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Cloning and functional expression in *Escherichia coli* of a cyanobacterial gene for lycopene cyclase, the enzyme that catalyzes the biosynthesis of β-carotene

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Carotenoids with cyclic end groups are essential components of the photosynthetic membrane in all known oxygenic photosynthetic organisms. These yellow pigments serve the vital role of protecting against potentially lethal photo-oxidative damage. Many of the enzymes and genes of the carotenoid biosynthetic pathway in cyanobacteria, algae and plants remain to be isolated or identified. We have cloned a cyanobacterial gene encoding lycopene cyclase, an enzyme that converts the acyclic carotenoid lycopene to the bicyclic molecule β-carotene. The gene was identified through the use of an experimental herbicide, 2-(4-methylphenoxy)triethylamine hydrochloride (MPTA), that prevents the cyclization of lycopene in plants and cyanobacteria. Chemically-induced mutants of the cyanobacterium *Synechococcus* sp PCC7942 were selected for resistance to MPTA, and a mutation responsible for this resistance was mapped to a genomic DNA region of 200 bp by genetic complementation of the resistance in wild-type cells. A 1.5 kb genomic DNA fragment containing this MPTA-resistance mutation was expressed in a lycopene-accumulating strain of *Escherichia coli*. The conversion of lycopene to β-carotene in these cells demonstrated that this fragment encodes the enzyme lycopene cyclase. The results indicate that a single gene product, designated *lcy*, catalyzes both of the cyclization reactions that are required to produce β-carotene from lycopene, and prove that this enzyme is a target site of the herbicide MPTA. The cloned cyanobacterial *lcy* gene hybridized well with genomic DNA from eukaryotic algae, thus it will enable the identification and cloning of homologous genes for lycopene cyclase in algae and plants

Carotenoid; CPTA; Herbicide, -(4-Methylphenoxy)-triethylamine hydrochloride; MPTA; *Synechococcus* sp. PCC 7942

1. INTRODUCTION

Carotenoid pigments are ubiquitous and essential components of all photosynthetic organisms and are found in many species of bacteria and fungi [1]. Their essential function in plants is that of protecting against photo-oxidative damage sensitized by chlorophyll in the photosynthetic apparatus [2,3] but they play a variety of other roles as well. They serve as accessory pigments in light-harvesting for photosynthesis and are integral components of photosynthetic reaction centers. Carotenoids are involved in the thermal dissipation of light energy captured by the light-harvesting antenna [4], are substrates for the biosynthesis of the plant growth regulator abscisic acid [5,6] and are the coloring agents of many flowers, fruits and animals [1]. Certain cyclic carotenoids, including β-carotene, are precursors of vitamin A in human and animal diets and are of current interest as potential anticancer agents [7,8].

The generally accepted pathway of carotenoid biosynthesis in plants [1,9,10] begins with the head-to-head condensation of two molecules of the soluble 20 carbon compound geranylgeranyl pyrophosphate to give the colorless, membrane-bound carotenoid phytoene (Fig. 1). This two-step reaction in plants and cyanobacteria is catalyzed by a single, soluble enzyme – phytoene synthase [11–14]. Two sequential desaturations of phytoene result in the formation of first phytofluene and then ζ-carotene. Both of these reactions are carried out by a single enzyme in plants and cyanobacteria –phytoene desaturase [15–17]. This enzyme, and enzymes catalyzing subsequent steps in the pathway are believed to be membrane-bound [18]. Two additional desaturations yield the symmetrical red carotenoid pigment lycopene, which is then converted to the yellow β-carotene via cyclization reactions at each end of the molecule (Fig. 1). Subsequent reactions in the pathway

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Abbreviations: CPTA, 2-(4-chlorophenylthio)triethylamine hydrochloride; EMS, ethane methylsulfonate; IPTG, isopropylthio-β-D-galactoside; Kan[r]; resistant to kanamycin; *lcy*, lycopene cyclase gene, MPTA, 2-(4-methylphenoxy)triethylamine hydrocylorıde; M[r]-5, MPTA-resistant mutant number five; *pds*, phytoene desaturase gene; *psy*, phytoene synthase gene

