APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2000, p. 64–72 Vol. 66, No. 1
0099-2240/00/$04.00+0

Increased Production of Zeaxanthin and Other Pigments by Application of Genetic Engineering Techniques to *Synechocystis* sp. Strain PCC 6803

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Received 28 June 1999/Accepted 17 October 1999

The *psbAII* locus was used as an integration platform to overexpress genes involved in carotenoid biosynthesis in *Synechocystis* sp. strain PCC 6803 under the control of the strong *psbAII* promoter. The sequences of the genes encoding the yeast isopentenyl diphosphate isomerase (*ipi*) and the *Synechocystis* β-carotene hydroxylase (*crtR*) and the linked *Synechocystis* genes coding for phytoene desaturase and phytoene synthase (*crtP* and *crtB*, respectively) were introduced into *Synechocystis*, replacing the *psbAII* coding sequence. Expression of *ipi*, *crtR*, and *crtP* and *crtB* led to a large increase in the corresponding transcript levels in the mutant strains, showing that the *psbAII* promoter can be used to drive transcription and to overexpress various genes in *Synechocystis*. Overexpression of *crtP* and *crtB* led to a 50% increase in the myxoxanthophyll and zeaxanthin contents in the mutant strain, whereas the β-carotene and echinenone contents remained unchanged. Overexpression of *crtR* induced a 2.5-fold increase in zeaxanthin accumulation in the corresponding overexpressing mutant compared to that in the wild-type strain. In this mutant strain, zeaxanthin becomes the major pigment (more than half the total amount of carotenoid) and the β-carotene and echinenone amounts are reduced by a factor of 2. However, overexpression of *ipi* did not result in a change in the carotenoid content of the mutant. To further alter the carotenoid content of *Synechocystis*, the *crtO* gene, encoding β-carotene ketolase, which converts β-carotene to echinenone, was disrupted in the wild type and in the overexpressing strains so that they no longer produced echinenone. In this way, by a combination of overexpression and deletion of particular genes, the carotenoid content of cyanobacteria can be altered significantly.

Carotenoids are pigments synthesized by photosynthetic and nonphotosynthetic organisms. In photosynthetic membranes, they are essential components of the photosynthetic apparatus and play a protective role against oxidative damage by various mechanisms, including by quenching of chlorophyll triplets, which otherwise could give rise to highly reactive singlet oxygen species (10, 13). Carotenoids may also serve a light-harvesting antenna function: absorbed light energy may be transferred to chlorophyll or may be dissipated in the case of excess radiant energy, thus protecting the photosynthetic apparatus from photooxidation (25). In photosynthetic organisms, carotenoids bind noncovalently but specifically with membrane proteins (2). Their distribution and localization in the photosynthetic membrane vary from one organism to another, but predominantly carotenes rather than xanthophylls (carotene derivatives that contain one or more oxygen atoms incorporated in various functional groups) are associated with the photosynthetic reaction center complexes (13). In plants, xanthophylls are associated mainly with the antenna complexes. Moreover, carotenoids may be found in the cytoplasmic membrane, where they are thought to influence membrane fluidity (3, 12).

Carotenoids, like sterols and gibberellins, are part of a group of compounds named isoprenoids. Despite their structural and functional diversity, isoprenoids are synthesized via a common precursor, isopentenyl diphosphate (IPP). IPP is isomerized to dimethylallyl diphosphate by IPP isomerase. Several condensation reactions convert dimethylallyl diphosphate into geranylgeranyl pyrophosphate. The first specific step in the carotenoid biosynthesis pathway is phytoene synthesis by condensation of two molecules of geranylgeranyl pyrophosphate. Four desaturation steps convert phytoene into lycopene via phytofluene, ζ-carotene, and neurosporene. Cyclization reactions occur on lycopene, giving rise to carotenes, such as β-carotene. Xanthophylls, such as zeaxanthin, are oxygenation products of carotenes (for reviews, see references 3 and 31).

In *Synechocystis* sp. strain PCC 6803, several genes encoding enzymes involved in carotenoid biosynthesis have been identified (Fig. 1). The *crtB* gene codes for phytoene synthase (17), *crtP* codes for phytoene desaturase (18), *crtQ* codes for ζ-carotene desaturase (6), *crtO* codes for β-carotene ketolase (8), and *crtR* codes for β-carotene hydroxylase (19). Only four carotenoids accumulate significantly in *Synechocystis*; these are myxoxanthophyll [2′-(β-1-rhamnopyranosyloxy)-3′,4′-didehydro-1′,2′-dihydro-β,Ψ-carotene-3,1′-diol], β-carotene (β,β-carotene), echinenone (β,β-caroten-4-one), and zeaxanthin (β,β-carotene-3,3′-diol) (Fig. 1).

Naturally occurring carotenoids are of commercial interest as coloring agents for food, pharmaceuticals, cosmetics, and animal feed. Because of their antioxidant properties (20), some of them have been proposed to act in the prevention of chronic diseases (27). Several of these pigments are currently produced for commercial purposes by total chemical synthesis (4, 24). The recent genetic elucidation of carotenoid biosynthetic pathways in several bacterial organisms may offer new opportunities for genetic engineering of carotenoid production in vivo (see reference 3 for a review). The first reports on synthesis of carotenoids in genetically altered noncarotenogenic bacteria

