

Functional Analysis of the β and ϵ Lycopene Cyclase Enzymes of Arabidopsis Reveals a Mechanism for Control of Cyclic Carotenoid Formation

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Carotenoids with cyclic end groups are essential components of the photosynthetic membranes in all plants, algae, and cyanobacteria. These lipid-soluble compounds protect against photooxidation, harvest light for photosynthesis, and dissipate excess light energy absorbed by the antenna pigments. The cyclization of lycopene (ψ,ψ -carotene) is a key branch point in the pathway of carotenoid biosynthesis. Two types of cyclic end groups are found in higher plant carotenoids: the β and ϵ rings. Carotenoids with two β rings are ubiquitous, and those with one β and one ϵ ring are common; however, carotenoids with two ϵ rings are rare. We have identified and sequenced cDNAs that encode the enzymes catalyzing the formation of these two rings in Arabidopsis. These β and ϵ cyclases are encoded by related, single-copy genes, and both enzymes use the linear, symmetrical lycopene as a substrate. However, the ϵ cyclase adds only one ring, forming the monocyclic δ -carotene (ϵ,ψ -carotene), whereas the β cyclase introduces a ring at both ends of lycopene to form the bicyclic β -carotene (β,β -carotene). When combined, the β and ϵ cyclases convert lycopene to α -carotene (β,ϵ -carotene), a carotenoid with one β and one ϵ ring. The inability of the ϵ cyclase to catalyze the introduction of a second ϵ ring reveals the mechanism by which production and proportions of β,β - and β,ϵ -carotenoids may be controlled and adjusted in plants and algae, while avoiding the formation of the inappropriate ϵ,ϵ -carotenoids.

INTRODUCTION

Carotenoid pigments with cyclic end groups are essential components of all known oxygenic photosynthetic organisms and serve an extraordinary variety of different functions in plants (Goodwin, 1980). The essential nature of these pigments derives from their ability to prevent the formation of and to deactivate the toxic singlet oxygen and free radicals that form in photosynthetic reaction centers as a result of electron transfer from long-lived triplet chlorophyll to molecular oxygen (Koyama, 1991; Frank and Cogdell, 1993). An inability to form cyclic carotenoids is eventually lethal in oxygen-evolving photosynthetic organisms (Britton, 1979).

In any one higher plant species, there are typically five or six different carotenoids found in appreciable quantity in the photosynthetic membranes. β -Carotene (β,β -carotene), ubiquitous in oxygen-evolving photosynthetic organisms, is intimately associated with chlorophyll in the photosynthetic reaction centers (Young, 1993). Lutein (β,ϵ -carotene-3,3'-diol), violaxanthin (5,6,5,6'-diepoxy-5,6,5,6'-tetrahydro- β,β -carotene-3,3'-

diol), and neoxanthin (5',6'-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro- β,β -carotene-3,5,3'-triol) are associated with the accessory light-harvesting chlorophyll antenna system and serve as antioxidants and light-harvesting pigments (Siefermann-Harms, 1987). Zeaxanthin (β,β -carotene-3,3'-diol) is involved in the thermal dissipation of excess light energy captured by the light-harvesting antenna (Yamamoto, 1979; Demmig-Adams and Adams, 1993).

The levels of specific carotenoid pigments in the photosynthetic membranes of plants and algae are tightly regulated and closely coordinated with the biogenesis and assembly of the photosynthetic apparatus. During chloroplast development, the accumulation of the individual carotenoid pigments closely approximates the accumulation of the antenna and reaction center polypeptides with which they are associated (Young, 1993). Inhibition of carotenoid biosynthesis profoundly affects the synthesis, assembly, and stability of these carotenoid-chlorophyll binding proteins (Plumley and Schmidt, 1987, 1995; Humbeck et al., 1989; Markgraf and Oelmüller, 1991; Bishop et al., 1995) and has a significant influence on the expression of nuclear genes that encode proteins of the chloroplast (Taylor, 1989).

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Little is known of the regulatory mechanisms that serve to control and coordinate the biosynthesis and accumulation of carotenoid pigments and carotenoid binding proteins in the photosynthetic membranes.

The biosynthesis of carotenoids occurs within the chloroplasts of plants and algae. The pathway (Spurgeon and Porter, 1980; Sandmann, 1994), illustrated in Figure 1, is a branch of the well-known isoprenoid pathway that begins with the conversion of mevalonic acid to the five-carbon compound isopentenyl pyrophosphate. In a series of prenyltransferase reactions, the 40-carbon carotenoid phytoene (7,8,11,12,7',8',

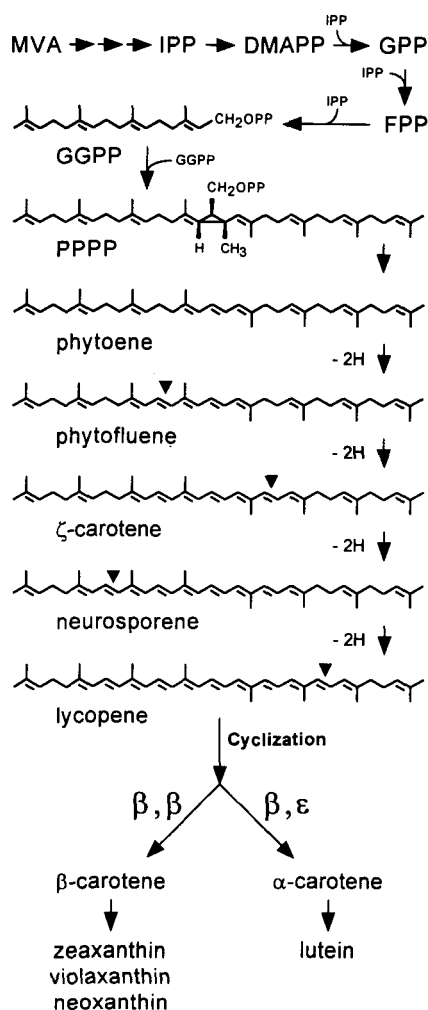


Figure 1. Early Steps in the Carotenoid Biosynthesis Pathway in Plants.