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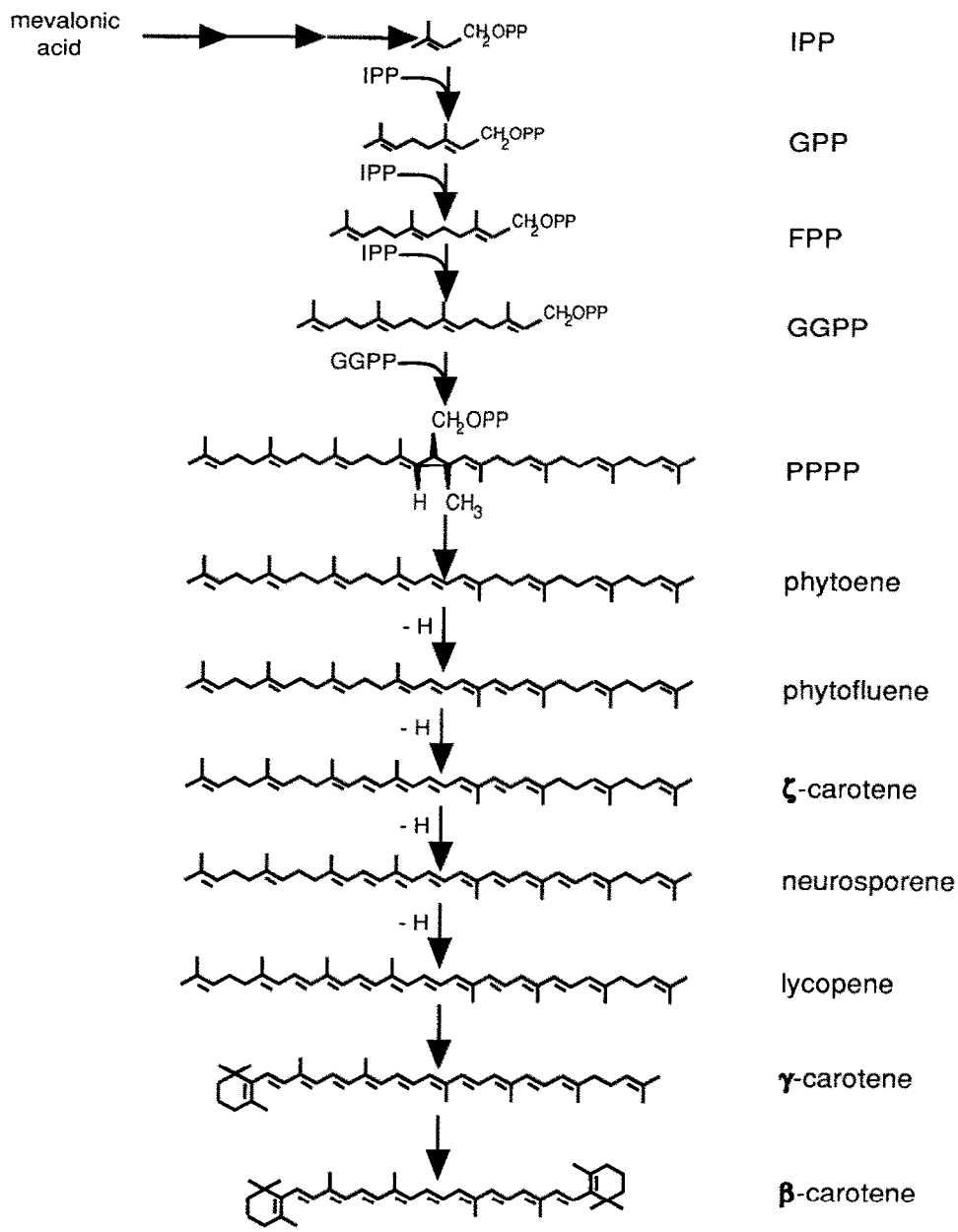

Fig. 1. Carotenoid biosynthetic pathway in cyanobacteria and plants. IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; PPPP, prephytoene pyrophosphate.

involve the addition of various oxygen functions to form the xanthophylls or oxygenated carotenoids.

Despite many efforts, few of the enzymes or genes of the carotenoid biosynthetic pathway have been identified and isolated in oxygenic photosynthetic organisms. The difficulties of preserving catalytic activity during purification of these largely membrane-bound enzymes have proved formidable, and the unavailability of labelled substrates for enzyme assay is also an obstacle. Genes for the complete carotenoid biosynthetic pathways in the photosynthetic bacterium *Rhodobacter capsulatus* [19] and the nonphotosynthetic bacteria *Erwinia uredovora* [20] and *Erwinia herbicola* [21] have been cloned and sequenced. It was initially thought that such genes would provide molecular probes enabling the identification of homologous genes in oxygen-evolving photosynthetic organisms (cyanobacteria, algae, and plants). However, this approach has not proven fruitful. Two genes, those for phytoene synthase [12,22] and phytoene desaturase [16,17,23] have now been cloned from oxygenic photosynthetic organisms using other approaches. There is high conservation in the amino acid sequences of the cyanobacterial phytoene desaturase and the homologous gene product of eukaryotic

algae and higher plants [16,24], but very little resemblance to the known bacterial or fungal phytoene desaturases.

