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Short Communication

β-Carotene Hydroxylase Gene from the Cyanobacterium *Synechocystis* sp. PCC6803

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The ORF sll1468 of *Synechocystis* sp. PCC6803 was identified as a gene for β-carotene hydroxylase by functional complementation in a β-carotene-producing *Escherichia coli*. The gene product of ORF sll1468 added hydroxyl groups to the β-ionone rings of β-carotene (β,β-carotene) to form zeaxanthin (β,β-carotene-3,3′-diol). This newly identified β-carotene hydroxylase does not show overall amino acid sequence similarity to the known β-carotene hydroxylases. However, it showed significant sequence similarity to β-carotene ketolases of marine bacteria and a green alga.

Key words: β-Carotene — Cyanobacterium — Hydroxylase — *Synechocystis* PCC6803 — Zeaxanthin.

Carotenoids are synthesized in all of the photosynthetic bacteria, cyanobacteria, algae, higher plants and also in some non-photosynthetic bacteria, yeasts and fungi (Britton 1988). The first step of carotenoid biosynthesis is the formation of phytoene from two molecules of geranylgeranyl diphosphate (GGPP). Phytoene is then converted to various carotenes such as neurosporene, lycopene, γ-carotene, β-carotene, and α-carotene, which are the substrates for the biosynthesis of the tremendous diversity of xanthophylls. Functional analysis of carotenoid biosynthesis genes has clarified many steps in the carotenoid biosynthetic pathway (Hirschberg and Chamovitz 1994, Scolnik and Bartley 1996, Armstrong 1997, Misawa 1997), e.g., the steps catalyzed by β-carotene hydroxylases and ketolases, corresponding the pathway from β-carotene to zeaxanthin, canthaxanthin, or astaxanthin (Kajiwara et al. 1995, Misawa et al. 1995a, b, Sun et al. 1996, Fraser et al. 1997, Pasamontes et al. 1997). In cyanobacteria, genes coding for phytoene synthase (*crtB/psy*), phytoene desaturase (*crtP/pds*), ζ-carotene desaturase (*crtQ/zds*), lycopene cyclase (*crtL/lcy*), and β-carotene ketolase (*crtO*) have been isolated and identified (Chamovitz et al. 1991, 1992, Martinez-Ferez and Vioque 1992, Cunningham et al. 1994, Linden et al. 1994, Martinez-Ferez et al. 1994, Fernandez-Gonzalez et al. 1997), while in the cyanobacterium *Synechocystis* sp. PCC6803, of which the whole genome has been sequenced (Kaneko et al. 1996), only *crtB*, *crtP*, and *crtO* have been identified (Fig. 1). This cyanobacterium is known to contain large amounts of β-carotene, zeaxanthin, echinenone and myxoxanthophyll (2′-*O*-rhamnosylmyxol), and a small amount of β-cryptoxanthin (β,β-caroten-3-ol) (Bramley and Sandmann 1985) (Fig. 1).

As we now know the whole sequence of the genome of *Synechocystis* sp. PCC6803, it should be a suitable organism for analysis of carotenoid biosynthetic pathway and the genes involved in the pathway. The present paper shows the identification of a β-carotene hydroxylase gene from this cyanobacterium through functional complementation experiments in *E. coli*. The β-carotene hydroxylase gene has been clarified for the first time in cyanobacteria.

Standard methods for recombinant DNA were used (Sambrook et al. 1989). The plasmid expressing a β-carotene hydroxylase gene was constructed as follows: the gene ORF sll1468, present in a cosmid clone of *Synechocystis* sp. PCC6803 (cs0827) (Kaneko et al. 1996), was amplified by PCR. The PCR reaction mixture contained 300 ng of the cosmid DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.125 mM each of dNTPs, 2 units of Taq polymerase (Takara, Japan), and 50 pmol each of the following sets of primers; forward primer, 5′-TCCTCGAGCGTGTGCCAGGAGTCCG-3′; reverse primer, 5′-ACTCTAGAGCTAGGGCTTGTCAGATG-3′ in a 50 μl final volume. The conditions for the PCR amplification were 94°C for 120 s, followed by 30 cycles of the temperature shift consisting of 94°C for 30 s, 60°C for 30 s, 72°C for 120 s in a PJ9600 thermal cycler (Perkin Elmer). A PCR product was double-digested with *Xho*I and *Xba*I in order to obtain a 0.94 kb DNA fragment which includes whole part of the structural gene of the ORF sll1468. The DNA fragment was ligated into the *Xho*I-*Xba*I sites of pBluescriptII KS+ (Stratagene) to construct a plasmid pBS-crtR. In this plasmid, the additional 24 amino acid residues translated from LacZ N-end is designed to be added

Abbreviations: ORF, open reading frame; dNTPs, deoxynucleoside triphosphates; GGPP, geranylgeranyl diphosphate.

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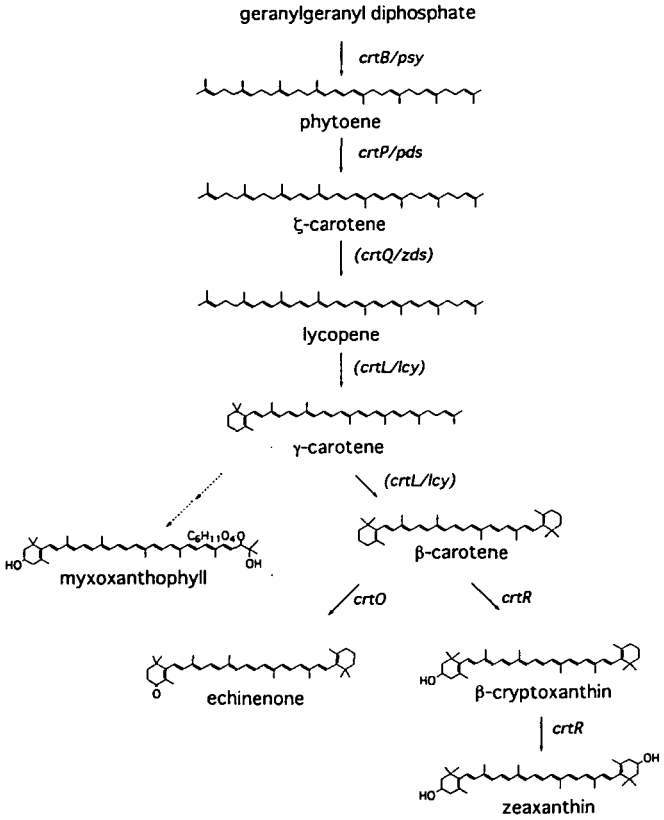

Fig. 1 Postulated carotenoid biosynthetic pathway and structures of carotenoids in the cyanobacterium *Synechocystis* sp. PCC6803. Genetic names indicate steps identified in this cyanobacterium or other cyanobacteria (parenthesized). The carotenoid biosynthesis genes of cyanobacteria had been designated in the same manner to those of higher plants (*psy, pds, zds, and lcy*). Hirschberg and Chamovitz (1994) have recommended that the cyanobacterial carotenogenic genes should follow the *crt* nomenclature, which has been used in the bacterial corresponding genes.

to the original N-end of the ORF sll1468 to generate a fusion protein by using the *lac* promoter.

Previously, the carotenoid biosynthesis genes, *crtE, crtB, crtI*, and *crtY*, were isolated from the epiphytic bacterium *Erwinia uredovora* (Misawa et al. 1990), and their functions were identified (Misawa et al. 1995b). The *crtE* gene codes for prenyl transferase to synthesize GGPP from farnesyl diphosphate. Two molecules of GGPP is condensed to phytoene by *crtB* (Fig. 1). Next, one enzyme encoded by *crtI* is needed for the conversion of phytoene to lycopene. In cyanobacteria, this desaturation is divided to two steps by two enzymes, CrtP and CrtQ, via ζ-carotene (Fig. 1). Finally, the *Erwinia crtY* gene product (CrtY) cyclizes lycopene to β-carotene via γ-carotene. This enzyme corresponds to CrtL in cyanobacteria (Fig. 1). Plasmid pACCAR16ΔcrtX carried the *E. uredovora* four *crt* genes on vector pACYC184 to confer a yellow pigmentation on *Escherichia coli* due the accumulation of β-carotene, as described (Misawa et al. 1995a).

The two plasmids pBS-crtR and pACCAR16ΔcrtX were used to co-transform *E. coli* JM101. The resultant *E. coli* transformant was grown to stationary phase in 2× YT medium (Sambrook et al. 1989), including 150 μg ml^{-1} ampicillin, 30 μg ml^{-1} chloramphenicol and 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 30°C. Carotenoid pigments were extracted from cells with acetone and the extracts were dried and re-extracted with chloroform/methanol (9 : 1). Pigments obtained were separated by high-performance liquid chromatography (HPLC) with a C18 column (300 × 3.9 mm, Nova-pak HR 6 μm [Waters]) under isocratic flow (acetonitrile/methanol/2-propanol (90 : 6 : 4), 1 ml min^{-1}), and were detected with a Waters photodiode array detector 996. Pigments obtained were also subjected to thin-layer chromatography (TLC) on silica gel ($60F_{254}$), which was developed with chloroform/methanol (15 : 1). Authentic carotenoids (β-carotene, zeaxanthin, canthaxanthin) were obtained as described (Misawa et al. 1990, 1995b).

The database of amino acid sequences deduced from genes and ORFs of the genome of *Synechocystis* sp. PCC6803 was taken from the ftp site (ftp://ftp.kazusa.or.jp/pub/cyano/cyano.p.aa.z) of Kazusa DNA Research Institute (Kaneko et al. 1996). This database together with SwissProt (rel.32.0) and PIR (rel.51.0) were searched for amino acid sequences similar to that deduced from sll1468 using the FASTA program (Person and Lipman 1988) in the GCG package (Devereux et al. 1984). A multiple alignment of the amino acid sequences was constructed with CLUSTALW (Thompson et al. 1994), and was refined visually to increase the sequence similarity. Statistical significance of the detected sequence similarity was examined by a jumbling test (Needleman and Wunsch 1970) with 100 pairs of randomized sequences. The alignment score of a pair of the aligned sequences was calculated by summing up the similarity score (Schwartz and Dayhoff 1978) of the residue pair at each alignment site. When a gap was observed, a given gap penalty was used as the score for the gap. Continuous gaps were considered as a single insertion/deletion event, thus treated as a single gap. After the calculation of average and standard deviation (SD) of alignment scores for the random sequences, the deviation of the alignment score for the original sequences from the average was devided by SD. Then, a normalized alignment called Z-score was obtained. When the Z-score was greater than 4.0, the similarity of the aligned sequences was considered to be statistically significant.

Sequence comparison revealed that the amino acid sequence encoded by ORF sll1468 of *Synechocystis* sp. PCC6803 showed significant similarity to the sequences of bacterial β-carotene ketolases. Therefore, we postulated that the ORF product should have β-carotene ketolase activity, which is the reason why we performed functional