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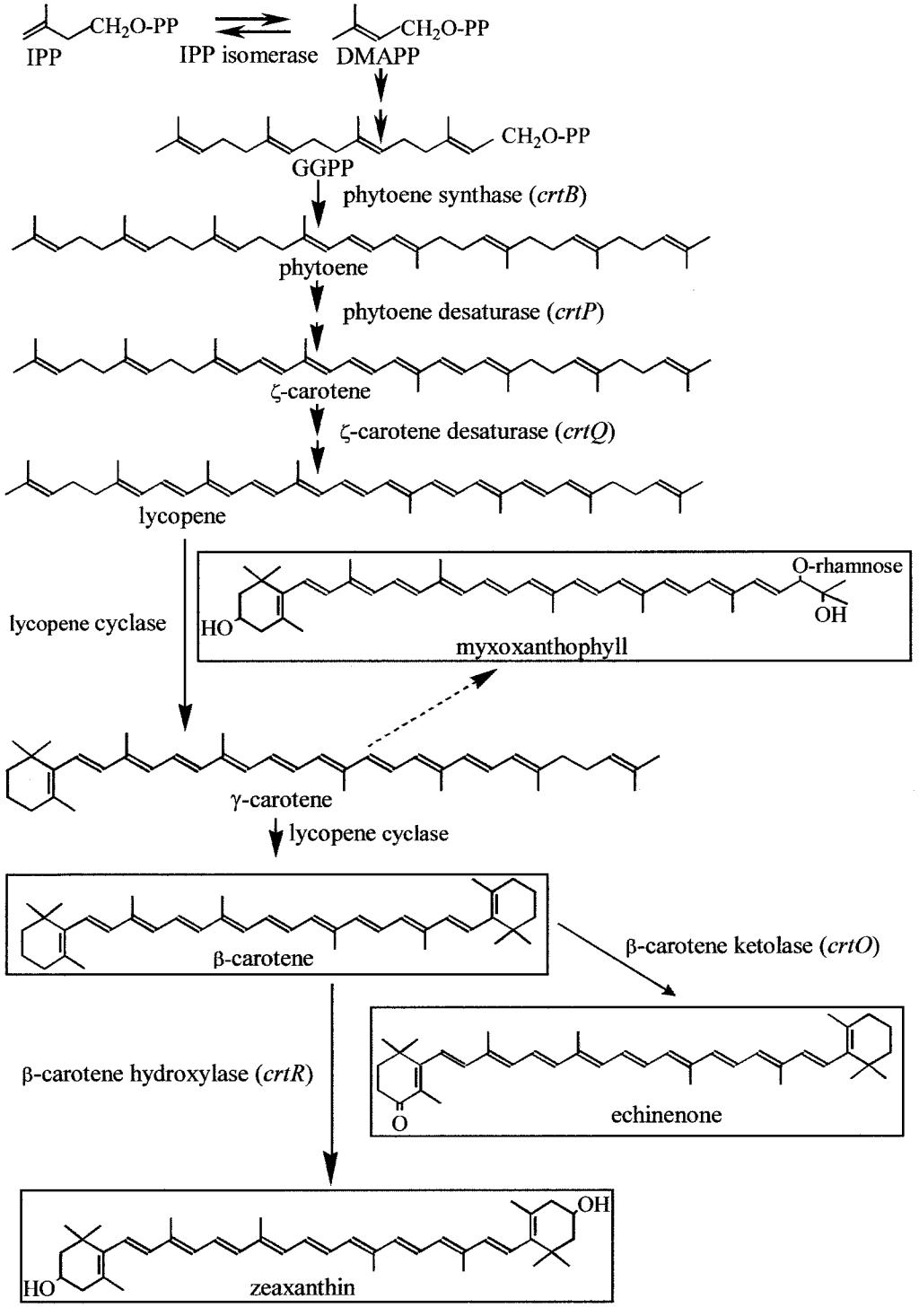

FIG. 1. Simplified biosynthesis pathway for the carotenoids (boxed) that significantly accumulate in *Synechocystis* sp. strain PCC 6803. Gene names for biosynthetic enzymes that have been identified in the genome are in parentheses next to the enzymes that they encode. Arrowheads indicate pathways that remain hypothetical. DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate.

have appeared (30, 32, 36). In an attempt to increase zeaxanthin accumulation in a photoautotrophic prokaryote, *Synechocystis* sp. strain PCC 6803, a system has now been designed to overexpress genes involved in carotenoid synthesis in this organism. This system employs the *psbAII* gene, which encodes the highly expressed D1 protein of photosystem II and which has a strong promoter in *Synechocystis* sp. strain PCC 6803 (23). The *Synechocystis* genome contains three genes coding for the D1 protein, *psbAI*, *psbAII*, and *psbAIII*, the latter two of which are expressed and by themselves individually can support normal photoautotrophic growth in the absence of the other two *psbA* genes (22). Therefore, the *psbAII* locus can be

TABLE 1. Plasmids used in this study

Plasmid	Description	Reference or source
pRL271	Contains the *sacB* gene	5
pSL1180		Pharmacia Biotech
pUC4K	Contains the *aphX* gene	26
pPSBA2	Contains the upstream and downstream regions of the *psbAII* gene, cloned into pSL1180	This study
pPSBA2KS	Contains an *aphX-sacB* construct inserted into pPSBA2	This study
pIPI	Contains the *ipi* gene from *S. cerevisiae* cloned as an *Nde*I-*Bam*HI fragment in pPSBA2	This study
pCrtPB	Contains the *crtP* and *crtB* coding sequences from *Synechocystis* cloned as an *Nde*I-*Bgl*II fragment in pPSBA2	This study
pCrtR	Contains the *crtR* coding sequence from *Synechocystis* cloned as an *Nde*I-*Bam*HI fragment in pPSBA2	This study
pCrtO	Contains the *crtO* gene from *Synechocystis* cloned as a *Bam*HI-*Eco*RI fragment in pSL1180	This study
pΔCrtO	Contains a disrupted *crtO* gene: a *Bcl*I-*Bcl*I fragment was removed from pCrtO	This study
pΔCrtOKS	Contains an *aphX-sacB* construct inserted into pΔCrtO	This study

used as an integration platform to overexpress genes in *Synechocystis*.

To further extend the usefulness of this integration platform, we chose to develop a system that would not lead to the presence of antibiotic resistance cassettes in overexpressing strains. For this purpose, the *sacB* gene (33), which encodes a levan sucrase, was used as a conditionally negative marker. Expression of the *sacB* gene is lethal in the presence of sucrose. Introduction of *sacB* together with an antibiotic resistance gene first allows a positive selection for the mutant genotype by using antibiotic resistance as a screenable phenotype. After segregation of wild-type and mutant genome copies, the antibiotic resistance-*sacB* cassette can be removed by transformation with a markerless construct, followed by selection for sucrose resistance and screening for a strain that no longer is antibiotic resistant (34).

MATERIALS AND METHODS

Strains and growth conditions. *Synechocystis* sp. strain PCC 6803 was cultivated at 30°C in modified BG-11 medium (29) buffered with 10 mM TES-NaOH (pH 8.0), at a photon flux density of 50 μmol of photons · m^{-2} · s^{-1} unless otherwise indicated. The BG-11 modification consisted of partial substitution of $NaNO_3$ with an equal concentration of NH_4NO_3 (the final concentration of ammonia was 4.5 mM). For growth on plates, 1.5% (wt/vol) agar and 0.3% (wt/vol) sodium thiosulfate were added. BG-11 medium was supplemented with 50 μg of kanamycin ml^{-1} for kanamycin-resistant strains. For initial transformant selection, the DNA-cell mixture was plated on a BG-11 plate (50 ml), and the next day 2.5 mg of kanamycin (dissolved in sterile water) was added to the bottom of the plate. This procedure allows a gentle exposure of transformants to increasing kanamycin concentrations. To remove the kanamycin resistance-*sacB* cassette from the genomes of mutant strains, mutant cells were transformed with a markerless construct carrying *Synechocystis* sequences from immediately upstream and downstream of the kanamycin resistance-*sacB* cassette. This construct may contain a gene that is to be overexpressed. After transformation, the *Synechocystis* cells were grown in BG-11 medium for 4 days. Transformants were then plated and selected for growth in the presence of 5% (wt/vol) sucrose. Sucrose-resistant colonies were then checked for kanamycin sensitivity.