We are using the transformable cyanobacterium *Synechococcus* sp. PCC7942 as a model organism to study the carotenoid biosynthetic pathway in oxygen-evolving organisms. We earlier cloned genes for phytoene synthase (*psy*, formerly called *pys*) and phytoene desaturase (*pds*) in this organism [12,23] and subsequently identified, cloned and sequenced plant and algal genes using the cyanobacterial genes as probed 16,23–25]. The gene *pds* was identified with the aid of a bleaching herbicide, norflurazon, that specifically inhibits the desaturation of phytoene by interacting with the enzyme phytoene desaturase [26]. We reasoned that a point mutation in the gene encoding the enzyme, leading to an amino acid substitution in the polypeptide, could confer resistance to the herbicide. By selecting for chemically-induced mutants which are resistant to norflurazon, and then mapping these mutations by genetic complementation of the resistance in the wild-type strain, we located the gene for phytoene desaturase [23,27].

A number of substituted triethylamine compounds inhibit the formation of cyclic carotenoids and result in the accumulation of lycopene in plants, algae, and cyanobacteria (reviewed in [28]). A particularly effective inhibitor of this class is the compound 2[(4-methylphenoxy)triethylamine hydrochloride (MPTA) [29,30]. The target site of MPTA and related compounds is believed to be the enzyme lycopene cyclase [28]. In this communication we describe the isolation of MPTA-resistant mutants of *Synechococcus* sp. PCC 7942 and the cloning of a gene that contains one of the mutations. Expression of this gene in a lycopene-accumulating strain of *E. coli* showed unequivocally that the MPTA-resistance gene encodes lycopene cyclase. DNA hybridization analysis indicates that this gene is conserved among other photosynthetic eukaryotes.

2. MATERIALS AND METHODS

2.1 *Organisms and growth conditions*

Cultures of *Synechococcus* sp PCC7942 (*Anacystis nidulans* R2) were grown in BG11 medium at 35°C as described previously [31]. For selection of MPTA-resistant mutants and transformants, cultures were spread on solid BG11 medium containing 1.5% agar (Bacto) and 20 or 30 μM MPTA. The MPTA was added to molten agar at 50°C from a 20 mM stock solution in MeOH immediately before pouring into petri plates Where required, kanamycin was incorporated in the BG11 agar plates at a concentration of 10 μg/ml

Escherichia coli strain XL1-Blue was used as host for genomic libraries of *Synechococcus* in the plasmid vector pBR329K [27] with kanamycin at 30 μg/ml for selection, and as a host for the plasmid pBluescript II KS$^+$ (Stratagene) and other plasmids with ampicillin at 100 μg/m! and/or chloramphenicol at 50 μg/ml Cultures of *E. coli* were grown in darkness at 37°C in LB medium [32].

2.2 *Selection of MPTA-resistant mutants*

Cultures of *Synechococcus* were treated with the chemical mutagen ethane methylsulfonate (EMS; from Sigma) as described previously [31], and were allowed to grow for 24 h in liquid culture before selection on BG11 agar plates containing 20 or 30 μM MPTA Herbicide tolerance in the selected mutants was examined by spotting 3 μl of dilute cultures on a master petri plate of BG11 agar After two weeks of growth, replicas were made on plates containing different concentrations of MPTA and allowed to grow for two more weeks Mutants were maintained on BG11 agar plates containing 4 μM MPTA. The MPTA was a generous gift of Dr. Henry Yokoyama, Fruit and Vegetable Chemistry Laboratory, Agricultural Research Service, United States Department of Agriculture, Pasadena, CA 91106

2.3. *Molecular cloning*

Genomic DNA was extracted [33] from MPTA-resistant *Synechococcus* mutant number five (M^r-5), completely digested with *Eco*RI, and used to construct a genomic library in the *Eco*RI site of pBR329K [27] The parent wild-type strain was transformed [33] with DNA of this M^r-5 library, and transformants were selected on BG11 agar plates containing both MPTA (20 or 30 μM) and kanamycin (10 μM). The strategy described in Chamovitz et al. [27] was employed to recover the plasmid vector along with flanking genomic DNA. A 2.1 kb *Eco*RI-*Sal*I genomic DNA fragment, which was recovered in this way, was used as a molecular probe for Southern DNA analysis. ^{32}P-Labelling was carried out by the random priming method. An 8.5 kb genomic clone, identified by colony hybridization [32] using this probe, was subcloned in the plasmid pBluescript II KS$^+$ (Stratagene) and was designated pM5EE. Various fragments of this clone were subcloned in the vector pBluescript II KS$^+$ and their ability to transform the wild-type strain of *Synechococcus* sp. PCC7942 to MPTA resistance was tested. Plasmid DNA minipreps were prepared using the procedure of Del Sal et al. [34].