The pathway branches with the cyclization of lycopene to form bicyclic β,β- or β,ε-carotenoids. Some of the more common plant β,β- and β,ε-carotenoids are listed below each branch. Locations of double bonds introduced as a consequence of the desaturation reactions are indicated by inverted triangles. DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; MVA, mevalonic acid; PP, pyrophosphate; PPPP, prephytoene pyrophosphate.

11',12'-octahydro-ψ,ψ-carotene) is assembled from eight molecules of isopentenyl pyrophosphate. Phytoene subsequently undergoes a series of four desaturation or dehydrogenation reactions that result in the formation of first phytofluene (7,8,11,12,7',8'-hexahydro-ψ,ψ-carotene) and then, in turn, ζ-carotene (7,8,7',8'-tetrahydro-ψ,ψ-carotene), neurosporene (7,8,-dihydro-ψ,ψ-carotene), and lycopene (ψ,ψ-carotene). These desaturation reactions serve to lengthen the conjugated series of carbon-carbon double bonds that constitutes the chromophore in carotenoid pigments; the reactions thereby transform the colorless phytoene into the pink-colored lycopene. The enzymes catalyzing the desaturation reactions and subsequent steps in the pathway, as well as the carotenoids themselves, are intrinsic to the membranes of the chloroplast (Bramley, 1985).

The cyclization of lycopene is an important branch point in the pathway of carotenoid biosynthesis. Cyclization at both ends of the symmetrical, linear lycopene leads to β-carotene, an essential carotenoid that also serves as a precursor for the production of several other carotenoids with roles in photosynthesis (Figure 1). The rings at each end of the symmetrical β-carotene are referred to as β rings. The formation of the β ring from the linear ψ end group of lycopene is illustrated in Figure 2. Most of the carotenoids common in plants and algae have two β or modified β rings. A second type of cyclic end group, the ε ring (Figure 2), is also common. Lutein, the predominant carotenoid in the photosynthetic tissues of many plants and algae, has one β ring and one ε ring. Carotenoids with two ε groups are, however, rarely observed in plants and algae, and when detected, they are usually found in only trace amounts (Goodwin, 1980).

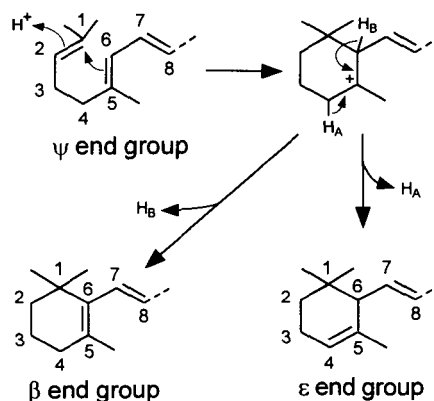


Figure 2. Formation of β and ε Rings from the Linear ψ End Group.

The linear ψ end groups, found in carotenoid precursors such as lycopene, are converted into the β or ε cyclic end groups or rings typical of most carotenoids in plants. A common carbonium ion intermediate has been suggested for these cyclization reactions (Britton, 1988). Whether a β or an ε ring is formed depends on which hydrogen atom, H_A or H_B, participates in the reaction. Conventional numbering of the carbon atoms is shown for each end group.

The conversion of lycopene to β -carotene is catalyzed by a single-gene product: the β cyclase (Cunningham et al., 1994; Hugueney et al., 1995; Scolnik and Bartley, 1995; Pecker et al., 1996). The ϵ ring differs from the β ring only in the position of the double bond within the cyclohexene ring, and it is thought that formation of β and ϵ rings proceeds via the same carbenium ion intermediate as shown in Figure 2 (after Britton, 1988). Whether an ϵ or a β ring is formed depends on the choice of the proton to be eliminated from this transient intermediate. Because of the similar ring structures and the likelihood of a common reaction mechanism, it has been expected that the enzymes catalyzing formation of ϵ and β rings would prove to be the products of related genes. However, a gene encoding an ϵ cyclase has not yet been reported.

In this paper, we describe the identification of cDNAs encoding the ϵ and β cyclases of Arabidopsis and examine the function and substrate specificity of the products of these two cDNAs. The ϵ and β cyclase enzymes of Arabidopsis are indeed the products of related genes, but the ϵ cyclase adds only one ϵ ring to lycopene, whereas the β cyclase can add two β rings. The inability of the ϵ cyclase to add a second ϵ ring suggests a mechanism for the control of cyclic carotenoid biosynthesis in plants and algae.

RESULTS

A Heterologous *Escherichia coli* System for Functional Analysis of Carotenoid Cyclase Enzymes

To identify and examine the catalytic activity of the ϵ and β cyclases of Arabidopsis, we sought to develop a convenient *in vivo* assay for these membrane-associated enzymes. Using carotenoid biosynthetic genes from several prokaryotic organisms, we have constructed a series of plasmids such that the cells of *E. coli* that contain them manufacture and accumulate a variety of different carotenoid pigments (Cunningham et al., 1993, 1994; see Methods). The vector used to construct these plasmids contains a p15a origin of replication that allows it to coexist in *E. coli* with cloning vectors that contain the more common ColE1 origin of replication. The addition of one or more β and/or ϵ cyclic end groups to the pink-colored lycopene will result in products that are yellow or orange-yellow in color. Therefore, the functioning of the β and ϵ lycopene cyclase enzymes may be detected by a change in the color of *E. coli* cultures that accumulate lycopene. We call such assays color complementation assays.