Integration platform and plasmids. Plasmids used in this study are listed in Table 1. The genome sequence of *Synechocystis* sp. PCC 6803 (15) was consulted through CyanoBase (http://www.kazusa.or.jp/cyano/cyano.html) to design the primers (Table 2) needed to amplify the *Synechocystis* sequences used to construct the integration platform and the different plasmids. The published sequence of the IPP isomerase gene (*ipi*) from *Saccharomyces cerevisiae* (1) was used to design primers and amplify the coding sequence of *ipi*. Sequences were amplified by PCR with *Taq* DNA polymerase. The integration platform, pPSBA2, contains regions upstream and downstream of the *psbAII* gene. It was constructed as follows: a 500-bp *Pst*I-*Nde*I fragment upstream of and including the *psbAII* ATG start codon and a 500-bp *Bam*HI-*Eco*RI fragment downstream of and including the *psbAII* gene stop codon were cloned by PCR and introduced into plasmid pSL1180 (Pharmacia Biotech), resulting in plasmid pPSBA2. The introduction of an *Nde*I site (CATATG) at the *psbAII* start codon allows the cloning of any coding sequence under the control of the *psbAII* promoter, with its start codon replacing the *psbAII* ATG. Plasmid pPSBA2KS was created by inserting the kanamycin resistance gene (*aphX*) from pUC4K (26) and the *sacB* gene from pRL271 (5, 33) between the *Hpa*I and *Bam*HI sites in the integration platform, pPSBA2 (Fig. 2).

A 1-kb *Nde*I-*Bam*HI fragment beginning at the translational start site of the *ipi* gene of *S. cerevisiae* was amplified by PCR with yeast genomic DNA as a template (the *ipi* gene does not contain introns and therefore can be used directly

TABLE 2. Sequences of the primers used in this study and their relative positions in the *Synechocystis* sp. strain PCC 6803 genome[a]

Primer	Sequence	Position
5′psbAII$_{up}$	5′ gagagagag**ctgcag**AGCGTTCCAGTGGAT 3′	6733–6747
3′psbAII$_{up}$	5′ gagagagag**CATaTG**GTTATAATTCCTTATG 3′	7210–7231
5′psbAII$_{down}$	5′ gagagagag**ggatcc**TAATTCCTTGGTGTAATGCC 3′	8309–8328
3′psbAII$_{down}$	5′ gagagagag**gaattC**GATCGCCTTGGCAAAACAAC 3′	8781–8801
5′IPP	5′ aacaga**catATG**ACTGCCGACAACAATAG 3′	
3′IPP	5′ aacaga**ggatcc**TTATAGCATTCTATGAATTTGCC 3′	
5′crtPB	5′ AGACAGTAA**CatATG**CGCGTTGTG 3′	1397807–1397830
3′crtPB	5′ ATCAACCAT**aGATCt**GTGGTCGGA 3′	1400319–1400342
5′crtR	5′ AAGTGCGTCCCAT**cataTG**TGCCAGGAGT 3′	982617–982645
3′crtR	5′ GGAAAGCAA**ggAtCC**TAGCTAGGG 3′	981673–981696
5′crtO	5′ ACCGCAATG**GGatCC**GACAAATTG 3′	2894607–2894630
3′crtO	5′ TTGATCAGAT**gAATTc**GAGCCTGG 3′	2896875–2896898
5′IPP$_p$	5′ TGAAGTCTCTAACTTCATTG 3′	
3′IPP$_p$	5′ TGAAAAGGGTTTACTACATC 3′	
5′crtP$_p$	5′ CATTGCCATGAGTAAGGCGT 3′	1398325–1398344
3′crtP$_p$	5′ TGGAGGTTAATGACCGGGAC 3′	1398719–1398738
5′crtB$_p$	5′ AAGGAGGGCCATTTGGGCGA 3′	1399519–1399538
3′crtB$_p$	5′ ATTGGTTCGCCACCCCCAAG 3′	1399921–1399940
5′crtR$_p$	5′ AATCCCAACGTGGCCATGTT 3′	982511–982530
3′crtR$_p$	5′ AAAAGATCTCATGGTAGAAG 3′	982170–982189

[a] Nucleotides in lowercase show changes that were made in the sequence to engineer restriction sites used for cloning. Recognition sites for restriction enzymes are in boldface. Base numbering in the *Synechocystis* genome was as in CyanoBase (see Materials and Methods). In the case of the yeast IPP isomerase gene, primers were designed according to the published sequence (1). Primers with a name ending with a subscript "p" were used to make probes for Northern analysis.

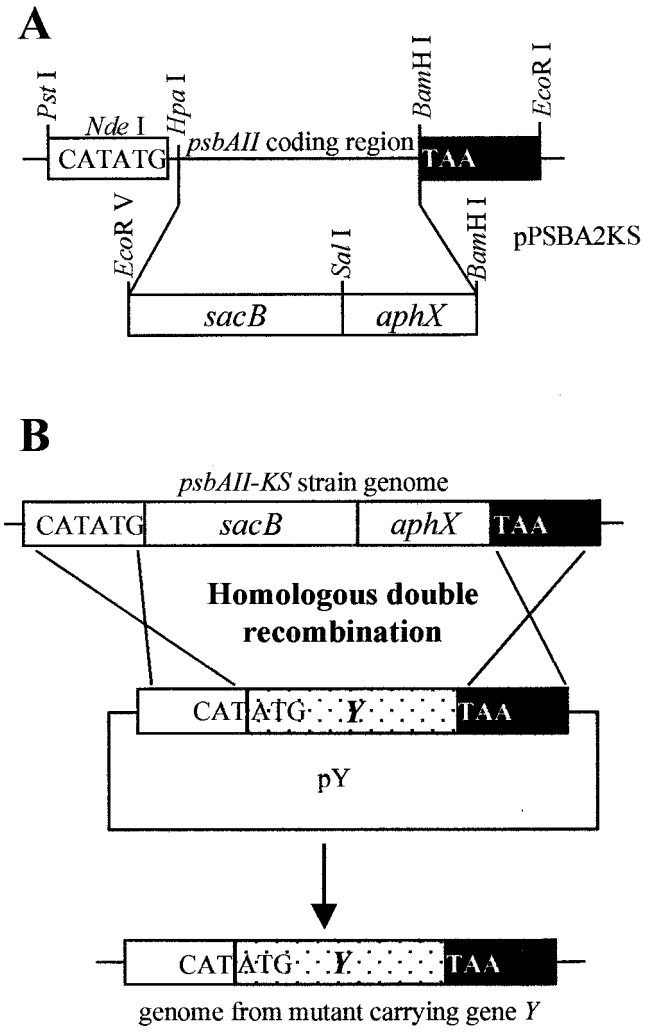

FIG. 2. Integration platform and its use to overexpress any gene in *Synechocystis* sp. strain PCC 6803. (A) Construction of the integration platform by replacement of the *psbAII* coding region with two selectable markers: the *sacB* gene, encoding a levan sucrase, and *aphX*, a gene conferring kanamycin resistance. (B) Use of the integration platform to express a generic gene, *Y*, in the *Synechocystis* sp. strain PCC 6803 genome, in lieu of *psbAII*; an *Nde*I site at the translation start site allows a simple in-frame introduction of gene *Y* into the integration platform, under the control of the *psbAII* promoter.

for expression in prokaryotic cells). This fragment was cloned between the *Nde*I and *Bam*HI sites of pPSBA2, leading to pIPI. A 2.5-kb *Nde*I-*Bgl*II fragment beginning at the translational start site of *crtP* and containing both the *crtP* and *crtB* coding sequences was cloned between the *Nde*I and *Bam*HI sites of pPSBA2, giving rise to pCrtPB. A 0.9-kb fragment containing the coding region of *crtR* was amplified by PCR, introducing an *Nde*I site at the start codon, changing it from GTG to ATG. Plasmid pCrtR was constructed by cloning this fragment between the *Nde*I and *Bam*HI sites in pPSBA2.