2 4 *Construction of a lycopene-accumulating strain of* E. coli

A cluster of genes encoding carotenoid biosynthesis enzymes has been cloned from *Erwinia uredovora* [20]. A 2.26 kb *Bst*EII-*Sna*BI fragment was deleted from the plasmid pCAR16 [20], and a 3.75 kb *Asp*^{718}-*Eco*RI fragment, carrying *crtE*, *crtB* and *crtI*, was subcloned in the *Eco*RV site of the plasmid vector pACYC184. The recombinant plasmid was designated pACCRT-EIB (Fig 2).

2 5. *Functional expression of lycopene cyclase in* E coli

A 7.2 kb *Xba*I-*Eco*RI DNA fragment, containing the MPTA resistance mutation, was cloned in the multiple cloning site of the vector pBluescript II SK$^+$ (Stratagene) and then excised as a *Sac*I-*Eco*RI fragment slightly larger in size. This *Sac*I-*Eco*RI fragment was then cloned in the IPTG-inducible expression vector pTrcHisB (Invitrogen) and the resulting plasmid, designated pLYCBV-M5XE, was used to transform competent cells of *E coli* strain XL1-Blue containing the

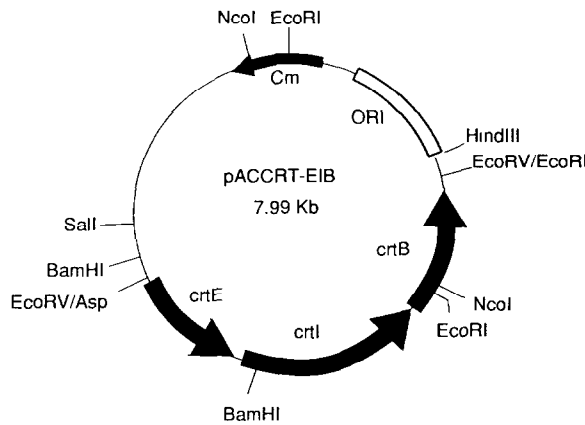

Fig 2 Structure of the plasmid pACCRT-EIB.

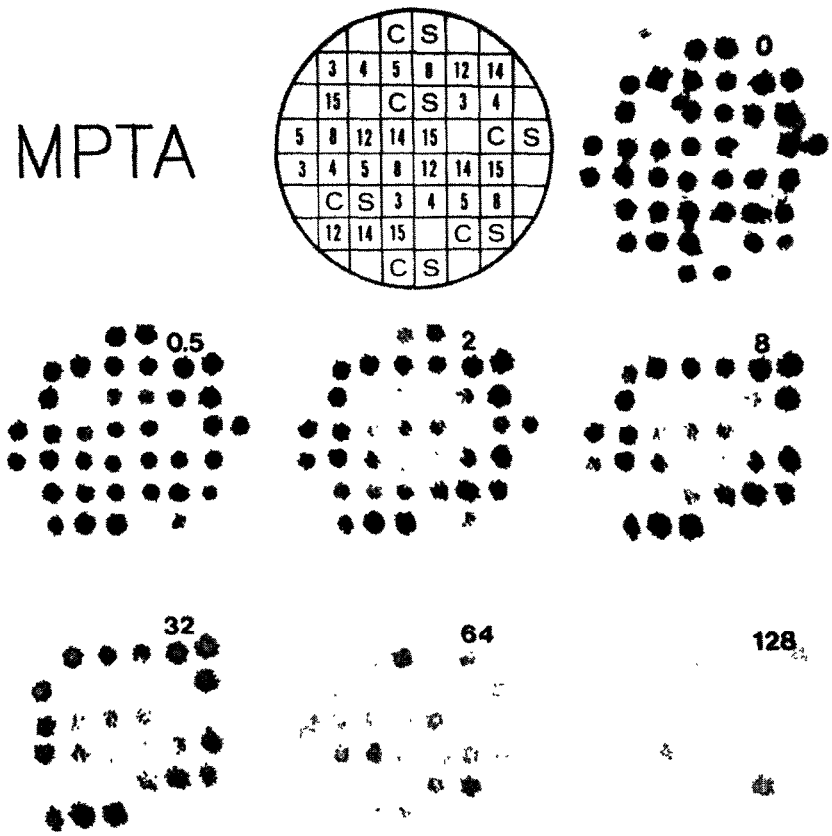

Fig. 3. MPTA-resistance in mutants (numbered) and two wild-type strains (C and S) of the cyanobacterium *Synechococcus* sp. PCC 7942. The wild-type strain indicated by the letter C was the parent strain from which the mutants were derived. The concentration of MPTA, in micromolar, is given in the upper right corner of each petri plate. Each strain was spotted in several places on each petri plate because of a modest positional influence on survival.

plasmid pACCRT-EIB Transformed cells were plated on LB agar plates containing ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml), and spread with 10 μl of a 100 mM aqueous solution of isopropylthio-β-D-galactoside (IPTG) one hour before cells were plated. Several other, smaller genomic DNA fragments were also cloned in the pTrcHis vector and examined for lycopene cyclase activity.