Cloning the ϵ and β Cyclases of Arabidopsis by Color Complementation in *E. coli*

We exploited the pink-to-yellow color change that accompanies carotenoid cyclization to visually screen for cDNAs

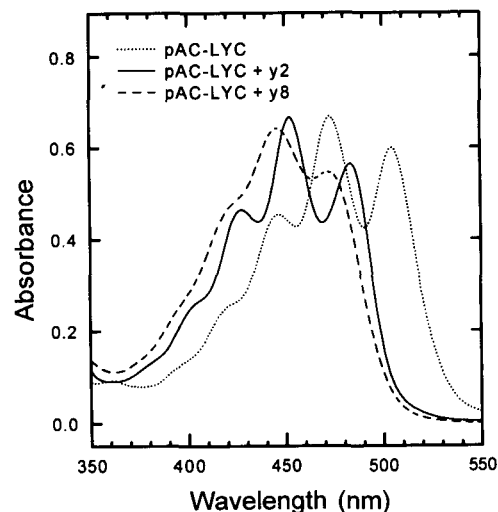


Figure 3. Absorption Spectra of Ethanolic Extracts of Two Yellow Transformants Selected in a Screen of an Arabidopsis Library for cDNAs Encoding Carotenoid Cyclase Enzymes.

The spectrum labeled pAC-LYC is of the host lycopene-accumulating strain containing the empty cloning vector. The other two spectra are for extracts of cultures containing pAC-LYC plus either plasmid y2 (containing an ϵ cyclase cDNA) or plasmid y8 (containing a β cyclase cDNA).

encoding carotenoid cyclase enzymes. A lycopene-accumulating strain of *E. coli* was transformed with an Arabidopsis cDNA library, and the transformants were spread on agar plates containing the appropriate antibiotics to select for library plasmids and to maintain the plasmid (pAC-LYC) containing the genes necessary for lycopene production. The plates were examined for the presence of rare yellow colonies in a background of pink colonies.

Two classes of deep yellow-colored colonies were selected with this visual screening protocol. Absorption spectra of pigments extracted from a representative of each of these two classes are shown in Figure 3. An absorption spectrum of lycopene, extracted from a control culture containing the plasmid pAC-LYC plus the empty library cloning vector (pBluescript SK+), is also included in Figure 3 for comparison. The absorption spectrum of the yellow extract obtained from the culture of *E. coli* containing both cDNA library plasmid y8 and plasmid pAC-LYC closely resembles that of the bicyclic β -carotene in both the wavelengths and the low spectral persistence of the absorption maxima (Britton, 1995). The absorption spectrum of the extract obtained from a culture containing library plasmid y2 and plasmid pAC-LYC is consistent with the introduction of a single ϵ ring to form the monocyclic δ -carotene (ϵ, ψ -carotene). The lack of absorbance at longer wavelengths indicates that little or no lycopene remained in the cultures containing y2 or y8. From the spectral data, we tentatively concluded that the cDNA in plasmid y2 encodes an ϵ cyclase enzyme and the cDNA in plasmid y8 encodes

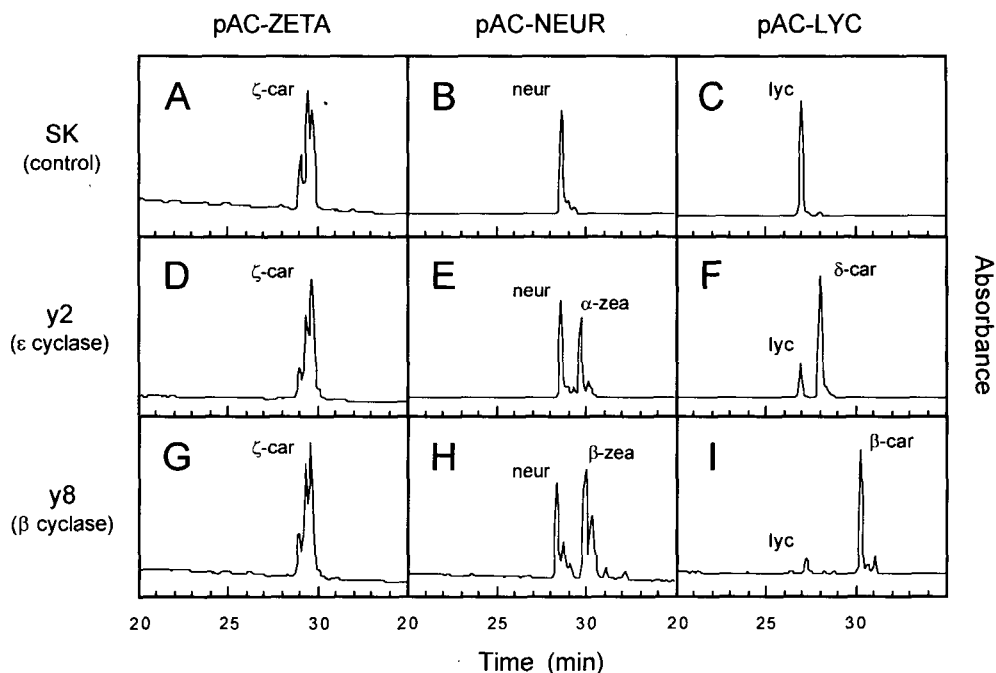


Figure 4. Substrate Specificity of Arabidopsis Carotenoid Cyclase Enzymes Determined by HPLC.

Carotenoid pigment composition was examined in cultures of *E. coli* containing the plasmids indicated above and to the left. HPLC detection was by absorption at 440 nm.

(A) to (C) Elution profiles of control cultures containing the empty cloning vector pBluescript SK+ (SK) in addition to the plasmids pAC-ZETA (A), pAC-NEUR (B), or pAC-LYC (C).

(D) to (F) Elution profiles of cultures containing Arabidopsis cDNA library plasmid y2 (with the ϵ cyclase cDNA) in addition to the plasmids pAC-ZETA (D), pAC-NEUR (E), or pAC-LYC (F).

(G) to (I) Elution profiles of cultures containing Arabidopsis cDNA library plasmid y8 (with the β cyclase cDNA) in addition to the plasmids pAC-ZETA (G), pAC-NEUR (H), or pAC-LYC (I).