A 2.3-kb *Bam*HI-*Eco*RI fragment containing the *crtO* gene was introduced between the *Bcl*I and *Eco*RI sites in pSL1180, leading to pCrtO. A 1.37-kb *Bcl*I-*Bcl*I fragment was removed from pCrtO so that a large part of the *crtO* coding sequence was deleted; the resulting plasmid was named pΔCrtO. A plasmid with the same deletion but with a kanamycin resistance-*sacB* construct in its place was named pΔCrtOKS.

Segregation of all mutants was confirmed by PCR. Primers used for PCR are listed in Table 2.

Northern blot analysis. Total RNA was isolated from *Synechocystis* sp. strain PCC 6803 as previously described (22). A sample of total RNA (10 μg per lane) was separated by electrophoresis on formaldehyde gels containing 1.2% agarose and transferred to GeneScreen Plus according to the manufacturer's instructions. Probes were prepared by hot PCR with [α-^{32}P]dATP by using the cloned genes as templates. Hybridization was performed at 37°C for 12 h in a buffer containing 30% (vol/vol) formamide, 1% (wt/vol) sodium dodecyl sulfate (SDS), 10% (wt/vol) dextran sulfate, 1 mM Na_2EDTA, 30 mM Tris-HCl (pH 7.5), and 3× SSC (1× SSC is 0.15 M NaCl plus 1.5 mM sodium citrate).

Pigment analysis. *Synechocystis* sp. strain PCC 6803 cells were harvested from cultures in exponential growth phase (optical density at 730 nm ≈ 0.5, measured with a Shimadzu UV-160A spectrophotometer). Pigments were extracted with 100% methanol and extracts were kept under nitrogen. Carotenoids were separated by high-performance liquid chromatography (HPLC) on a Spherisorb ODS2 4.0- by 250-mm C_{18} column by using a 15-min gradient of ethyl acetate (0 to 100%) in acetonitrile-water-triethylamine (9:1:0.01, vol/vol/vol) at a flow rate of 1.5 ml/min. Absorption spectra for individual peaks were obtained with a photodiode array detector. Carotenoid species were identified by their absorption spectra and by their typical retention times. The content of each carotenoid was determined by using the following equation: $C_{car} = C_{chl} \times [(\varepsilon_{chl} \times A_{car})/(\varepsilon_{car} \times A_{chl})]$, where C_{chl} is the chlorophyll concentration in the pigment extract (calculated from the absorbance of the pigment extract at 663 nm and the extinction coefficient of chlorophyll *a* at the same wavelength: $E^{1\%} = 820$) and ε_{chl} and ε_{car} are the specific extinction coefficients of chlorophyll α and the carotenoids, respectively, at 440 nm. The extinction coefficients of the chlorophyll and the carotenoid are the same in methanol, ethanol, and acetonitrile, as the absorbance values and the shape of the absorption spectra in these solvents are the same (data not shown). Therefore, the specific extinction coefficients used in this study were those reported previously with methanol or ethanol (16). They were assumed to remain constant regardless of the ethyl acetate concentration in the HPLC eluent. A_{chl} and A_{car} are the peak areas on the chromatogram (recorded at 440 nm) of chlorophyll *a* and the carotenoid species, respectively.

Protein analysis. The presence of IPP isomerase was determined by SDS-polyacrylamide gel electrophoresis (PAGE). Soluble fractions were prepared from *Synechocystis* strains by breaking cells in 25 mM HEPES-NaOH (pH 7.0)–5 mM $MgCl_2$–15 mM $CaCl_2$–10% (vol/vol) glycerol–0.5% (vol/vol) dimethyl sulfoxide and collecting the supernatant fluid. Samples of these fractions (25 μg per lane) were loaded and polypeptides were separated on an SDS–10% PAGE gel. Proteins were visualized by staining the gel with Coomassie brilliant blue.

RESULTS

Construction of recombinant *Synechocystis* strains. DNA from plasmid pPSBA2KS was used to transform *Synechocystis* sp. strain PCC 6803 (wild type). Transformants were selected in the presence of kanamycin and were sucrose sensitive due to the presence of the *sacB* gene (28). The complete segregation of the *psbAII-KS* strain was confirmed by PCR assays with primers upstream and downstream of the *psbAII* gene (Fig. 3). This strain was then transformed with pIPI, pCrtR, or pCrtPB DNA to remove the kanamycin resistance-*sacB* cassette and replace it with the coding sequences of *ipi* (the IPP isomerase gene of *S. cerevisiae*), *crtR* (the β-carotene hydroxylase gene in *Synechocystis* sp. strain PCC 6803), and *crtP* and *crtB* (genes involved in earlier carotenoid biosynthesis steps in *Synechocystis*). Complete segregation of the *crtR*^{2} and *crtPB*^{2} strains (i.e., strains overexpressing these genes and having a gene copy under the control of the *psbAII* promoter along with the native one) was confirmed by PCR assays with the same set of primers (Fig. 3). The correct insertion of the *ipi* gene in the *ipi*^{Sc} strain (i.e., the strain overexpressing the *ipi* gene of *S. cerevisiae* under the control of the *psbAII* promoter) was confirmed by sequencing of a PCR product from the *Synechocystis ipi*^{Sc} strain that covered *ipi* and the flanking regions (data not shown).

The *psbAII-KS* strain of *Synechocystis* already contains a wild-type copy of *crtR*. Homologous single recombination at this site with pCrtR DNA would result in insertion of plasmid DNA and duplication of *crtR*. Even though single-recombination events are rare in *Synechocystis* sp. strain PCC 6803 (35), we chose to probe the *crtR* locus by PCR. Primers were designed upstream and downstream of the *crtR* gene to amplify only the wild-type copy of *crtR*. Indeed, as expected, the nor-