2.6. *Carotenoid pigment analysis*

Cultures of *E coli* were grown for 18 h at 37°C in LB medium containing 1 mM IPTG. Bacterial cells from 50 ml of the suspension culture were harvested by centrifugation and carotenoid pigments were extracted by dissolving the pellet in 90% acetone. The carotenoids were analyzed by HPLC on a Spherisorb ODS1 25 cm reverse phase column as previously described [12]. A Merck/Hitachi HPLC apparatus, consisting of a L6200 pump, L300 multichannel photodetector and D6000 interphase was used, employing an isocratic solvent system of acetonitrile/methanol/isopropanol (85:10:5). The Hitachi DAD Manager software allowed for the simultaneous detection of phytoene and colored carotenoids. Individual carotenoids were identified on the basis of online absorption spectra and typical retention times in comparison to reference standards of lycopene and β-carotene.

3. RESULTS

3.1. *Selection and mapping of MPTA-resistant mutants*

Wild-type cells of the cyanobacterium *Synechococcus* sp. PCC7942 did not grow on BG11 agar plates containing more than 2 μM of the bleaching herbicide MPTA. Following mutagenesis of the wild-type strain with EMS and selection for growth on agar plates containing 30 μM MPTA, we selected a number of herbicide-tolerant mutants. Mutant number five (M^r-5) exhibited the highest resistance and grew on agar plates containing 50 μM MPTA (Fig. 3). This mutant was used for all subsequent experiments described in this report.

The genetic basis of MPTA resistance in M^r-5 was established by transformation experiments. Genomic DNA was extracted from M^r-5, digested with the endonuclease *Eco*RI and transferred to wild-type cells of *Synechococcus* PCC 7942. The appearance of colonies at high frequency (ca. 10^4 times that for untransformed controls) on agar plates containing 30 μM MPTA demonstrated the genetic character of the resistance to MPTA and indicated that either a single lesion or closely-linked mutations were responsible for the resistance trait.

3.2. *Cloning the MPTA-resistance gene*

A genomic library of mutant M^r-5 was constructed in

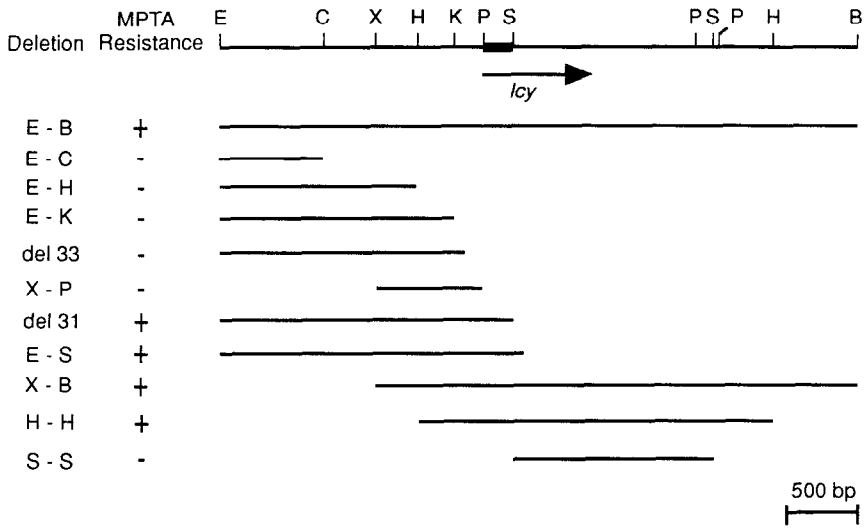

Fig. 4. Mapping the mutation for MPTA^r. Various DNA fragments (indicated below the restriction map) were transfected to cells of the wild-type strain of *Synechococcus* sp. PCC7942. Their ability to confer herbicide-resistance (indicated by +) was established by plating the transformants on MPTA containing medium. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xba*I.

the *Eco*RI site of the plasmid vector pBR329K [27]. DNA of this library was transfected to cells of the wild-type strain as closed circular plasmids, and transformants were selected for growth on BG11 agar plates containing both MPTA and kanamycin. Stable transformation in *Synechococcus* sp. PCC 7942 occurs by integration of the foreign DNA into the bacterial chromosome following homologous recombination [35,36]. The doubly-resistant transformants resulted from a single cross-over event between a plasmid containing genomic DNA with the mutation for MPTA resistance (MPTA^r), and its homologous sequence in the chromosome. Consequently, the pBR329K plasmid was integrated into the cyanobacterial chromosome adjacent to the MPTA-resistance gene (see Fig. 2 in [27] for detailed explanation).