α -zea, α -zeacarotene; β -car, β -carotene; β -zea, β -zeacarotene; δ -car, δ -carotene; lyc, lycopene; neur, neurosporene; SK, pBluescript SK+; ζ -car, ζ -carotene.

a β cyclase. HPLC (see below) and mass spectral data (not shown) provided additional support for identifying δ -carotene and β -carotene as the products formed from lycopene in the presence of the Arabidopsis cDNAs in plasmids y2 and y8, respectively.

Substrate Specificity of the β and ϵ Cyclases of Arabidopsis

Substrate specificities of the ϵ and β cyclases of Arabidopsis, encoded by cDNAs in plasmids y2 and y8, were examined by introducing the plasmids into *E. coli* strains that were engineered to accumulate a variety of different carotenoids and carotenoid precursors. HPLC and spectral analyses of some of these experiments are illustrated in Figures 4 and 5. The empty cloning vector pBluescript SK+, used as a control (Figures 4A to 4C), did not influence the composition of pigments accumulated by the different carotenoid-accumulating

strains of *E. coli* used in these experiments (Cunningham et al., 1994; data not shown).

When plasmids y2 and y8 were introduced into a ζ -carotene-accumulating *E. coli* strain (containing plasmid pAC-ZETA), the composition of the carotenoid pigments in the host was not affected, and the HPLC elution profiles (Figures 4D and 4G) and corresponding absorption spectra (Figure 5 at left) were indistinguishable from those of control cultures (Figure 4A and Figure 5 at left). The multiple peaks apparent in the HPLC elution profiles displayed in Figures 4A, 4D, and 4G are due to the presence of geometrical isomers of ζ -carotene (Zechmeister, 1962; Cunningham and Schiff, 1985; Cunningham et al., 1994).

With neurosporene as substrate (cells contained plasmid pAC-NEUR), different products were formed by the two cyclases (Figures 4E and 4H). HPLC retention times, absorption spectra (Figure 5 at center), and mass spectral analyses indicated that the products formed from neurosporene via the

action of the y2 and y8 cDNA products are the monocyclic compounds α -zeacarotene (7',8'-dihydro- ϵ , ψ -carotene) and β -zeacarotene (7',8'-dihydro- β , ψ -carotene), respectively. The structures of these carotenoids are illustrated in Figure 6.

With lycopene as the substrate (cells contained pAC-LYC), the introduction of the y8 plasmid resulted in formation of the bicyclic β -carotene (Figure 4I and Figure 5 at right). With plasmid y2, an ϵ ring was introduced, and the product formed (Figure 4F and Figure 5 at right) was the monocyclic δ -carotene rather than the expected bicyclic ϵ -carotene (ϵ , ϵ -carotene). These substrate utilization experiments indicate that both the β and ϵ cyclase enzymes require desaturation of the 7-8 or 7'-8' carbon-carbon double bond (see Figures 2 and 6 for numbering of the carbon atoms) before cyclization can take place.

We examined the products formed when both an ϵ and a β cyclase were present simultaneously in the lycopene-accumulating strain of *E. coli*. Figure 7A displays an HPLC elution profile of the carotenoid pigments accumulated in *E. coli* cells containing the *Erwinia herbicola* β cyclase gene in addition to plasmid pAC-LYC. As expected, the predominant pigment in these cells is β -carotene. The addition of the Arabidopsis ϵ cyclase (in plasmid y2) to cells containing pAC-LYC and the *E. herbicola* β cyclase resulted in the production and accumulation of a carotenoid with one ϵ and one β ring (i.e., α -carotene [β , ϵ -carotene]), but β -carotene remained the major product (Figure 7B). The high ratio of β -carotene to α -carotene probably reflects a more active and/or more plentiful bacterial β cyclase relative to the Arabidopsis ϵ cyclase.

In cells of *E. coli* containing pAC-LYC plus cDNAs encoding both the β and ϵ cyclases of Arabidopsis (y2 plus y8), a complex mixture of carotenoid pigments was observed (Figure 7C). The major product was δ -carotene, which is also the major product in cells containing pAC-LYC plus y2 alone (Figure 4F). A

small amount of β -carotene and a substantial amount of α -carotene were also detected (Figure 7C); the absorption spectra of these peak fractions are displayed in Figure 8. From the relatively low proportion of β -carotene, it appears that the ϵ cyclase is produced in greater amounts and/or exhibits a higher specific activity than the β cyclase under the conditions of this experiment.

The formation of α -carotene from lycopene in the simultaneous presence of β and ϵ cyclase enzymes likely proceeds by the addition of a β ring to δ -carotene (Figure 6). The alternative route, the addition of an ϵ ring to γ -carotene (β , ψ -carotene), does not seem likely because of the inability of the ϵ cyclase to add a second ϵ ring to the monocyclic δ -carotene. Our current understanding of carotenoid cyclization in Arabidopsis is summarized in Figure 6.

DNA and Predicted Amino Acid Sequences of the Arabidopsis β and ϵ Cyclase cDNAs

The DNA sequences of the cDNAs encoding the ϵ and β cyclases of Arabidopsis were determined (GenBank accession numbers U50738 and U50739, respectively). That of the β cyclase cDNA (in plasmid y8) is 1924 bp long, excluding the poly(A) tail, and was in the forward orientation in the cloning vector. A 5' untranslated region of 173 bp precedes the putative start codon, and 245 bp of 3' untranslated DNA follow the termination codon and precede the poly(A) tail. A possible polyadenylation signal (the sequence AATAA at base pairs 1895 to 1899) occurs 25 bp before the poly(A) tail. The predicted amino acid sequence of the β cyclase specifies a polypeptide of 501 amino acids in length with a molecular weight of 56,174 and a pI of 5.7.

The ϵ cyclase cDNA (in plasmid y2) is 1860 bp in length.

