putative flavin-binding domain. Some of those likely encode the same class of the new type of lycopene β-cyclases, which would account for the lycopene β-cyclases previously not identified in several cyanobacteria including *Synechocystis* sp. PCC6803 and *Nostoc* sp. PCC7120.

Novel functions for putative phytoene dehydrogenase homologues

Phytoene dehydrogenases (CrtI) are a diverse group of enzymes that introduce double bonds to the phytoene backbone. They could catalyze 2-step, 3-step, 4-step, and 5-step desaturation of phytoene. Several other desaturases, such as the diapophytoene dehydrogenase CrtN [48], ζ-carotene desaturase CrtQ [27], and the carotenoid 3,4-desaturase CrtD [3, 16], also show homology to phytoene dehydrogenases. The genetic, biochemical, and phylogenetic aspects of different types of phytoene dehydrogenases were previously reviewed [38]. Here, we would like to describe several examples of phytoene dehydrogenase homologues identified by sequence analysis, however, were demonstrated to carry out novel functions different from the homology prediction.

The CrtO-type of β-carotene ketolases

The *Synechocystis* CrtO is the first reported case [15] of a new type of β-carotene ketolase, which has homology to bacterial phytoene dehydrogenases but showed no such activity experimentally. The *Rhodococcus* CrtO and the *Deinococcus* CrtO also showed homology to phytoene dehydrogenases. Three lines of evidence indicated that they function as β-carotene ketolases [41]. First, knockout of the *crtO* gene in the native *Rhodococcus* host blocked the keto group addition. Second, heterologous expression of the *crtO* gene in *E. coli* accumulating β-carotene produced canthaxanthin. Third, the extract of *E. coli* cells expressing the *crtO* gene converted β-carotene to echinenone and canthaxanthin in vitro. The *Rhodococcus* and the *Deinococcus* CrtO both showed symmetrical-acting activity to add two keto groups to β-carotene to produce canthaxanthin, which was different from the asymmetric activity to produce echinenone by the *Synechocystis* CrtO. Search of the genomic sequence databases identified several more putative CrtO homologues from *Nostoc* sp., one of which was functionally confirmed recently [32]. Six conserved regions were identified in the CrtO by multiple sequence alignment. No other proteins in the databases contain all six regions. Presence and location of the six motifs may be a signature for the new CrtO-type of β-carotene ketolases. On the other hand, the four conserved regions previously identified in the CrtW-type of β-carotene ketolases [22] are not present in the CrtO ketolases. Phylogenetic analysis of the carotenoid ketolases and the phytoene dehydrogenases showed that the CrtO-type and the CrtW-type of ketolases belong to two different clades of the phylogenetic tree. The CrtO enzymes (> 500 amino acids) are almost twice the size as the CrtW enzymes and do not share significant sequence homology. CrtO and CrtW might have convergently evolved from different ancestors to achieve similar functions. Interestingly, the CrtO are more closely related to phytoene dehydrogenases CrtI. Two out of the six conserved regions in CrtO are also conserved in CrtI. CrtI and CrtO might have divergently evolved from the same ancestor to acquire different functions.

Novel carotenoid oxidases (CrtNb) for synthesis of C30 carotenoid aldehydes

Biosynthesis of C30 carotenoids is relatively uncommon in nature but has been described in *Staphylococcus* and in methylotrophic bacteria. The proposed pathways involve synthesis of C30 carotene backbone followed by functionalization of the C30 backbone. The genes responsible for synthesis of the C30 carotene backbone from *Staphylococcus* [48] were well characterized about a decade ago: *crtM* encoding diapophytoene synthase, and *crtN* encoding diapophytoene desaturase. The genes for functionalization of the C30 carotene backbone were only discovered very recently in 2005 [30, 43].