A portion of the pBR329K vector was recovered along with a fragment of the adjacent genomic DNA, by digestion of the genomic DNA of a doubly-resistant transformant with either *Sal*I or *Bam*HI endonucleases, followed by DNA ligation reaction, transfection to cells of *E. coli* and selection for kanamycin resistance (Kan^r). From one such Kan^r-MPTA^r transformant we recovered a 2.1 kb *Eco*RI-*Sal*I fragment of cyanobacterial genomic DNA. This fragment was used as a molecular probe to screen the original M^r-5 genomic DNA library, and identified a plasmid containing an 8.5 kb *Eco*RI genomic insert. Transfection of this 8.5 kb DNA fragment into cells of the wild-type strain of *Synechococcus* sp. PCC7942 resulted in a high frequency of herbicide-resistant colonies thus confirming that this fragment contained the mutation conferring MPTA^r. A similar test indicated that the mutation was located in a 4.6 kb *Eco*RI-*Bam*HI fragment, and subsequently it was mapped to a region of 0.2 kb that is delineated by the *Pst*I and *Sal*I restriction sites (Fig. 4).

3.3. *The MPTA resistance gene encodes lycopene cyclase*

Since MPTA inhibits lycopene cyclazation we expected that the MPTA resistance in strain M^r-5 was due to a change in lycopene cyclase, so that mapping the mutation would lead us to the gene for this enzyme. We therefore tested for the presence of this gene by expressing cyanobacterial genomic fragments containing the mutation, in cells of *E. coli* that produce lycopene.

We constructed the plasmid pACCRT-EIB, which contains genes from the bacterium *E. uredovora* encoding the enzymes GGPP synthase (*crtE*), phytoene synthase (*crtB*) and phytoene desaturase (*crtI*) (Fig. 2). Cells of *E. coli* carrying this plasmid accumulate lycopene (Fig. 6) and produce pink colonies on agar plates.

The plasmid pLCYB-M5XE, which contains a 7.2 kb genomic insert (Fig. 5), was introduced into cells of *E. coli* already containing pACCRT-EIB. In the presence of IPTG, colonies and cultures of cells containing both plasmids were yellow in color and contained β-carotene in addition to lycopene as revealed by HPLC analysis (Fig. 6). A number of other genomic fragments which contained the mutation, the smallest of which was a 1.5 kb *Pst*I-*Pst*I fragment in the vector pTrcHisB (plasmid pLCYB-M5PPF), gave similar results (data not shown). A truncated version of pLCYB-M5PPF (pLCYB-M5SP), lacking only the 0.2 kb *Pst*I-*Sal*I portion containing the MPTA resistance mutation, did not sustain lycopene cyclase activity in *E. coli* cells (data not shown).

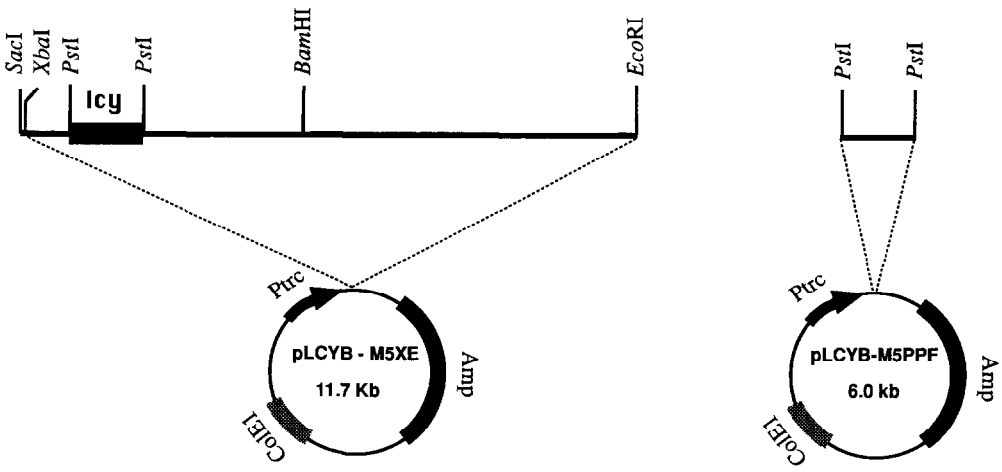

Fig. 5. Construction of plasmids pLCY-BM5XE and pLCYB-M5PPF. Putative localization of the *lcy* gene is based on expression experiments (see text).

Given the small size of the genomic fragment in pLCYB-M5PPF, it is likely that only a single, full-length open reading frame can be accommodated and accounts for the enzymatic activity observed. We conclude that this putative gene, which we have named *lcy*, encodes the enzyme lycopene cyclase, and that this single gene product is sufficient to catalyze both cyclization steps required to produce β-carotene from lycopene.