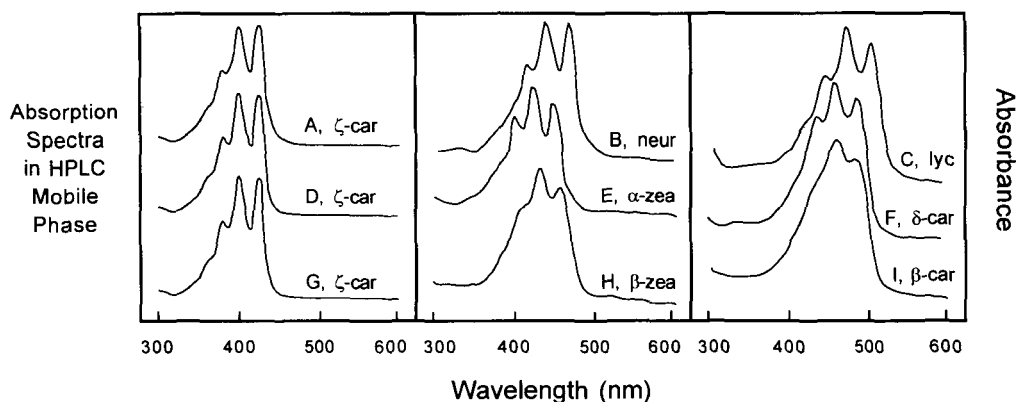


Figure 5. Absorption Spectra of Selected Peaks in the HPLC Elution Profiles of Figure 4.

Spectra are labeled with the letters A through I, corresponding to the profile in Figure 4 that contains the elution peak for which the absorption spectrum was obtained. α -zea, α -zeacarotene; β -car, β -carotene; β -zea, β -zeacarotene; δ -car, δ -carotene; ζ -car, ζ -carotene; lyc, lycopene; neur, neurosporene.

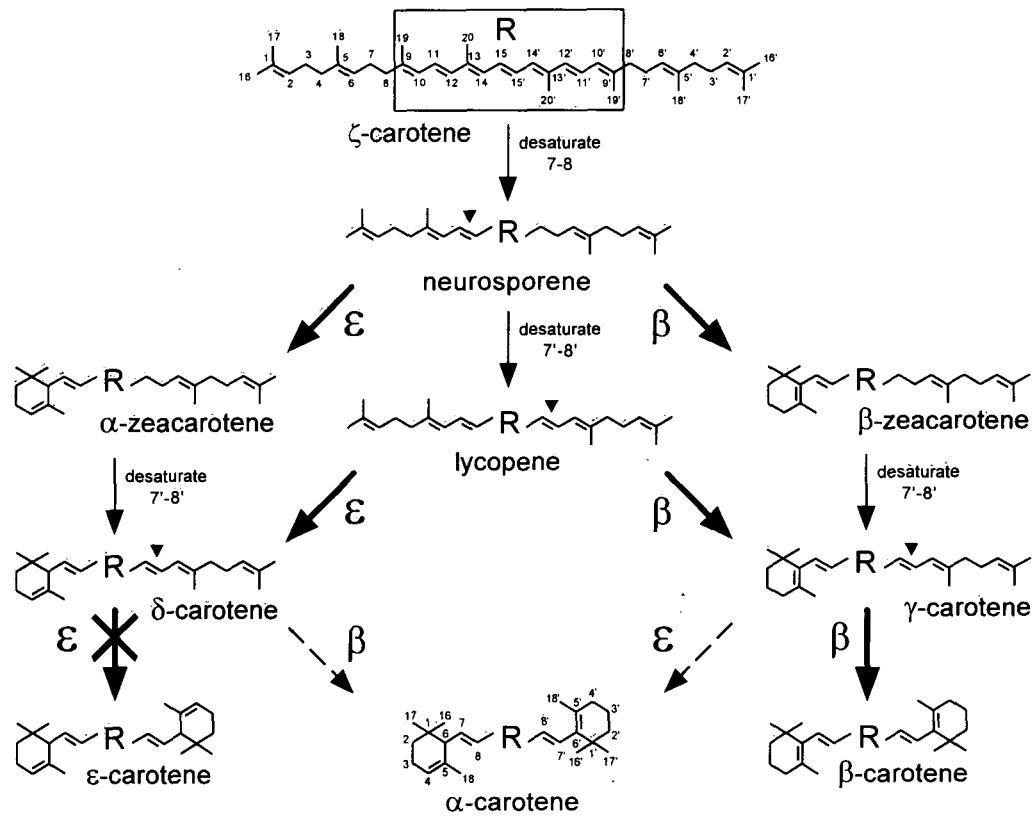


Figure 6. Routes of Carotenoid Cyclization in Arabidopsis.

Demonstrated activities of the β and ϵ lycopene cyclase enzymes of Arabidopsis are indicated by boldface arrows labeled with β or ϵ , respectively. The X over the arrow leading to ϵ -carotene indicates that the enzymatic activity was examined but no product was detected. The steps marked by an arrow with a dashed line have not yet been specifically examined by us. We have ascertained that α -carotene can be formed via the concerted action of the ϵ and β cyclase enzymes, but we do not yet know which of the two possible routes is followed. Conventional numbering of the carbon atoms is given for ζ -carotene and α -carotene. Inverted triangles mark positions of the double bonds introduced as a consequence of the desaturation reactions.

A 5' untranslated region of 108 bp precedes the putative start codon, and 180 bp of 3' untranslated DNA follow the termination codon. This cDNA was in the reverse orientation in the cloning vector and therefore was not downstream of the bacterial promoters present in the vector (pBluescript SK+). Yet plasmid y2 managed to provide for the production of sufficient enzyme for the conversion of lycopene to δ -carotene in *E. coli*. No poly(A) tail was observed for the y2 cDNA. cDNAs in the library that was the source of plasmid y2 (Stock CD4-14; Arabidopsis Biological Resource Center Seed and DNA Stock List) quite often lack the poly(A) tail and an additional 8 to 10 bp before it as well. A possible polyadenylation signal (the sequence AATAA at base pairs 1732 to 1736) was observed, but this does not occur close to the 3' end of the cDNA sequence, as would be expected. The predicted ϵ cyclase polypeptide, with a length of 524 amino acids, a molecular weight of 58,512, and a pI of 5.7, is similar in size and charge to the β cyclase

of Arabidopsis. For both the β and ϵ cyclases, we were able to detect only a single gene copy with DNA gel blot analysis at low to moderate stringency (data not shown).