complementation analysis for this ORF. An *E. coli* strain accumulating β-carotene, due to the presence of plasmid pACCAR16ΔcrtX, was transformed with the plasmid pBS-crtR that carries the ORF sll1468 of *Synechocystis* sp. PCC6803. Pigments were extracted from a resultant transformant, and four yellow pigments were separated by HPLC (Fig. 2). Pigments 1 and 4 were determined as zeaxanthin (all-*trans*) and β-carotene (all-*trans*), respectively, by their UV-VIS spectral data as well as HPLC and TLC properties, using authentic samples for comparison. Yellow pigment 2 was considered to be the *cis* form of zeaxanthin due to the occurrence of the *cis* peak (340 nm) in its UV-VIS spectrum. Yellow pigment 3 was considered to be β-cryptoxanthin (all-*trans*) by its UV-VIS spectral datum as well as HPLC and TLC properties. Zeaxanthin and β-cryptoxanthin accounted for 65% and 5% of total carotenoids, respectively. Except for these carotenoids, no other carotenoid pigments, including echinenone (retention time, 23 min), were detected. Thus, contrary to expectations, we have concluded that the ORF sll1468 of *Synechocystis* sp. PCC6803 is a gene that encodes a β-carotene hydroxylase (CrtR), which catalyzes the hydroxylation reactions of β-carotene to produce zeaxanthin via β-cryptoxanthin (Fig. 1). Little accumulation of mono-hydroxylated β-carotene (β-cryptoxanthin) (Fig. 2) suggests symmetrical induction of hydroxyl groups to β-carotene by the CrtR enzyme, which has the same properties to the *Erwinia*'s and *Arabidopsis*'s β-carotene hydroxylases (Misawa et al. 1990, Sun et al. 1996). Hirschberg and Chamovitz (1994) recommended that a β-carotene hydroxylase gene of cyanobacterium should be designated *crtR* based on the *crt* nomenclature of bacterial carotenoid biosynthesis genes.

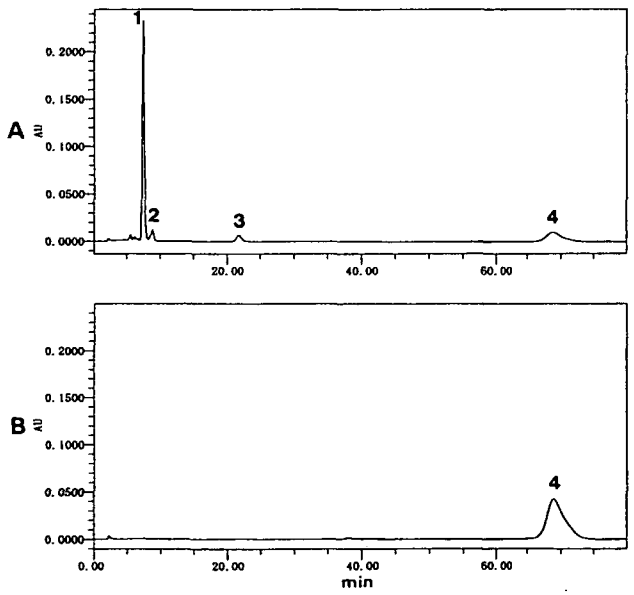

Fig. 2 HPLC profiles of carotenoid pigments extracted from *E. coli* cells carrying plasmids pBS-crtR and pACCAR16ΔcrtX (A) and plasmid pACCAR16ΔcrtX (B). Peaks numbered 1, 2, 3 and 4 correspond to zeaxanthin, *cis*-zeaxanthin, β-cryptoxanthin and β-carotene, respectively.

The amino acid sequence of the *Synechocystis* sp. PCC6803 CrtR (called SS-CrtR hereafter in this paper) was compared with amino acid sequences deduced from all the other ORFs of *Synechocystis* sp. PCC6803. Two open reading frames, sll1611 (hypothetical protein) and sll1441 (fatty-acid Δ^{15} desaturase), were found to be similar to SS-CrtR, but the sequence identities were less than 20%. Therefore, the gene of SS-CrtR was concluded to be a single copy gene.

Recent enzymatic analyses of β-carotene hydroxylases and β-carotene ketolases revealed that Fe^{2+} was required for the enzymes as one of the cofactors (Fraser et al. 1997). Amino acid sequence comparison of the hydroxylases and of the ketolases suggested the presence of histidine-rich iron-binding motifs (Fraser et al. 1997). The histidine-rich iron-binding motifs were also found in SS-CrtR (Fig. 3, boxed), suggesting Fe^{2+} requirement as a cofactor for the activity of the enzyme. All of these β-carotene hydroxylases and ketolases had three iron-binding motifs in their primary structures. However, the motifs of SS-CrtR were considerably different in residue and length from the corresponding motifs of the other β-carotene hydroxylases and ketolases, except for the conserved histidine residues.