3.4. *Lycopene cyclase is the target site for MPTA inhibition*

Suspension cultures of *E. coli* cells carrying plasmids pACCRT-EIB and pLCYB-M5XE were grown in the presence of 100 μM MPTA in order to examine the interaction between the herbicide and lycopene cyclization activity. HPLC analysis of the carotenoids, shown in Fig. 6, indicated that these cells accumulated significant amounts of monocyclic γ-carotene, in addition to lycopene and β-carotene, whereas no γ-carotene was detected in the absence of MPTA. We conclude that MPTA acts directly as an inhibitor of lycopene cyclase.

4. DISCUSSION

Our approach to cloning the gene for lycopene cyclase followed the strategy that we used earlier to identify the gene for phytoene desaturase in *Synechococcus* sp. PCC7942. The rationale for this approach derived from our assumption that the enzyme lycopene cyclase is the target site of the bleaching herbicide MPTA, and our conjecture that a subtle change in the primary structure of lycopene cyclase could produce an enzyme that was tolerant of MPTA and yet still retained adequate catalytic activity. We did not know, a priori, whether the resistant phenotype of a particular mutant was derived from a lesion in the gene for lycopene cyclase. The phenotype selected could also have been due to an increase in the rate of breakdown or detoxification of the inhibitor, or the result of a reduction in herbicide uptake. The functional expression in *E. coli* of a gene that contains the MPTA-resistance mutation of mutant M^r-5 demonstrated that this gene, designated *lcy*, does, in fact, encode the enzyme lycopene cyclase.

Lycopene is the primary substrate for the formation of cyclic carotenoids in plants and cyanobacteria. The conversion of lycopene to β-carotene requires that cyclization reactions occur at both ends of the symmetrical lycopene. Our results demonstrates that a single cyanobacterial enzyme efficiently catalyzes both cyclizations. We did not observe accumulation of the monocyclic species γ-carotene in cultures containing pACCRT-EIB and pLCYB-M5XE, even though a substantial amount of lycopene accumulated in these cells. This observation indicates that any molecule of lycopene which is cyclized at one end has a very high probability of being cyclized at the other end as well. The possibility that lycopene cyclase operates as a homodimeric complex is one explanation for these results.

We have observed (data not shown) that no cyclization of ζ-carotene takes place when *lcy* is expressed in cells of *E. coli* that accumulate ζ-carotene. This result implies that the cyclization reactions catalyzed by the *Synechococcus lcy* gene product require that the linear molecule is fully desaturated. It was shown in *Phycomyces* that neurosporene can undergo cyclization only in that half of the molecule which is desaturated to the level of lycopene [18].

The target site of action for MPTA and related compounds has been a matter of some dispute. MPTA was previously shown to induce accumulation of lycopene in grapefruit [29] and to prevent cyclization of lycopene in isolated chloroplasts of *Euglena gracilis* [30]. Much more work has been done with the structurally-related

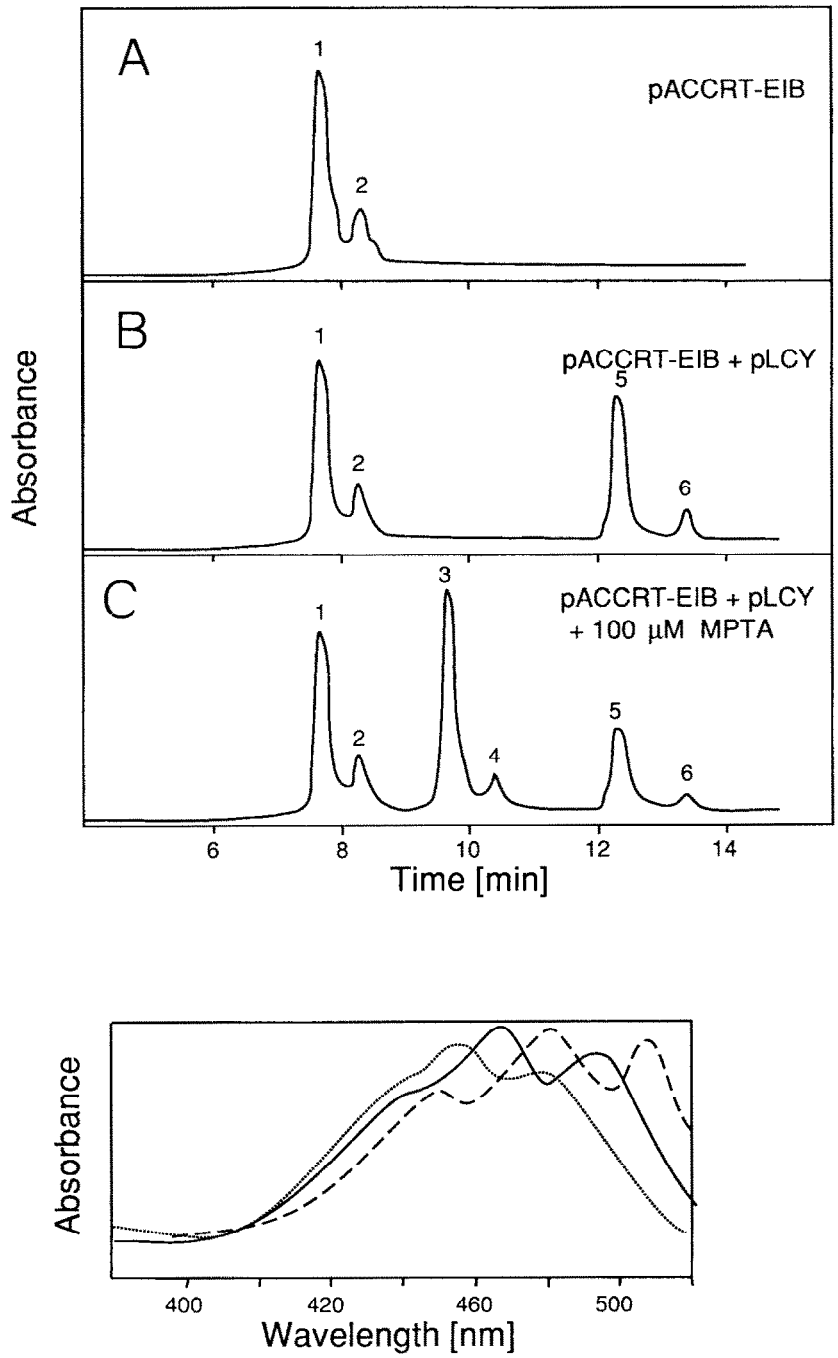

Fig. 6. HPLC analysis of carotenoid pigments extracted from cells of *E coli* carrying plasmids pCRT-EIB (panel A) or pCRT-EIB and pLCY-BM5XE (panels B and C) The effect of treatment of the *E. coli* cells with 100 mM MPTA is shown in panel C. The absorbance spectra of each peak are shown in the lower panel (– – –) peak 1 - lycopene; ( ) peak 3 - γ-carotene; (—) peak 5 - β-carotene.

compound 2-(4-chlorophenylthio)triethylamine hydrochloride (CPTA) [10,28] with similar results: lycopene accumulation in vivo. However, in contrast to the results obtained in vivo, CPTA has been reported to exert little or no effect on carotenoid biosynthesis in cell-free systems derived from acetone extracts of tomato plastids [37], from *Narcissus* chromoplasts [38], and from *Capsicum* chromoplasts [39]. Suggestions have been made that CPTA and related compounds act at the level of gene expression rather than by directly affecting enzyme function [37,40,41]. More recent work, using a cell-free system from the cyanobacterium *Aphanocapsa*, indicates that CPTA can be an effective inhibitor of lycopene cyclization in vitro and that it inhibits the

cyclization reaction in a noncompetitive manner (reviewed in [28]). The lack of inhibition by CPTA observed in many other cell-free systems suggests that the physical integrity of the membrane and/or an ordered structural association of the enzymes of carotenoid biosynthesis are important for effective herbicide action. The fragile nature of the carotenoid biosynthetic machinery in cell free systems is well known [42]. Our finding that a mutation in the gene for lycopene cyclase confers resistance to MPTA provides strong evidence that this enzyme is a target site of action for MPTA and related compounds. This conclusion is further supported by our observation that MPTA inhibits lycopene cyclase activity in cells of *E. coli* where no authentic carotenogenic apparatus is presumed. The accumulation of γ-carotene in MPTA treated cells of *E. coli* suggests that the second cyclization step carried out by lycopene cyclase is more sensitive to inhibition by the herbicide.

Southern hybridization analysis, using the putative cyanobacterial *lcy* sequence as a molecular probe, indicates that this gene is conserved with homologous DNA sequences in eukaryotic algae, and shows the feasibility of using *lcy* as a molecular probe to clone lycopene cyclase from algae and higher plants.

In the 'plant-type' pathway of carotenoid biosynthesis, we now have identified genes for all of the steps from geranylgeranyl pyrophosphate to β-carotene except the two desaturations which convert the symmetrical ζ-carotene to lycopene. Cloning of a putative gene for ζ-carotene desaturase has been recently reported [43]. The desaturation of ζ-carotene in plants and algae is inhibited by a number of experimental bleaching herbicides [28], and several of these compounds effectively inhibit the desaturation of ζ-carotene in *Synechococcus* PCC7942 (our unpublished observations). Our successes in cloning *psy*, *pds* and *lcy* suggest that the same strategy of mutagenesis and selection of herbicide-resistant mutants may enable the cloning of ζ-carotene desaturase.

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