DISCUSSION

A Mechanism for Control of Cyclic Carotenoid Formation in Plants

The cyclization of lycopene is a key branch point in the pathway of carotenoid biosynthesis in plants and algae. It is here that the pathway leading to carotenoids with one β and one ϵ ring (e.g., the abundant lutein) diverges from that which leads to carotenoids with two β rings (e.g., β -carotene, zeaxanthin, and violaxanthin; see Figure 1). Functional analysis of the β

and ϵ cyclases of Arabidopsis (Figure 4) has shown that the β cyclase will efficiently add rings to both ends of lycopene but the ϵ cyclase will add only a single ϵ ring to this symmetrical, linear carotenoid. The presence of a ring at one end of the molecule somehow prevents or inhibits ring formation by the ϵ cyclase enzyme at the other end. The inability of the ϵ cyclase to add more than one ϵ ring to lycopene has important implications for the control of cyclic carotenoid formation in plants and in algae that contain carotenoids with ϵ end groups. First, because the ϵ cyclase is a monocyclase, plants are thereby restrained from producing carotenoids with two ϵ end groups. Second, our data (Figure 7) suggest that the apportioning of substrate into the pathways leading to ϵ,β - and β,β -carotenoids could be determined quite simply by the relative amounts and/or activities of the ϵ and β cyclase enzymes.

The substrate utilization experiments were performed with the cyclase enzymes produced and examined in a heterologous *E. coli* assay system. We must consider whether the data obtained from these experiments reflect the functioning of the enzymes in their natural milieu. It is unlikely that the chloroplast

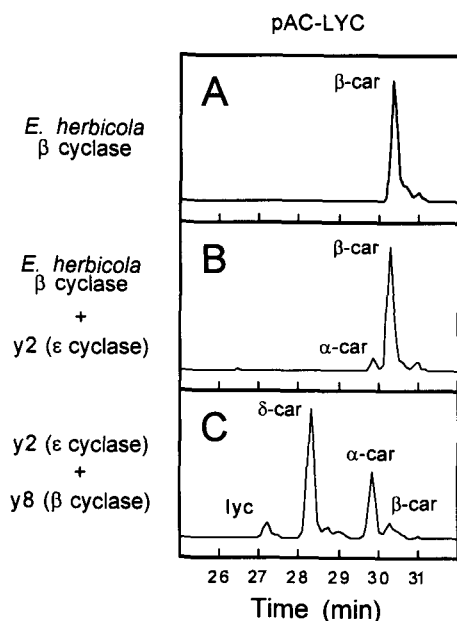


Figure 7. HPLC Analysis of Products Formed from Lycopene in the Simultaneous Presence of the Arabidopsis β and ϵ Cyclases.

Carotenoid pigment composition was examined in cultures of *E. coli* containing the plasmid pAC-LYC plus genes or cDNAs encoding carotenoid cyclase enzymes.

(A) pAC-LYC plus the *E. herbicola* β cyclase.

(B) pAC-LYC plus the *E. herbicola* β cyclase and the Arabidopsis ϵ cyclase.

(C) pAC-LYC plus the Arabidopsis β and ϵ cyclases, respectively. HPLC detection was by absorption at 440 nm. The *E. herbicola* β cyclase gene was cloned directly in the pAC-LYC plasmid (to give the plasmid pAC-BETA; see Methods). The y2 and y8 cDNAs were cloned together in a second plasmid (see Methods). α -car, α -carotene; β -car, β -carotene; δ -car, δ -carotene; lyc, lycopene.

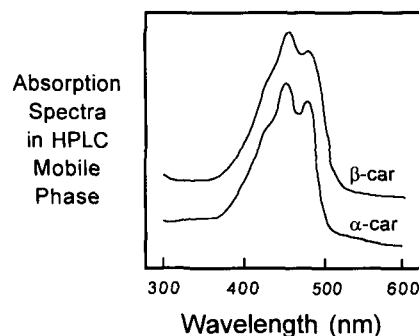


Figure 8. Absorption Spectra of Products Formed from Lycopene in the Simultaneous Presence of the Arabidopsis β and ϵ Cyclases.

The absorption spectra shown here are for selected peaks in the HPLC elution profile of Figure 7C. α -car, α -carotene; β -car, β -carotene.

transit sequences of the plastid-localized cyclase enzymes are cleaved at the appropriate position, if they are cleaved at all, in *E. coli*. And it is quite possible that the enzymes are not folded, processed, phosphorylated, or otherwise secondarily modified as they might be in Arabidopsis. The environment of the cyclase enzymes in *E. coli*, including the lipid composition of the membrane in which these enzymes reside, is almost certainly not the same as in Arabidopsis. An argument could be made that the observed inability of the ϵ cyclase to introduce a second ring is a consequence of the abnormal environment. Yet the β cyclase is not so impaired.

Despite the limitations inherent in the heterologous *E. coli* system, we conclude that the inability of the ϵ cyclase to add a second ring to lycopene and thereby form ϵ -carotene in *E. coli* reflects the biological specificity of the enzyme in the plant. Perhaps the most compelling support for this conclusion is provided by mutants of tomato and maize. The red color of the fruits of tomato is due primarily to the substantial amount of lycopene that accumulates during fruit ripening. A mutant of tomato known as the *delta* strain produces yellow fruit that contain large amounts of δ -carotene instead of lycopene (Kargl et al., 1960; Tomes, 1967). The *delta* allele is a dominant one (Tomes, 1963). Other δ -carotene-accumulating tomato strains are known, and a δ -carotene-accumulating mutant of maize has also been described (Horvath et al., 1972); however, we are not aware of any ϵ -carotene-accumulating mutants of plants. The existence of the high δ -carotene mutants persuades us that the ϵ cyclase is a monocyclase in vivo. To assume otherwise requires a more complicated explanation of the maize and tomato mutants.