A gene cluster involved in C30 carotenoid synthesis was identified in the genome of *Methylomonas* sp. 16a [43]. Two of the genes on this cluster, designated as *crtN* and *crtNb*, showed sequence homology (31–34% amino acid identities) to carotenoid desaturases. They shared 28% amino acid identity to each other. An aldehyde dehydrogenase gene (*ald*) was located between *crtN* and *crtNb* in the same gene cluster. Co-expression of this gene cluster with *Staphylococcus crtM* in *E. coli* produced a pink pigment of 4,4′-diapocarotenoic-4,4′-diacid. In vitro transposon mutagenesis was performed on the gene cluster in order to assign function of each individual gene. Transposon insertion in the *crtN* gene produced only the colorless C30 carotenoid precursor 4,4′-diapophytoene. This is consistent with the function of the *Methylomonas crtN* to encode a diapophytoene desaturase. Transposon insertion in the *ald* gene produced the 4,4′-diapolycopene-4,4′-dialdehyde in addition to the 4,4′-diapophytoene precursor. This confirmed the function of the *Methylomonas ald* to encode an aldehyde dehydrogenase. Transposon insertion in the *crtNb* gene produced the fully unsaturated C30 carotenoid backbone 4,4′-diapolycopene with some additional less unsaturated intermediates. The fact that only the C30 carotenoid backbones were produced in this mutant suggested that functionalization of the 3,4-didehydro-psi end group of 4,4′-diapolycopene was blocked in this mutant. The *crtNb* was not a diapophytoene desaturase gene, instead, it likely encodes an enzyme that oxidizes the terminal methyl group of the 4,4′-diapolycopene to produce 4,4′-diapolycopene-4,4′-dialdehyde, which is further oxidized by the aldehyde dehydrogenase to produce the C30-carboxy carotenoid, 4,4′-diapolycopene-4,4′-diacid. The *Staphylococcus crtNb* homologue was also identified, which was upstream of the *crtMN* genes with an unknown ORF in between. The function of CrtNb was further confirmed by overexpression of c*rtMN* and *crtNb* in *E. coli* to produce 4,4′-diapolycopene-4,4′-dialdehyde. The CrtNb was also tested on 4,4′-diapophytoene and phytoene substrates. No desaturated products were observed. This confirmed that CrtNb is not a carotenoid desaturase, inspite of its apparent sequence homology.

CrtNb appears to prefer the fully unsaturated linear end of the C30 carotenoid backbone. CrtNb from *Methylomonas* and *Staphylococcus* both produced 4,4′-diaponeurosporene-4-monoaldehyde from 4,4′-diaponeurosporene, and produced 4,4′-diapolycopene-4,4′-dialdehyde from 4,4′-diapolycopene [43]. CrtNb exhibited low reactivity with the linear end of the C40 carotenoids lycopene and neurosporene. When the fully unsaturated C40 carotenoid 2,4,2′,4′-tetradehydrolycopene was used as the substrate for CrtNb, 2,4,2′,4′-tetradehydrolycopene dialdehyde and monoaldehyde were observed [30], although considerable level of lycopene precursor was still present. The CrtNb (also named as CrtOx) was evolved in vitro [30] by error-prone PCR mutagenesis and three mutant enzymes significantly changed the product profiles without making any new

product. Higher yields of 2,4,2′,4′-tetradehydrolycopene dialdehyde and lower amount of lycopene precursor were obtained. In one of the mutants, 2,4,2′,4′-tetradehydrolycopene dialdehyde represented 85% of the total carotenoids.

Prolycopene isomerases CrtH/CRTISO

Prolycopene (7Z,9Z,7′Z,9′Z-tetra-*cis*-lycopene) isomerases is another example of a group of enzymes in the broad phytoene dehydrogenases family, but evolved to perform a different function. Bacterial phytoene dehydrogenases CrtI catalyze 4-step desaturation from phytoene to all E-lycopene, which can be cyclized to form β-carotene. Plants and cyanobacteria use 2-step phytoene desaturases combined with 2-step ζ-carotene desaturases, which produce poly-*cis* prolycopene [6]. Due to its steric configuration, poly-*cis* prolycopene cannot be cyclized to ionone end groups. Prolycopene isomerases were identified in cyanobacteria (CrtH) and plants (CrtISO) that converted poly-*cis* prolycopene to all E-lycopene. Prolycopene isomerases were structurally related to bacterial phytoene dehydrogenases (CrtI), and their function as isomerases were confirmed by heterologous gene expression, knockout in the native host and in vitro enzymatic studies.

Gene sll0033 (*crtH*) from *Synechocystis* sp. PCC6803 was confirmed to encode the prolycopene isomerase. *E. coli* expressing plant desaturases could produce prolycopene. Upon co-expression of *crtH*, formation of all-E lycopene was mediated [8]. A *Synechocystis crtH* mutant produced primarily *cis*-carotenes and small amount of all-E carotenes under dark conditions. Under light conditions photoisomerization could complement the CrtH-catalyzed enzymatic isomerization [29]. The plant carotenoid isomerases CrtISO were cloned from *Arabidopsis* [34] and tomato [21]. The tangerine tomatoes that accumulated prolycopene were attributed to loss of function or impaired function of CrtISO. In vitro enzymatic assays further confirmed the function of CrtISO as authentic prolycopene isomearases [7, 20].

New carotenoid hydroxylases

Cyclic and acyclic hydroxy carotenoids are common in native carotenogenic hosts and have also been synthesized in recombinant hosts. The gene *crtC*, encoding a hydratase that introduces water to C-1, 2 of a ψ-carotene end group, is responsible for synthesis of acyclic hydroxy carotenoids. The gene *crtZ or crtR*, encoding the 3,3′-β-ionone ring hydroxylase, is responsible for synthesis of most cyclic hydroxy carotenoids. CrtZ was recently found to also hydroxylate the ε-ionone ring to catalyze the direct conversion of α-carotene to lutein (PCT WO 01/66703). Here we would like to describe two new hydroxylases recently identified that hydroxylate 2,2′ of β-ionone rings of cyclic carotenoids or 3,3′ of aromatic rings of aryl carotenoids.

Novel 2,2′-β-ionone ring hydroxylase CrtG

Several bacteria were reported to produce highly polar carotenoids such as (2R,3S,3′S)-2-hydroxyastaxanthin [50] and (2R,3S,2R′,3′R)-4-ketonostoxanthin 3′-sulfate [49] in addition to astaxanthin. It was expected that they might possess a novel oxygenase encoding the 2,2′-β-ionone ring hydroxylase, in addition to the *crtW* encoding the 4,4′-β-ionone ring ketolase and the *crtZ* encoding the 3,3′-β-ionone ring hydroxylase. This structurally and functionally novel gene *crtG* encoding the 2,2′-β-ionone ring hydroxylase was identified recently in a carotenoid synthesis gene cluster from *Brevundimonas* sp. SD212 [33]. This enzyme, CrtG, composed of 257 amino acid residues showed no overall homology with any other proteins in the databases. However, it exhibited intriguingly partial homology with the middle region of animal sterol-C5-desaturase (Δ7-sterol 5-desaturase), which catalyzes the oxidation from lathosterol to 7-dehydrocholesterol in the cholesterol biosynthesis pathway. Expression of *crtG* in *E. coli* accumulating zeaxanthin, canthaxanthin, or astaxanthin produced dihydroxyzeaxanthin (nostoxanthin), dihydroxycanthaxanthin, or 2-hydroxyastaxanthin, respectively. Interestingly, expression of *crtG* in *E. coli* accumulating β-carotene did not produce detectable 2-hydroxy carotenoids. Therefore, CrtG likely requires an oxygenated β-ionone ring, such as a 3-hydroxy- or 4-keto-β-ionone ring as a substrate for the 2-hydroxylation reaction.

Isorenieratene hydroxylase

Brevibacterium linens, an orange bacterium involved in cheese ripening, synthesizes carotenoids with aromatic end groups, isorenieratene, 3-hydroxy isorenieratene, and 3,3′-dihydroxyisorenieratene. The genes responsible for synthesis of isorenieratene were previously well characterized including the *crtU* for formation of aromatic ring from β-ionone ring [23]. The subsequent hydroxylation of isorenieratene to hydroxyisorenieratene was hypothesized to be catalyzed by a cytochrome P450 enzyme encoded by a gene (*ORF10*) in the cluster. This has been confirmed recently by data presented by Dufosse [13] at the 14th international symposium on carotenoids. A P450 inhibitor tetcyclacis was used to investigate inhibition of isorenieratene hydroxylation. More isorenieratene and less hydroxyisorenieratene were obtained from *B. linens* grown with increasing concentrations of tetcyclacis. Hydroxylase mutants of *B. linens* that produce predominantly isorenieratene were obtained by chemical mutagenesis and UV mutagenesis [12]. Mutations were mapped to the cytochrome P450 gene *ORF10*, which most likely encode the isorenieratene hydroxylase.

Conclusion

Understanding of carotenoid biosynthesis has improved dramatically in the last several years through isolation and characterization of new carotenoid synthesis genes. The newly identified genes have further enriched the structural diversity and functional novelty of carotenoid synthesis genes, and shed light on their possible evolutionary origins. Functions of some of these new genes remain to be confirmed by experimental means. Cautions should be exercised to predict gene function based on homology analysis. One example is the CrtU homologues identified in several cyanobacteria, which do not produce aromatic carotenoids. The biological function of the cyanobacterial CrtU homologues, which most likely would be different from the CrtU desaturase in gram-positive bacteria for aromatic carotenoid synthesis, is yet unknown.

Regulation of carotenoid biosynthesis is still largely uncharacterized. Nevertheless, considerable progress has been made in engineering the recombinant production hosts such as *E. coli* to produce high titers of carotenoids [1]. The newly identified biosynthetic genes are useful in producing novel and desirable carotenoids by genetic engineering of carotenoid synthetic pathways in the native or recombinant production hosts.

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