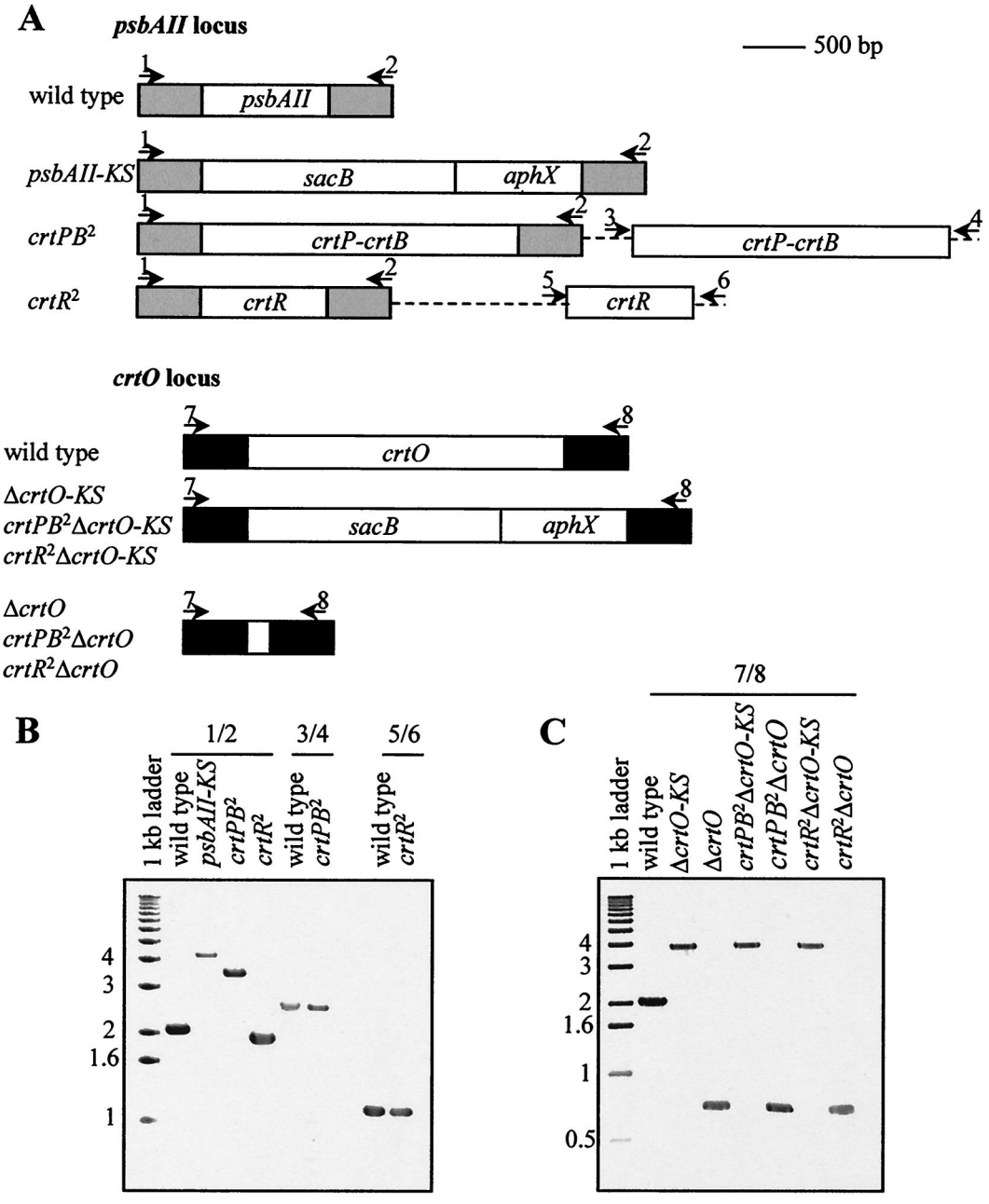

FIG. 3. Segregation of *Synechocystis* sp. strain PCC 6803 transformants. (A) Organization of the genomic DNA at the *psbAII* and *crtO* loci in the different strains. (B) PCR products indicating complete segregation of the *psbAII-KS*, *crtPB*^{2}, and *crtR*^{2} strains at the *psbAII* locus and the presence of an intact wild-type copy of *crtP*, *crtB*, and *crtR* in the *crtPB*^{2} and *crtR*^{2} strains. (C) PCR products indicating segregation of the appropriate strains at the *crtO* locus. The numbers 1/2, 3/4, 5/6, and 7/8 stand for the four different sets of primers used to amplify genes and check for complete segregation of the different strains. Primers are as follows: 1, 5′psbAII$_{up}$; 2, 3′psbAII$_{down}$; 3, 5′crtPB; 4, 3′crtPB; 5, 5′crtR; 6, 3′crtR; 7, 5′crtO; and 8, 3′crtO (Table 2). The hybridization locations of these primers are indicated in panel A.

mal wild-type *crtR* copy was present in the genome of the *crtR*^{2} strain and no foreign DNA fragment had been inserted at this locus (Fig. 3). Furthermore, PCR assays were performed with a primer designed upstream of the *crtR* gene combined with one designed downstream of the *psbAII* gene or with primers designed upstream of the *psbAII* gene and downstream of the *crtR* gene. In these cases, no PCR product was amplified (data not shown). This result excludes the possibility of single crossings over at the *crtR* locus. The presence of wild-type copies of *crtP* and *crtB* in the *crtPB*^{2} strain was checked in a similar fashion (Fig. 3).

Disruption of the β-carotene ketolase gene (*crtO*) has been reported to have no effect on the physiological functions of the resulting echinenoneless strain (8). The specific role of echinenone in *Synechocystis* remains unknown. Therefore, to simplify the carotenoid content of strains created in this study, the *crtO* gene was disrupted in the wild type and the *crtR*^{2} and *crtPB*^{2} strains. DNA from pΔCrtOKS was used to transform these strains. Complete segregation was confirmed by PCR assays with primers upstream and downstream of the *crtO* gene. The Δ*crtO-KS*, *crtPB*^{2}Δ*crtO-KS*, and *crtR*^{2}Δ*crtO-KS* strains, were then transformed with pΔCrtO DNA to remove the kana-

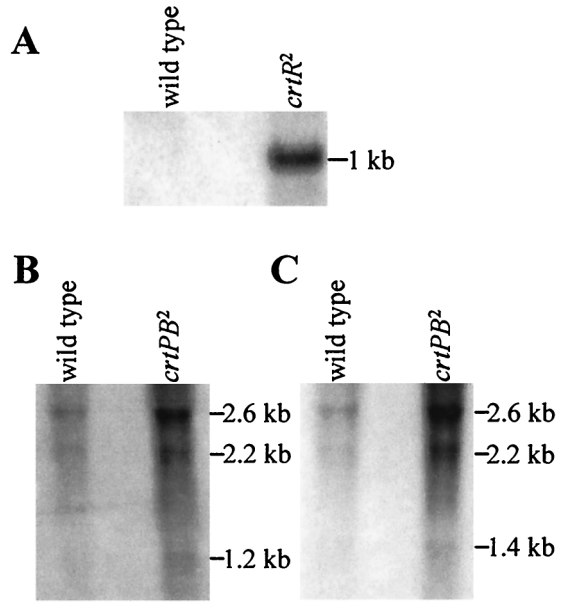

FIG. 4. Northern blot analysis of *crtR* and *crtPB*. Probes were prepared by PCR with the cloned genes as templates. (A) Steady-state level of the *crtR* transcript in the wild type and the *crtR*^{2} strain, determined by using a *crtR* gene-internal 400-bp fragment as a probe. (B) Northern blot analysis performed on the wild type and the *crtPB*^{2} strain by using a *crtB* gene-internal 400-bp fragment as a probe. (C) Northern blot analysis performed on the wild type and the *crtPB*^{2} strain by using a 400-bp fragment inside *crtP* as a probe.

mycin resistance-*sacB* construct and to select strains without an antibiotic resistance marker (Fig. 3A and C).