All of the β-carotene hydroxylases from the bacteria, *Synechocystis* sp. PCC6803 and *Arabidopsis* showed the hydroxylation activity of β-ionone ring at position 3(3′) in common. However, the SS-CrtR protein did not show amino acid sequence similarity to the other bacterial and plant enzymes except for the histidine-rich motifs, while these bacterial and plant enzymes were homologous to each other, e.g., 53–56% identity between those of the *Erwinia* species and the marine bacteria and 28–36% identity between the bacteria and the plant *Arabidopsis* (Misawa et al. 1995b, Sun et al. 1996).

It is surprising that SS-CrtR had significant amino acid sequence similarity to the bacterial β-carotene ketolases (27% identity) and the green algal one (21% identity) (Fig. 3) for almost entire region, although SS-CrtR did not show any β-carotene ketolase activity. SS-CrtR and the ketolases of the marine bacteria or the green alga shared three highly conserved segments (Fig. 3, alignment sites, 184 to 194, 267 to 272, and 317 to 322) as well as the iron-binding motifs. Statistical significance of the similarity was examined for the alignment sites from 127 to 331 of each sequence pair after excluding highly diverged N- and C-terminal regions. The obtained Z-scores ranged from 4.2 to 5.3, which suggested that SS-CrtR and the ketolases are homologous, that is, they have evolved from a common ancestor in spite of their different activity. These results may suggest that the evolutionary origin of SS-CrtR is different from that of the β-carotene hydroxylases derived

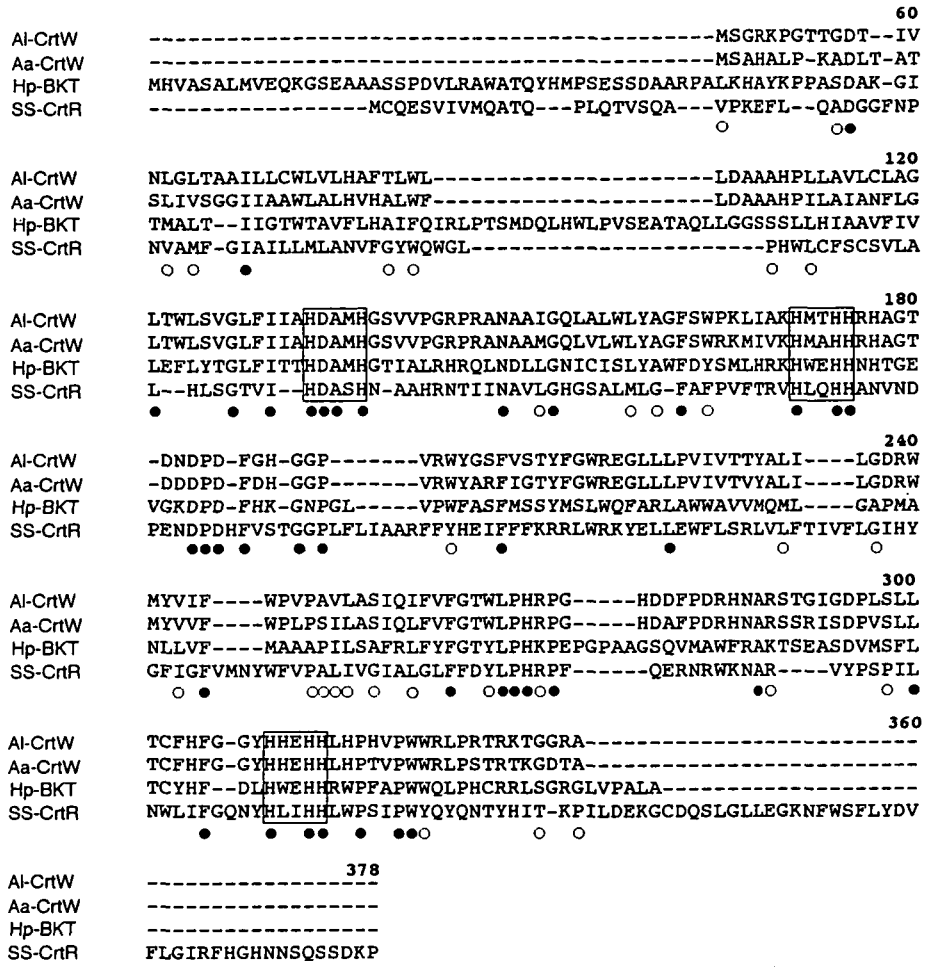

Fig. 3 Alignment of amino acid sequences of *Synechocystis* sp. PCC6803 β-carotene hydroxylase (SS-CrtR), and *Haematococcus pluvialis* β-carotene ketolase (Hp-BKT; SwissProt accession number, Q39982), *Agrobacterium aurantiacum* β-carotene ketolase (Aa-CrtW; SwissProt accession number, P54972), *Alcaligenes* PC-1 β-carotene ketolase (Al-CrtW; SwissProt accession number, Q44261). The alignment sites occupied by the identical amino acid residues and chemically similar amino acid residues among the four enzymes were represented by ● and ○, respectively. Conserved iron-binding motifs were boxed.

from the bacteria and *Arabidopsis*.

Recently β-carotene ketolase was identified in *Synechocystis* sp. PCC6803, which was encoded by slr0088 (Fernandez-Gonzalez et al. 1997). This enzyme did not show amino acid sequence similarity to any other β-carotene ketolases or β-carotene hydroxylases including SS-CrtR over its entire length. It is therefore considered that the *Synechocystis* sp. PCC6803 ketolase has evolved from an ancestor distinct from the ketolase and hydroxylase enzymes of other organisms. The cyanobacterium *Synechocystis* sp. PCC6803 seems to be unique with respect to evolutionary point of view on carotenoid biosynthesis.

We also introduced plasmid pBS-crtR into a neurosporene accumulating *E. coli* (data not shown). A resultant transformant was not able to convert neurosporene, an acyclic carotenoid, into a hydroxylated compound. *Synechocystis* sp. PCC6803 includes myxoxanthophyll, the xanthophyll with hydroxy groups in the acyclic end group (Fig. 1). It is therefore likely that another hydroxylase remained unidentified in this cyanobacterium.

References

Armstrong, G.A. (1997) Genetics of eubacterial carotenoid biosynthesis: a colorful tale. *Annu. Rev. Microbiol.* 51: 629–659.

Bramley, P.M. and Sandmann, G. (1985) In vitro and in vivo biosynthesis of xanthophylls by the cyanobacterium *Aphanocapsa*. *Phytochemistry* 24: 2919–2922.

Britton, G. (1988) Biosynthesis of carotenoids. *In* Plant Pigments. Edited by Goodwin, T.W. pp. 133–182. Academic Press, London.

Chamovitz, D., Misawa, N., Sandmann, G. and Hirschberg, J. (1992) Molecular cloning and expression in *Escherichia coli* of a cyanobacterial gene coding for phytoene synthase, a carotenoid biosynthesis enzyme. *FEBS Lett.* 296: 305–310.

Chamovitz, D., Pecker, I. and Hirschberg, J. (1991) The molecular basis of resistance to the herbicide norflurazon. *Plant Mol. Biol.* 16: 967-974.

Cunningham, F.X., Jr., Sun, Z., Chamovitz, D., Hirschberg, J. and Gantt, E. (1994) Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp. PCC7942. *Plant Cell* 6: 1107-1121.

Devereux, J., Haerberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for VAX. *Nucl. Acids Res.* 12: 387-395.

Fernandez-Gonzalez, B., Sandmann, G. and Vioque, A. (1997) A new type of asymmetrically acting β-carotene ketolase is required for the synthesis of echinenone in the cyanobacterium *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* 272: 9728-9733.

Fraser, D.P., Miura, Y. and Misawa, N. (1997) In vitro characterization of astaxanthin biosynthetic enzymes. *J. Biol. Chem.* 272: 6128-6135.

Hirschberg, J. and Chamovitz, D. (1994) Carotenoids in cyanobacteria. *In* The Molecular Biology of Cyanobacteria. Edited by Bryant, D.A. pp. 559-579. Kluwer Academic Publishers, Dordrecht.

Kajiwara, S., Kakizono, T., Saito, T., Kondo, K., Ohtani, T., Nishino, N., Nagai, S. and Misawa, N. (1995) Isolation and functional identification of a novel cDNA for astaxanthin biosynthesis from *Haematococcus pluvialis*, and astaxanthin synthesis in *Escherichia coli*. *Plant Mol. Biol.* 29: 343-352.

Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M. and Tabata, S. (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res.* 3: 109-136.

Linden, H., Misawa, N., Saito, T. and Sandmann, G. (1994) A novel carotenoid biosynthesis gene coding for ζ-carotene desaturase: functional expression, sequence and phylogenic origin. *Plant Mol. Biol.* 24: 369-379.

Martinez-Ferez, I., Fernandez-Gonzalez, B., Sandmann, G. and Vioque, A. (1994) Cloning and expression in *Escherichia coli* of the gene coding for phytoene synthase from the cyanobacterium *Synechocystis* sp. PCC6803. *Biochim. Biophys. Acta* 1218: 145-152.

Martinez-Ferez, I. and Vioque, A. (1992) Nucleotide sequence of the phytoene desaturase gene from *Synechocystis* sp. PCC6803 and characterization of a new mutation which confers resistance to the herbicide norflurazon. *Plant Mol. Biol.* 18: 981-983.

Misawa, N. (1997) Carotenoid biosynthesis at the gene level. *In* Dyanmic Aspects of Natural Products Chemistry—Molecular Biological Approaches—. Edited by Ogura, K. and Sankawa, U. pp. 49-70. Kodansya, Tokyo.

Misawa, N., Kajiwara, S., Kondo, K., Yokoyama, A., Satomi, Y., Saito, T., Miki, W. and Ohtani, T. (1995a) Canthaxanthin biosynthesis by the conversion of methylene to keto groups in a hydrocarbon β-carotene by a single gene. *Biochem. Biophys. Res. Commun.* 209: 867-876.

Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K. and 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., Kajiwara, S., Saito, T., Ohtani, T. and Miki, W. (1995b) 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.

Needleman, S.B. and Wunsch, C.D. (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. *J. Mol. Biol.* 48: 443-453.

Pasamontes, L., Hug, D., Tessier, M., Hohmann, H.-P., Schierle, J. and van Loon, A.P.G.M. (1997) Isolation and characterization of the carotenoid biosynthesis genes of *Flavobacterium* sp. strain R1534. *Gene* 185: 35-41.

Person, W.R. and Lipman, D.J. (1988) Improved tool for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* 85: 2444-2448.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schwartz, R.M. and Dayhoff, M.O. (1978) Matrices for detecting distant relationships. *In* Atlas of Protein Sequence and structure. Edited by Dayhoff, M.O. pp. 353-359. National Biomedical Research Foundation, Washington DC.

Scolnik, P.A. and Bartley, G.E. (1996) A table of some cloned plant genes involved in isoprenoid biosynthesis. *Plant Mol. Biol. Rep.* 14: 305-319.

Sun, Z., Gantt, E. and Cunningham, F.X., Jr. (1996) Cloning and functional analysis of the β-carotene hydroxylase of *Arabidopsis thaliana*. *J. Biol. Chem.* 271: 24349-24352.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* 22: 4673-4680.

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