Plant Carotenoid Cyclases Are the Products of Related Genes

The predicted amino acid sequence of the Arabidopsis β cyclase is quite similar to those of the three other known plant

β cyclases (from pepper, Hugueney et al., 1995; and from tomato and tobacco, Pecker et al., 1996) and also resembles the β cyclase from the cyanobacterium *Synechococcus* sp strain PCC7942 (Cunningham et al., 1994). Table 1 lists amino acid sequence identities and similarities for pairwise comparisons of these and other carotenoid cyclase enzymes, and Figure 9 graphically illustrates the degree of sequence similarity and the locations of the more highly conserved regions. The sequence comparisons in Table 1 and Figure 9 reveal that the *Arabidopsis* ϵ cyclase is similar in sequence to the plant and cyanobacterial β cyclase enzymes and that both β and ϵ cyclases are related to a third type of carotenoid cyclase enzyme: the capsanthin-capsorubin synthase (CCS) of pepper. The known bacterial genes encoding β cyclases bear little overall resemblance to the plant and cyanobacterial cyclases: sequence identities of pairwise comparisons are only ~ 18 to 25% (Table 1). However, some local regions of sequence similarity can be seen in the homology plots of Figure 9.

The reactions catalyzed by the β and ϵ cyclases of *Arabidopsis* are similar (Figure 2). Each enzyme utilizes a ψ end group as substrate, the products differ only in the position of the carbon-carbon double bond within the six-carbon ring, and the proposed reaction mechanisms (Britton, 1988) are essentially the same. The CCS enzyme of pepper catalyzes the formation of the unusual five-carbon κ ring (Bouvier et al., 1994), shown in Figure 10, from the 3-hydroxy-5,6-epoxy-modified β ring present in the common carotenoids antheraxanthin (5,6-epoxy-5,6-dihydro- β , β -carotene-3,3'-diol) and violaxanthin. This reaction would seem, at first, to be very different from formation of ϵ and β end groups, but mechanistically it may proceed in much the same way (Hugueney et al., 1995). Interestingly, the pepper CCS exhibits a β cyclase activity when lycopene is provided as the substrate (Hugueney et al., 1995).

Carotenoids with β rings are found in all oxygenic photosynthetic organisms without exception, as far as is known (Goodwin, 1980). Carotenoids with ϵ rings are widespread in the higher plants and are also found in a number of algal classes, but they are not present in cyanobacteria. The κ ring has been found so far only in fruits of some species in the dicot genus *Capsicum*, floral parts of some species of the monocot Liliaceae, and in the pollen and anthers of a plant in the horse chestnut family, *Aesculus rubicunda* (Goodwin, 1980). The phylogenetic distribution of the κ ring and the much higher percentage sequence identity and similarity between the pepper CCS and the higher plant β cyclases ($\sim 58/75$; Table 1 and Figure 9) suggest that this enzyme is of relatively recent origin compared with the ϵ cyclase and that it may have arisen by adaptive modification of a duplicated β cyclase gene (in pepper, at least). We have found only a single copy of the β cyclase gene in *Arabidopsis*, but there are two β cyclase genes in tomato (Pecker et al., 1996), a species considered to be more closely related to pepper. In any case, the three different carotenoid cyclase enzymes, the ϵ cyclase, the β cyclase, and the CCS, are the products of related genes and likely arose from the same ancestral enzyme, arguably a β cyclase.

Identification of Polypeptide Regions and Amino Acid Residues of Functional Significance

The related ϵ and β cyclase enzymes of *Arabidopsis* differ in two fundamental respects: the type and the number of rings added when lycopene is provided as the substrate. A careful analysis and comparison of the amino acid sequences of the ϵ and β cyclases may suggest particular regions or residues that participate in enzyme function or that differentiate their respective activities. A consensus sequence for the four known

Table 1. Percentage of Amino Acid Sequence Identity/Similarity for Carotenoid Cyclases^a

| Cyclase Enzyme | Arab ϵ | Arab β | Tob β | Tom β | Pep β | Pep CCS | Syn β | <i>E.h.</i> β | <i>E.u.</i> β |
|-------------------------------|-----------------|--------------|-------------|-------------|-------------|---------|-------------|---------------------|---------------------|
| <i>Arabidopsis</i> β | 36/58 | | | | | | | | |
| Tobacco β | 38/59 | 82/90 | | | | | | | |
| Tomato β | 37/59 | 84/92 | 90/95 | | | | | | |
| Pepper β | 38/59 | 85/92 | 91/95 | 96/99 | | | | | |
| Pepper CCS | 38/58 | 59/75 | 58/75 | 58/75 | 58/76 | | | | |
| <i>Synechococcus</i> β | 36/57 | 36/56 | 35/55 | 36/56 | 35/56 | 33/54 | | | |
| <i>E. herbicola</i> β | 21/45 | 25/50 | 22/48 | 25/49 | 23/49 | 24/48 | 23/47 | | |
| <i>E. uredovora</i> β | 18/44 | 24/49 | 21/49 | 23/53 | 23/52 | 23/50 | 23/47 | 58/74 | |
| <i>A. aurianticum</i> β | 21/48 | 25/53 | 21/48 | 23/47 | 23/49 | 21/48 | 23/48 | 46/66 | 45/65 |

^a The plant sequences used to generate the values listed in this table were truncated to begin at the same relative position as the initial methionine residue in the *Synechococcus* sp strain PCC7942 (*Syn*) β cyclase (e.g., beginning with residue 109 in the alignment displayed in Figure 11). A gap weight of 3.0 and length weight of 0.1 were used for sequence alignment with the GAP program of the Genetics Computer Group sequence analysis software. GenBank accession numbers for the sequences compared in this table are given in the legends to Figures 9 and/or 11. The *E. herbicola* β cyclase sequence used for the comparisons in this table is that for the strain Eho10 predicted by the DNA sequence listed under GenBank accession number M87280. The predicted amino acid sequence for the β cyclase of *E. herbicola* strain Eho13 (GenBank accession number M90698) closely resembles that of *E. uredovora* (percentage of identity/similarity equal to 89/93) and was not used for the comparisons listed here. Arab, *Arabidopsis*; Tob, tobacco; Tom, tomato; Pep, pepper; CCS, capsanthin-capsorubin synthase; *Syn*, *Synechococcus* sp strain PCC7942; *E.h.*, *Erwinia herbicola*; *E.u.*, *E. uredovora*; *A. aurianticum*, *Agrobacterium aurianticum*.

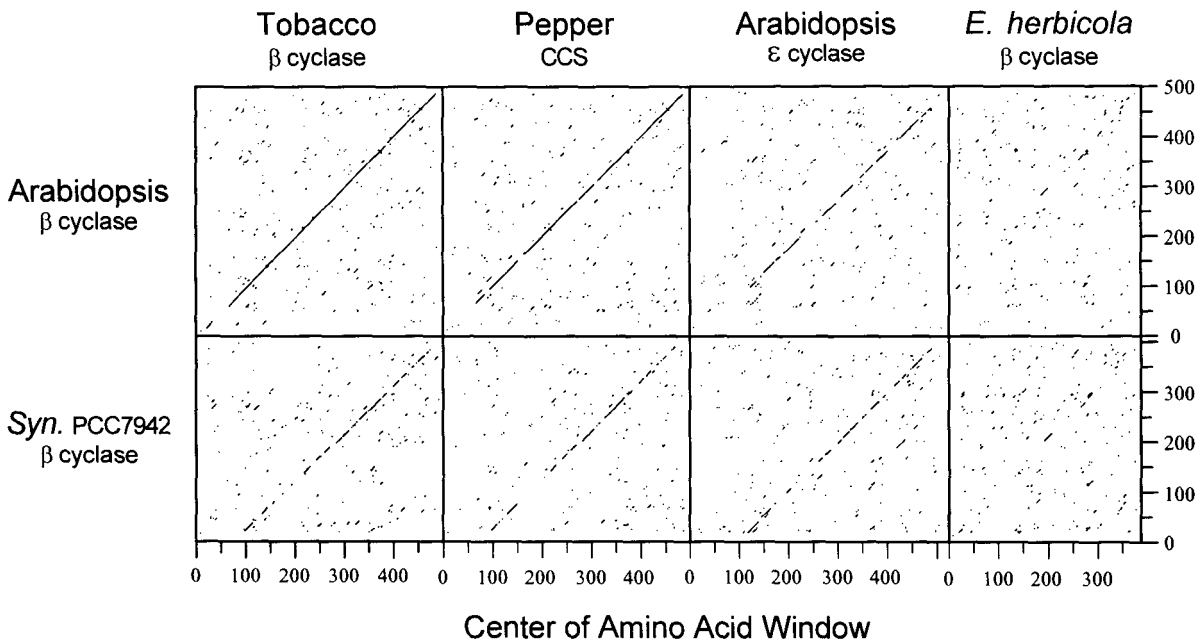


Figure 9. Protein Homology Plots of Carotenoid Cyclase Enzymes.

Amino acid sequences were compared over the entire length for identities or similarities within a window of 10 residues. Seven identical or similar amino acids were scored as a dot. GenBank accession numbers are as follows: Arabidopsis ϵ cyclase, U50738; Arabidopsis β cyclase, U50739; pepper CCS, X76165; *E. herbicola* Eho10 β cyclase, M87280; tobacco β cyclase, X81787; *Synechococcus* (*Syn.*) sp strain PCC7942 β cyclase, X74599.

plant β cyclases was determined and is displayed in Figure 11, where it is aligned with the predicted amino acid sequence of the Arabidopsis ϵ cyclase. There are only a few small gaps in the alignment, and a number of highly conserved regions are evident. A consensus sequence is given in which the amino acids are identical in the plant ϵ and β cyclases, and several features of interest are underlined below this consensus. Amino acid residues conserved in all of the known β cyclases (from plants, cyanobacteria, and bacteria) are also indicated below the consensus sequence.

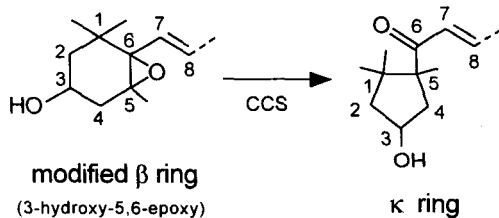


Figure 10. Formation of the κ Ring.

In fruits of certain species of *Capsicum* and in floral parts of a few other species of plants, the five-carbon κ ring may be formed from 3-hydroxy-5,6-epoxy-modified β end groups. Conventional numbering of the carbon atoms is shown for each ring.

An amino acid sequence signature indicative of a dinucleotide-binding motif is present in all of the known carotenoid cyclases (Figure 11). This motif is defined by a characteristic β sheet- α helix-loop- β sheet configuration referred to as the Rossmann or dinucleotide binding fold (Rossmann et al., 1974; Wierenga et al., 1986). Polypeptides with this motif bind such dinucleotides as NADP, NAD, ADP, and flavin adenine dinucleotide (FAD). For the cyclases, this sequence signature resembles that found at the N terminus of enzymes that are known to bind FAD (Van Beeumen et al., 1991; Cunningham et al., 1994). Whether the carotenoid cyclases bind FAD (or NAD, NADP, or ADP) and what role such cofactors might play in the reactions catalyzed by these enzymes are not known. There is no net oxidation or reduction (the molecular formulas of the products are the same as those of the substrates). The proposed reaction mechanisms for the cyclization reactions (Britton, 1988; Hugueney et al., 1995) do not require the presence of a redox cofactor. We suggest that FAD may play a role in stabilizing the envisioned carbonium ion intermediate. Alternatively, it may be that bound FAD is simply a relic of the ancestral enzyme from which the cyclase enzymes were evolved. It could be retained out of necessity for the structural integrity of the enzyme rather than because of any specific catalytic requirement. There are other enzymes that bind FAD and catalyze reactions that result in no net oxidation or reduction (LaRossa and Schloss, 1984; