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Short Communication

Identification of a Gene Required for *cis*-to-*trans* Carotene Isomerization in Carotenogenesis of the Cyanobacterium *Synechocystis* sp. PCC 6803

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A disruptive mutant of the *sll0033* gene of the cyanobacterium *Synechocystis* sp. PCC 6803 produced primarily *cis* carotenes and small amounts of all-*trans* carotenes, but no xanthophylls, under dark conditions. Under light conditions, however, it produced normal carotenoids, that were the same as those produced by wild-type cells grown under both light and dark conditions. When the mutant cells cultured under dark conditions were irradiated, *cis*-isomers of carotenes were converted to all-*trans* lycopene. These findings demonstrate that this gene, designated *crtH*, is involved in the isomerization of *cis*-carotenes to all-*trans* forms in dark conditions, and that *cis*-carotenes were also converted to all-*trans* forms under light conditions by photoisomerization.

Key words: Carotene isomerase — *crtH* — Cyanobacteria — *cis*-Lycopene — Photoisomerization — *Synechocystis* sp. PCC 6803.

Abbreviations: LAHG, light-activated-heterotrophic-growth; PMG, photomixotrophic-growth; PAG, photoautotrophic-growth.

The first carotenoid 15-*cis* phytoene in carotenogenesis is converted to carotenes and xanthophylls depending on the organism. In photosynthetic bacteria, carotenogenic bacteria and fungi, 15-*cis* phytoene is converted to all-*trans* neurosporene or all-*trans* lycopene by phytoene desaturase CrtI (Armstrong 1997, Sandmann 1994, Sandmann 2001) (see Fig. 1 for the carotene components). In contrast to these organisms, in cyanobacteria and higher plants, these steps are catalyzed by two enzymes, phytoene desaturase CrtP/PDS and ζ-carotene desaturase CrtQ/ZDS (Cunningham and Gantt 1998, Sandmann 1994, Sandmann 2001, Hirschberg and Chamovitz 1994). Accumulation of poly-*cis* carotenes has been observed in some mutants of green algae, *Scenedesmus obliquus* C-6D (Sandmann 1991, Humbeck 1990), *Chlorella* 5/520 (Claes 1961), and *Euglena* W_3BUL (Cunningham and Schiff 1985), all grown in darkness. The accumulated poly-*cis* carotenes have been photoisomerized to *trans* forms in vivo and in vitro. The accumulation of *cis*-carotenes (Bartley et al. 1999, Breitenbach et al. 1998, Chamovitz et al. 1992, Linden et al. 1991) and their photoisomerization (Bartley et al. 1999) have also been observed in a heterologous expression of CrtP/PDS and CrtQ/ZDS in *Escherichia coli*. Hence, these steps are suggested as the *cis*-pathway for all-*trans* lycopene synthesis (the conversion of 15-*cis* phytoene to all-*trans* lycopene) (Bartley et al. 1999). We would, therefore, expect the presence of a *cis*-to-*trans* carotene isomerase for all-*trans* lycopene synthesis in cyanobacteria and higher plants.

In *Synechocystis* sp. PCC 6803, the genes *slr0088*, *slr0940* and *sll0033* have a weak homology to the genes of bacterial phytoene desaturases (*crtI*) but the encoded polypeptides of the genes have no phytoene desaturase activity. The gene *slr1254* encodes a phytoene desaturase CrtP (Martinez-Ferez and Vioque 1992), while *slr0088* is a gene for β-carotene ketolase CrtO (Fernandez-Gonzalez et al. 1997) and *slr0940* is a gene for ζ-carotene desaturase CrtQ-2 (Breitenbach et al. 1998). The function of *sll0033* has not yet been identified, however, because the disruptant of *sll0033* showed the same phenotype as that of the wild type under photosynthetic growth conditions (Breitenbach et al. 1998).

The present study demonstrates that the gene *sll0033* of *Synechocystis* sp. PCC 6803, designated *crtH*, is required for the biosynthesis of normal carotenoids in darkness, and suggests that CrtH encodes a *cis*-to-*trans* carotene isomerase.

Growth profiles of the wild-type and mutant cells under PAG, PMG, or LAHG conditions were almost the same (data not shown), showing that disruption of the *sll0033* gene in the mutant (*crtH* mutant) has no effect on growth.

The wild-type cells grown under LAHG conditions had the same pigment composition as those grown under PMG conditions (Fig. 2A, 2B); the major pigments were myxol dimethy-fucoside, zeaxanthin, Chl *a*, echinenone and β-carotene (Takaichi et al. 2001). The *crtH* mutant cells grown under PMG conditions contained the same pigments as those of the wild-type cells grown under PMG or LAHG conditions, but

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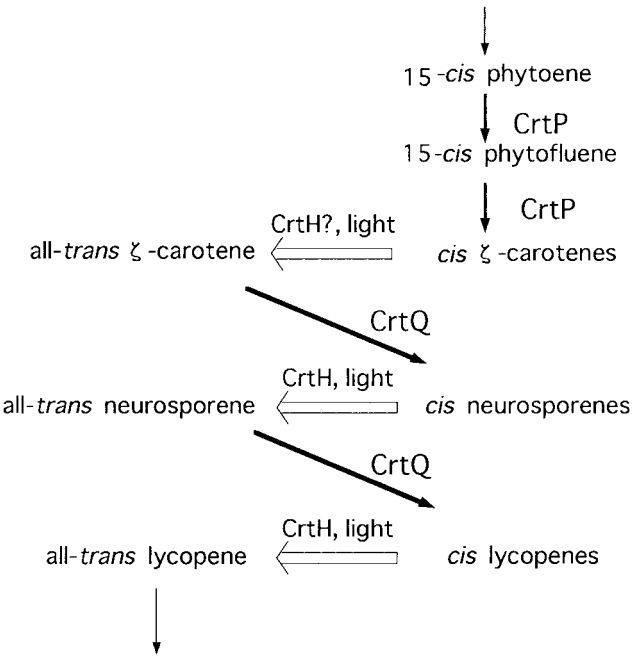

Fig. 1 A putative carotenogenic pathway from 15-*cis* phytoene to all-*trans* lycopene of the cyanobacterium *Synechocystis* sp. PCC 6803 (*cis*-pathway). Phytoene desaturase CrtP converts 15-*cis* phytoene to *cis* ζ-carotenes (black arrows). ζ-Carotene desaturase CrtQ converts *trans* forms of carotenes to *cis* isomers of carotenes (wide black arrows). *Cis* carotenes are converted to *trans* forms by *cis*-to-*trans* carotene isomerase CrtH or by light (white arrows). See the text for a detailed explanation. CrtP and CrtQ in cyanobacteria correspond to PDS and ZDS in higher plants, respectively.

additional small peaks of unknown carotenes were detected just before the β-carotene peak (Fig. 2C). When the *crtH* mutant was grown under LAHG conditions, the mutant cells did not have normal carotenoids, while unknown carotene peaks S1, S3, and S5 appeared (Fig. 2D).

The carotene fraction of the *crtH* mutant cells grown under LAHG conditions was analyzed by HPLC with the Novapak C_{18} column (Fig. 3, Table 1). The elution profile showed many carotene peaks. All-*trans* lycopene, all-*trans* neurosporene, *cis* ζ-carotene and all-*trans* ζ-carotene, β-zeacarotene, 15-*cis* phytofluene, all-*trans* β-carotene, and 15-*cis* phytoene were identified based on the absorption spectra and the retention times on HPLC (Takaichi 2000). Furthermore, all-*trans* ζ-carotene, 15-*cis* phytofluene, and all-*trans* β-carotene were also confirmed based on the analysis of the relative molecular masses (Table 1). Overall, those carotene contents except all-*trans* ζ-carotene were very small. Since substantial amounts of all-*trans* β-carotene were found under PMG and PAG conditions with the *crtH* mutant, the β-carotenes might be produced from all-*trans* lycopene by lycopene cyclase, and consequently *crtH* is not a lycopene cyclase gene.

Absorption spectra of the N1, N2, N3, and N4 peaks showed the *cis* form of carotenes (Fig. 3, Table 1). Because it is difficult to separate all of the *cis* forms in this HPLC system and to determine the *cis* position from the absorption spectra, we indicated them as *cis* carotenes in this study including mono- and poly-*cis* forms. The components in the N1 to N4 peaks were collected from the HPLC. The relative molecular mass of the N1, N2, and N4 peaks was 536 (Table 1), which corresponds to that of lycopene, and the relative molecular mass of the N3 peak was 538, neurosporene. Therefore, the major component in peaks N1, N2, and N4 seems to be *cis* isomers of lycopene (more than 70% of the total carotenes; Table 1), and that in peak N3 seems to be *cis* neurosporene.

Compared with the elution profiles and absorption spectra of HPLC with both the Novapak C_{18} column (Fig. 3, Table 1) and the Supersphere RP-18 column (Fig. 2D), the major component in peaks S1 and S3 seemed to be *cis* isomers of lycopene. Peak S4 appeared to contain *cis* lycopene isomer, all-*trans* neurosporene, and *cis* ζ-carotene, and peak S5 contained *cis* lycopene isomer and all-*trans* ζ-carotene (Table 1).

When the *crtH* mutant cells grown under LAHG conditions were irradiated, peaks S1, S3, and S5 (*cis* lycopene isomers, *cis* neurosporene and all-*trans* ζ-carotene) disappeared within 20 min with a concomitant increase in all-*trans* lycopene (Fig. 4; broken line). Such a photoisomerization has been reported to be facilitated by the presence of Chl both in vivo and in vitro experiments in *Chlorella* (Claes 1961). Photoisomerizations of pro-lycopene to *trans*-lycopene (Humbeck 1990) and *cis* ζ-carotene to *trans* ζ-carotene (Sandmann 1991, Cunningham and Schiff 1985) of dark-grown *Scenedesmus* C-6D and *Euglena* W_3BUL mutants seem to be the same as those of *Synechocystis* sp. PCC 6803 *crtH* mutant observed in the present study.

From the results described above, we concluded that the product of the *sll0033* gene of *Synechocystis* sp. PCC 6803 might be *cis*-to-*trans* carotene isomerase. The substrates were mono- and poly-*cis* carotenes. We named it CrtH. Although Schnurr et al. (1991) named β-carotene hydroxylase from *Erwinia* CrtH, the enzyme has already been named CrtZ (Misawa et al. 1990), and CrtH is not used at present.

We searched for CrtH homologues in the genome databases of cyanobacteria and *Arabidopsis thaliana*, and found only one gene in each genome with high homology: 74% identity to that of *Nostoc punctiforme* ATCC 29133 (Contig552); 70% to *Anabaena* sp. PCC 7120 (C251A); 68% to *Synechococcus* sp. WH8102 (Contig48); 58% to *Prochlorococcus marinus* MED4 (Contig25); and 48% to *A. thaliana* (AAF63149.1). *Cis*-to-*trans* carotene isomerase may be widely distributed.

A putative carotenogenic pathway from 15-*cis* phytoene to all-*trans* lycopene of cyanobacteria is proposed in Fig. 1. Phytoene desaturase CrtP converts 15-*cis* phytoene to *cis* ζ-carotenes, since expression of *Synechococcus* CrtP in *E. coli* with a phytoene background accumulates *cis* ζ-carotenes

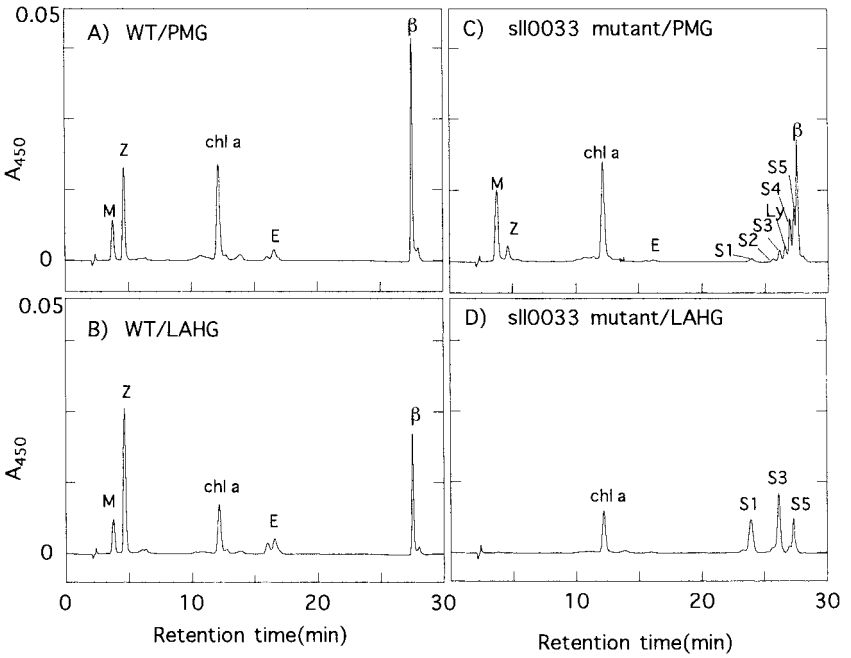

Fig. 2 HPLC profiles with a Supersphere RP-18 column of the pigment extracts of the wild-type and *crtH* mutant cells grown under PMG or LAHG conditions. The carotenoids were: M, myxol dimethyl-fucoside; Z, zeaxanthin; E, echinenone; and β, β-carotene. The carotene identification is summarized in Table 1.

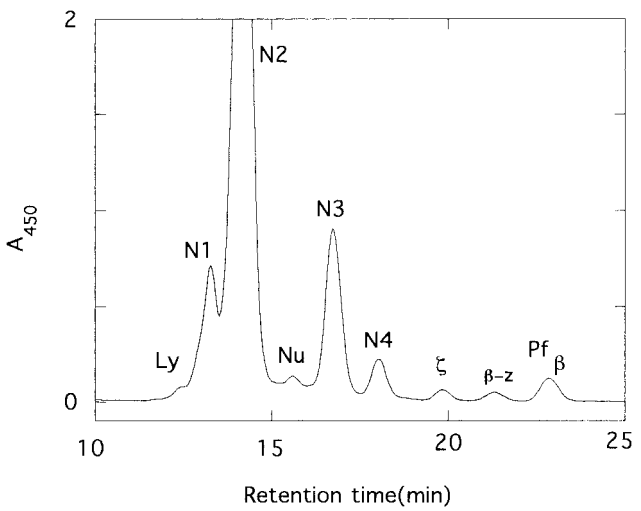

Fig. 3 HPLC profile with a Novapak C_{18} column of the carotene fraction of the *crtH* mutant cells grown under LAHG conditions. The all-*trans* carotenes identified were: Ly, lycopene; Nu, neurosporene; ζ, ζ-carotene; β-z, β-zeacarotene; and β, β-carotene. Absorption of the N2 peak was ca. 6.6. Identification of each peak is summarized in Table 1.

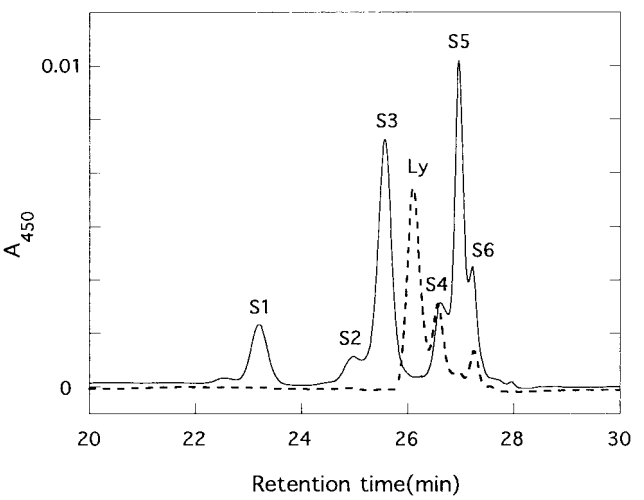

Fig. 4 Photoisomerization of carotenes in the *crtH* mutant cells grown under LAHG conditions. The pigments were extracted from the cells before (solid line) and after (broken line) irradiation for 20 min at 30°C. HPLC conditions were the same as those in Fig. 2. The largest peak in the profile (broken line) was all-*trans* lycopene.

(Sandmann 1994, Linden et al. 1991). Expression of PDS and ZDS of Arabidopsis in *E. coli* with the phytoene background under dark conditions accumulates mainly pro-lycopene and small amounts of *cis* forms of ζ-carotene, neurosporene and lycopene (Bartley et al. 1999). Consequently, ζ-carotene desaturase CrtQ may convert all-*trans* ζ-carotene to *cis* neurosporenes and all-*trans* neurosporene to *cis* isomers of lycopene. *Cis*-to-*trans* carotene isomerase CrtH and light convert *cis* ζ-carotenes to all-*trans* ζ-carotene, *cis* neurosporenes to all-*trans* neurosporene, and *cis* lycopenes including pro-lycopene

Table 1 Characterization of carotenes of the *crtH* mutant cells grown under LAHG conditions

Retention time (min) [a]	Absorption maxima (nm) [b]					Major molecular mass (*m/z*) [c]	mol%	Major carotenoid	Peak position on Supersphere C_{18} HPLC [d]
12.4 (Ly)	295	364	446	471	502	(536)	<1	all-*trans* lycopene	Ly
13.3 (N1)	296	364	447	470	*494*	536	10	*cis* lycopene isomer	
14.1 (N2)	297	*347*	*423*	442	*463*	536	60	*cis* lycopene isomer	S1, S3
15.6 (Nu)	267	332	418	439	468	(538)	2	all-*trans* neurosporene	
16.7 (N3)		333	412	434	463	538	15	*cis* neurosporene isomer	S3
18.0 (N4)	284	355	*441*	461	*484*	536	2	*cis* lycopene isomer	S5
18.9		296	377	399	422	(540)	<1	*cis* ζ-carotene isomer	
19.8 (ζ)		294	380	402	426	540	10	all-*trans* ζ-carotene	S5
21.3 (β-z)			406	428	454	(538)	<1	β-zeacarotene	
22.6 (Pf)		255	332	349	368	542	2	15-*cis* phytofluene	
22.9 (β)	278		*432*	454	479	536	1	all-*trans* β-carotene	β
25.1		292	380	402	426	(540)	<1	7,8-dihydro-β-zeacarotene	
27.5			*277*	287	*297*	(544)	2	15-*cis* phytoene	

[a] Retention time on the Novapak C_{18} HPLC (Fig. 3). Parentheses indicate the peak names in Fig. 3.
[b] Main absorbance maximum in the HPLC eluent is underlined, and absorption shoulders are shown in italics as calculated by differentiation.
[c] Values in parentheses were obtained from the authentic samples.
[d] Fig. 2D and 4.

to all-*trans* lycopene. We speculate that carotene is isomerized by the same *cis*-pathway in higher plants. In bacteria, phytoene desaturase CrtI converts and isomerizes 15-*cis* phytoene to all-*trans* lycopene (Linden et al. 1991, Sandmann 1994).

The *crtH* mutant cells grown under LAHG conditions accumulated significant amounts of all-*trans* ζ-carotene (Table 1). This must be synthesized by isomerization of *cis* ζ-carotene. There are two possibilities for carotene isomerization. The first is that *cis* ζ-carotenes are photoisomerized to the *trans* form, since the mutant cells were irradiated for 10 min d^{-1} under LAHG conditions, although the irradiation was insufficient for the photoisomerization of *cis* lycopenes. The second is that the other isomerase, which catalyzes the isomerization of *cis* ζ-carotenes to all-*trans* ζ-carotene, is present in *Synechocystis* sp. PCC 6803.

The *crtH* gene is demonstrated to be responsible for, at least, isomerization of *cis* lycopene isomers to all-*trans* lycopene under LAHG conditions in this study. Biochemical characterization of CrtH in the isomerization of *cis* carotenes is in progress.

The wild-type cells of *Synechocystis* sp. PCC 6803 were grown at 30°C for 3–4 d in a BG-11 medium supplemented with 4 mM HEPES buffer (pH 7.5) under photoautotrophic growth (PAG), photomixotrophic growth (PMG) or light-activated-heterotrophic-growth (LAHG) conditions. Cultures grown in PAG and PMG conditions were irradiated (40 μmol photons m^{-2} s^{-1}) with white fluorescent lamps. Five mM glucose was added to the growth medium in cultures in PMG and LAHG conditions. Cultures grown in LAHG conditions were incubated in darkness with light pulses supplied for 10 min d^{-1} with an intensity of 20 μmol photons m^{-2} s^{-1}. Except for the light pulse, the cultures were kept in complete darkness (Anderson and McIntosh 1991). The *crtH* mutant described below was also grown under PAG, PMG or LAHG conditions in the same medium with the addition of 20 μg ml^{-1} kanamycin.

A plasmid vector pBS-*sll0033* was constructed essentially as reported previously (Masamoto et al. 1998). A kanamycin-resistant gene (Km^r) cartridge, prepared from pUC4KIXX (Amersham, U.K.) as a 1.6 kb-*Hin*dIII-fragment as reported previously (Okumura et al. 1997), was inserted into the *Hin*dIII site in the coding region of the *sll0033* in pBS-*sll0033* (0.34 kb away from the 5′-end of ORF). The constructed plasmid with the disrupted *sll0033* gene (pBS-*sll0033::Km*^{r}) was used for transforming the wild-type cells, as reported (Williams 1988). Disruption of the *sll0033* gene in the mutant (*crtH* mutant) was confirmed by PCR and genomic Southern blot hybridization analysis as reported previously (Okumura et al. 1997).

For investigating photoisomerization, the *crtH* mutant cells grown under LAHG conditions were harvested and suspended in the above growth medium supplied with 5 μM norflurazon and 1 mM nicotine, incubated for 1 h in the dark, and then irradiated for 20 min at an intensity of 700 μmol photons m^{-2} s^{-1}.

Organic solvent soluble pigments from the washed cells were extracted with 90% acetone under dim light and were separated by HPLC with a Supersphere 100 RP-18 column (250 × 4 mm, Merck, Germany). The pigments were eluted using 100% methanol for the first 2 min, followed by a 21-min linear gradient of 0–20% ethanol in methanol, and then a 4-min linear gradient of 20–100% ethanol in methanol, which was con-

tinued isocratically until the completion of the 30-min separation. The flow rate was 1.0 ml min^{-1}. They were monitored at 450 nm and absorption spectra were obtained with a photodiode array detector (SPD-M10AV, Shimadzu, Japan).

The carotenes of the *crtH* mutant grown under LAHG conditions were identified as follows (Takaichi 1993, Takaichi 2000). The pigments were extracted with acetone-methanol (7 : 2, v/v), and the carotene fraction was then obtained by silica gel 60 (Merck) column chromatography. The fraction was analyzed by HPLC with a Novapak C_{18} column (100 × 8 mm, RCM type, Waters, U.S.A.) eluted by acetonitrile-methanol-tetrahydrofuran (58 : 35 : 7, by vol.). Absorption spectra were recorded with a photodiode-array detector (MCPD-3600, Otsuka Electronics, Japan). The carotenes in the main peaks were collected from the HPLC system, and their relative molecular masses were analyzed with FD-MS (M-2500, Hitachi, Japan).

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Note Added in Proof

We realized after finishing our manuscript that the identifications of carotene isomerases in the cyanobacterium and higher plants appeared in the following papers: Breitenbach, J., Viouq, A. and Sandmann, G. (2001) Gene *sll0033* from *Synechocystis* 6803 encodes a carotene isomerase involved in the biosynthesis of all-*E* lycopene. *Z. Naturforsch.*, 56c: 915–917. Park, H., Kreunen, S.S., Cuttriss, A.J., DellaPenna, D. and Pogson, B.J. (2002) Identification of the carotenoid isomerase provides insight into carotenoid biosynthesis, prolamellar body formation and photomorphogenesis. *Plant Cell* 14: (in press). Isaacson, T., Ronen, G., Zamir, D. and Hirschberg, J. (2002) Cloning of tangerine from tomato reveals a carotenoid isomerase essential for production of β-carotene and xanthophylls in plants. *Plant Cell* 14: (in press).

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