Overaccumulation of transcripts. The steady-state levels of *ipi*, *crtPB*, and *crtR* transcripts in overexpressing and control strains were determined by Northern blot analysis with a 400-bp gene internal DNA probe for each of these genes. In the wild-type strain, no transcript was detected with the *crtR* probe, indicating a very low level of transcript accumulation. However, in the *crtR*^{2} strain, a 1-kb *crtR* transcript accumulated to significant levels (Fig. 4A). These results show that the *psbAII* promoter indeed is suitable for overexpression and that the integration of a gene into the *psbAII* locus leads to overexpression of the inserted gene.

Accumulation of the *crtPB* transcript in the wild type and the *crtPB*^{2} strain was determined by using two different probes that were specific for *crtB* and *crtP*. Indeed, with both probes a three- or fourfold-stronger signal was detected in the *crtPB*^{2} strain than in the wild-type strain (Fig. 4B and C). With the *crtB* probe, transcripts of 1.2, 2.2, and 2.6 kb were detected in the *crtPB*^{2} strain (Fig. 4B). The smallest transcript was accumulated at a low level (which was barely reproduced on the photograph) and had a size close to that expected for the *crtB* transcript. The 2.6-kb band may correspond to a transcript that includes both *crtP* and *crtB*. The 2.2-kb transcript may be a processing product of the 2.6-kb transcript. With the *crtP* probe, 2.2- and 2.6-kb transcripts were detected along with a 1.4-kb transcript, the last being a very weak band presumably corresponding to a *crtP* transcript (Fig. 4C). The two gene-specific transcripts found in the overexpressing strain presumably are processing products of the 2.6-kb transcript.

In *Synechocystis* sp. strain PCC 6803, the gene coding for IPP isomerase has not been identified: in CyanoBase (see Materials and Methods) no sequence with convincing similarity to yeast *ipi* was found. As expected, no transcript was detected in the wild-type *Synechocystis* strain with the *ipi* probe. In the *ipi*^{Sc} strain a 1-kb *ipi* transcript was easily detected and was accumulated to a significant level (Fig. 5A). Therefore, as expected, the *psbAII* promoter can drive overexpression of various genes, including genes from other organisms.

Accumulation of IPP isomerase in the *ipi*^{Sc} strain. The presence of IPP isomerase in the *ipi*^{Sc} strain was determined by SDS-PAGE. Proteins were visualized by staining the gel with Coomassie brilliant blue (Fig. 5B). Two proteins with molecular masses close to 40 and 20 kDa were detected only in the *ipi*^{Sc} strain and not in the wild type. The *ipi*^{Sc}-specific band at around 40 kDa has an apparent molecular mass consistent with that determined by SDS-PAGE for IPP isomerase purified from *S. cerevisiae* (1). The smaller, 20-kDa band (barely visible on the photograph) may represent an IPP isomerase degradation product.

Carotenoid accumulation. To determine whether the carotenoid content and composition had been affected by introduc-

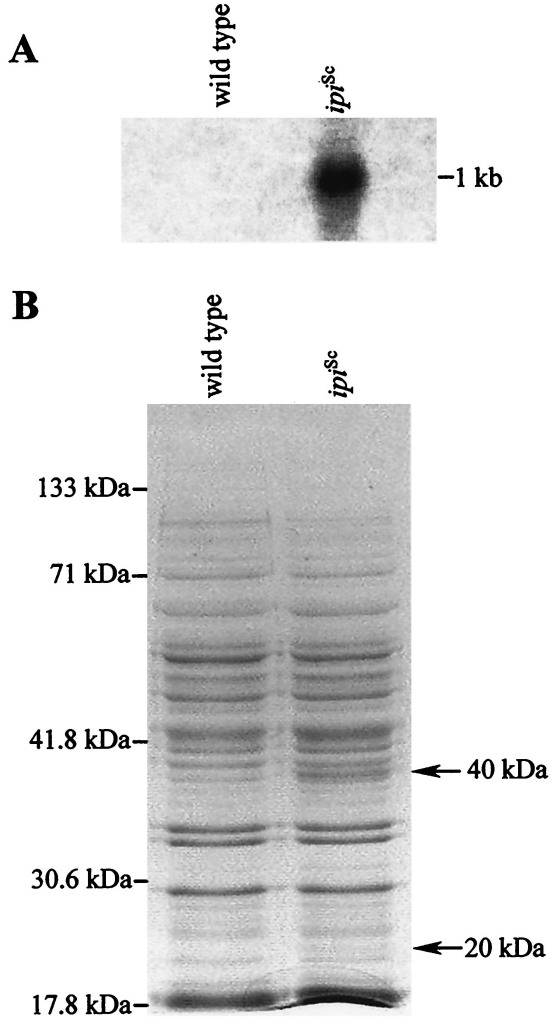

FIG. 5. Detection of the *ipi* transcript and IPP isomerase. (A) Steady-state level of the yeast *ipi* transcript in the wild type and the *ipi*^{Sc} strain. The probe was prepared by PCR with an *ipi* gene-internal 400-bp fragment as a template. (B) SDS-PAGE analysis of the soluble cell fraction of the wild type and the *ipi*^{Sc} strain. This fraction, prepared by breaking cells and collecting the supernatant, was loaded (20 μg protein per lane) on an SDS gel containing 10% acrylamide. Proteins were visualized by staining the gel with Coomassie brilliant blue. Arrows show two proteins that appear to be present in the *ipi*^{Sc} strain and not in the wild type.

TABLE 3. Carotenoid contents in wild-type and mutant *Synechocystis* sp. strain PCC 6803[a]

Strain description	Carotenoid content (μg/ml/OD_{730} unit)				
	Myxoxanthophyll	Zeaxanthin	Echinenone	β-Carotene	Total
Wild type	0.45 ± 0.02	0.39 ± 0.02	0.25 ± 0.01	0.53 ± 0.02	1.62
crtR^2	0.42 ± 0.04	0.98 ± 0.02	0.12 ± 0.01	0.20 ± 0.01	1.72
crtB^2	0.75 ± 0.02	0.55 ± 0.02	0.27 ± 0.02	0.54 ± 0.02	2.11
ipi^{Sc}	0.47 ± 0.03	0.41 ± 0.02	0.25 ± 0.01	0.53 ± 0.02	1.66
Δ*crtO*	1.05 ± 0.05	0.44 ± 0.02	0	0.56 ± 0.03	2.05
crtR^2 Δ*crtO*	0.35 ± 0.01	0.95 ± 0.02	0	0.19 ± 0.01	1.49
crtPB^2 Δ*crtO*	1.19 ± 0.10	0.62 ± 0.02	0	0.68 ± 0.03	2.49

[a] Values are averages from nine determinations performed on three different cultures. Pigments were extracted from cells with methanol. Methanol extracts were analyzed by HPLC and the carotenoid content was determined as described in Materials and Methods. OD_{730}, optical density at 730 nm.

tion of the various mutations, methanol extractions were performed on intact cells and the carotenoid content of the extracts was analyzed by HPLC. The results are shown in Table 3 (see also Fig. 6). Overexpression of *crtR* in the *crtR*^{2} strain led to a 2.5-fold increase in zeaxanthin accumulation and a 2-fold reduction in β-carotene and echinenone content. The myxoxanthophyll content was barely affected by overexpression of *crtR*. Overexpression of *crtP* and *crtB* induced a 60% increase in myxoxanthophyll content and also a significant increase in zeaxanthin content in the *crtPB*^{2} strain compared to those in the wild-type strain. Echinenone and β-carotene accumulations were unaffected. In contrast to the results of *crtR* and *crtPB* overexpression, introduction of yeast *ipi* did not lead to any significant change in the carotenoid content.

The carotenoid content in the Δ*crtO*, *crtR*^{2}Δ*crtO*, and *crtPB*^{2}Δ*crtO* strains was analyzed as well (Table 3). Disruption of the *crtO* gene and an absence of echinenone in a *crtO*-deficient strain were reported to have no effect on the growth rate, the photosynthetic oxygen evolution, or the carotenoid content (besides the absence of echinenone) (8). As expected, in the three Δ*crtO* strains echinenone was absent. In the Δ*crtO* strain in a wild-type background, β-carotene and zeaxanthin contents were insignificantly changed compared to those in the wild-type strain, in agreement with previous observations (8). However, in our study, in the Δ*crtO* strain myxoxanthophyll accumulated to levels more than twice that of the control. Disruption of *crtO* in the *crtPB*^{2} strain resulted in a slight increase in β-carotene and zeaxanthin content and a more substantial increase (30%) in myxoxanthophyll content. On the other hand, the nonechinenone carotenoid content in the *crtR*^{2} Δ*crtO* strain was little modified from that in the *crtR*^{2} strain.

Physiological effects. Growth and carotenoid composition of the different mutant strains were studied after growth at three photon flux densities: 50, 100, and 200 μmol of photons · m^{-2} · s^{-1}. At these three photon flux densities, the doubling times of the *crtPB*^{2} and *crtR*^{2} strains were similar to that of the wild-type strain (between 12 and 13 h [data not shown]). Similarly, increasing the photon flux density did not significantly affect carotenoid content in the wild-type strain or in the *crtPB*^{2} or *crtR*^{2} strain (data not shown).

DISCUSSION

The *psbAII* gene is known to have a strong promoter in *Synechocystis* sp. strain PCC 6803 (23). In this study, we show that this promoter can be used to drive transcription and to overexpress various genes in *Synechocystis*: introduction of coding sequences of genes involved in carotenoid biosynthesis in lieu of the *psbAII* coding sequence led to a stable overaccumulation of the corresponding transcripts. The degree of overaccumulation appeared to depend on the gene that was introduced. Overexpression of *Synechocystis* genes involved in carotenoid biosynthesis appeared to lead in most cases to an increased synthesis of the corresponding enzymes, because the carotenoid content of the *crtPB*- and *crtR*-overexpressing strains was modified. This overexpression system thereby provides a way to better understand carotenoid biosynthesis regulation.

Overexpression of the IPP isomerase gene from *S. cerevisiae* in the *ipi*^{Sc} strain led to the accumulation of the corresponding transcript and of a protein with a molecular mass of about 40 kDa. This molecular mass is consistent with the molecular mass determined by SDS-PAGE for IPP isomerase purified from *S. cerevisiae* (1). Expression of the yeast IPP isomerase gene was reported to enhance carotenoid biosynthesis in *Escherichia coli* (14). In this system, increases in carotenoid content were consistent with quantitative increases in IPP isomerase activity. In our study, *ipi* overexpression had no effect on the carotenoid content of the *ipi*^{Sc} strain. IPP isomerase activity will have to be examined to determine if the exogenous IPP isomerase is functionally active in the mutant strain. The absence of an effect of yeast IPP isomerase on carotenoid composition may indicate that either this enzyme is too different

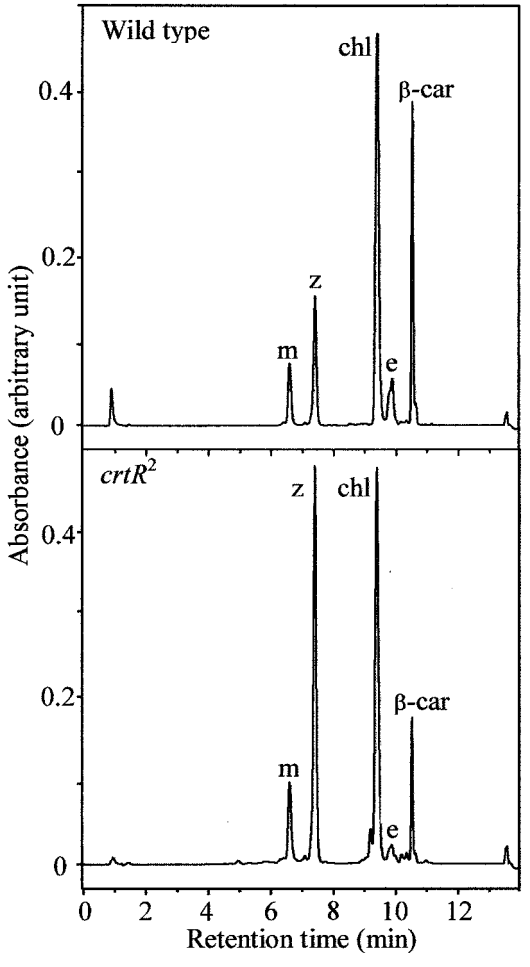

FIG. 6. HPLC analysis of pigments extracted from wild-type and *crtR*^{2} cells of *Synechocystis* sp. strain PCC 6803. Chromatograms were recorded as a function of the absorbance at 440 nm. m, myxoxanthophyll; z, zeaxanthin; chl, chlorophyll *a*; e, echinenone; β-car, β-carotene.

from the *Synechocystis* IPP isomerase to be functional in this organism (for example, it may need other polypeptides for function in vivo) or isomerization of IPP is not a rate-limiting step in carotenoid biosynthesis in *Synechocystis* sp. strain PCC 6803.

Overexpression of genes encoding phytoene synthase and phytoene desaturase (*crtB* and *crtP*, respectively) in the *crtPB*^{2} strain was indicated by an overaccumulation of a 2.6-kb transcript. This transcript is likely to include both the *crtP* and *crtB* coding sequences. A transcript of this size was also detected in the wild-type strain, which suggests that the *crtP* and *crtB* genes are part of the same operon. The presence of two small (1.2- and 1.4-kb) transcripts specific to *crtB* and *crtP* in the *crtPB*^{2} strain may indicate either processing of the longer transcript or the possibility of transcription of the two genes independently. Earlier data showing that disruption of *crtP* did not greatly affect the expression of a reporter gene placed in lieu of *crtB* were interpreted to indicate that *crtB* may have its own promoter (17). This result was confirmed recently (9) by detection of *crtB* and *crtP* transcripts with sizes smaller than 2.5 kb. However, judging from the sizes on the Northern blots, the majority of the *crtB*-containing transcripts in our study appear to be dicistronic *crtPB* transcripts.

Phytoene desaturation has been reported to be a rate-limiting step in carotenoid biosynthesis in *Synechococcus* sp. strain PCC 7942 (7). However, overexpression of the phytoene desaturase gene (*crtP*) by itself did not induce an increase in the carotenoid content of the corresponding overexpressing strain (data not shown). On the other hand, overexpression of both *crtP* and *crtB* (the latter being the phytoene synthase gene) resulted in a 50% increase in the myxoxanthophyll and zeaxanthin content. As these two carotenoids are terminal biosynthesis products in *Synechocystis*, this result is consistent with an increased carotenoid biosynthesis capacity. Therefore, we suggest that phytoene synthase is slightly rate limiting in wild-type *Synechocystis* sp. strain PCC 6803 under the conditions we used.

The myxoxanthophyll biosynthesis pathway remains unknown. However, as indicated in Fig. 1, this carotenoid is thought to be synthesized from γ-carotene (2). Indeed, overexpression of *crtP* and *crtB* may lead to increased levels of γ-carotene, part of which would be converted to myxoxanthophyll. In the *crtPB*^{2} strain, the increased γ-carotene synthesis would be expected to also lead to an increase in β-carotene levels if more of this pigment could be accommodated. The steady-state β-carotene amount remained unchanged, but the level of zeaxanthin, which is formed by hydroxylation of β-carotene, was increased in the *crtPB*^{2} strain. Interestingly, the only mutant in which β-carotene levels were significantly increased in comparison to that in the wild type is the *crtPB* overexpresser strain that lacks *crtO*: in this strain, it is possible that β-carotene occupied some of the carotenoid binding sites that were left vacant due to the lack of echinenone. These results suggest that the β-carotene level cannot be increased very much but that zeaxanthin or myxoxanthophyll levels are less strictly regulated. Similar observations have been reported with transgenic *Synechococcus* sp. strain PCC 7942, in which expression of an algal gene encoding β-C-4-oxygenase led to the production of various ketocarotenoids (which normally do not accumulate in this cyanobacterium), with a decrease in zeaxanthin content but no change in β-carotene accumulation (11).

As levels of zeaxanthin and myxoxanthophyll could be increased almost threefold compared to wild-type levels, there may be more unoccupied binding sites available for zeaxanthin and myxoxanthophyll than for β-carotene. In addition, zeaxanthin and myxoxanthophyll may also be present as free molecules in the membrane or cell wall. In the membrane, dipolar carotenoids, like zeaxanthin or myxoxanthophyll, that are not bound to protein are anchored in the bilayer, with their long axis almost perpendicular to the membrane plane, thus essentially spanning the membrane. However, β-carotene is distributed homogeneously within the lipophilic part of the membrane without well-defined orientation. As a result, dipolar carotenoids decrease membrane fluidity whereas β-carotene tends to increase it (12). These different effects of carotenoids on membrane fluidity may explain why zeaxanthin and myxoxanthophyll accumulate in the *crtPB*^{2} strain whereas β-carotene does not.

Overexpression of the gene coding for β-carotene hydroxylase in the *crtR*^{2} strain led to a large increase in zeaxanthin accumulation in this strain. In logarithmically growing cultures of the wild-type strain, the amount of zeaxanthin represents a quarter of the total carotenoid content and β-carotene is the major carotenoid (a third of the total). In the *crtR*-overexpressing strain, zeaxanthin is the major carotenoid (more than half of the total amount) and β-carotene becomes a minor component of the cells. This result suggests that β-carotene hydroxylation is a rate-limiting step in zeaxanthin biosynthesis in *Synechocystis* sp. strain PCC 6803: the conversion of β-carotene into zeaxanthin is regulated by the amount of β-carotene hydroxylase available. This result is consistent with what was previously reported for a noncarotenogenic microorganism: the amount of production of zeaxanthin in *E. coli* was related to the expression levels of β-carotene hydroxylase (30).

Disruption of the β-carotene ketolase gene (*crtO*) has been reported not to affect the *Synechocystis* carotenoid content, except that echinenone, which is produced by β-carotene ketolase activity, is absent in a strain with such a disruption (8). In the same work, the myxoxanthophyll content in the wild type and in the *crtO*-deficient strain appeared to be very small compared to the content of other carotenoids. In our study a higher myxoxanthophyll content was observed in the Δ*crtO* and *crtPB*^{2} Δ*crtO* strains than in the wild type and the *crtPB*^{2} strain. The content of other carotenoids (β-carotene and zeaxanthin) remained virtually unchanged compared to that in the wild-type background. In our study, disruption of the *crtO* gene in the wild type and in the *crtPB*^{2} mutant led to an increase in overall carotenoid levels, mainly due to an increase in myxoxanthophyll amounts. The difference observed in myxoxanthophyll amounts between the previous study (8) and our work may be due to the use of different methods to extract and analyze carotenoids.

How this apparent regulation of echinenone versus myxoxanthophyll accumulation works is as yet unknown, but it may be related to the relative accumulation of one of the intermediates in the biosynthesis pathway. In the *crtR*^{2} strain, the echinenone content was reduced because overexpression of β-carotene hydroxylase induced a higher conversion of β-carotene into zeaxanthin and left less substrate available for conversion to echinenone. Consistent with the reasoning above, disruption of *crtO* in the *crtR*^{2} strain did not lead to an increase in myxoxanthophyll content.

Carotenoid biosynthesis gene clusters have been isolated and the functions of individual genes have been identified for several carotenogenic bacteria (3). Metabolic engineering, the modification of metabolic networks in living cells to produce desirable chemicals with superior yields and productivity by recombinant DNA techniques, allows the production of large amounts of useful carotenoids in vivo (see reference 21 for a review). In this study, we show that such a metabolic engineering approach can be used with *Synechocystis* sp. strain PCC

6803 to overproduce desirable carotenoids, such as zeaxanthin. Furthermore, the system developed in this study allows for gene replacement without introduction of antibiotic resistance cassettes in the final overexpressing strains. The absence of cassettes in such strains is a positive feature highlighting the increasing desire of the biotechnology industry to avoid spreading antibiotic resistance cassettes.

ACKNOWLEDGMENTS

We are grateful to G. Ajlani for kindly providing plasmid pRL271. We also thank the members of the Vermaas laboratory, particularly Jason W. Cooley and Crispin A. Howitt, for useful discussions.

Funding for materials and supplies in the Vermaas laboratory was provided by a grant from the National Science Foundation (MCB-9728400).

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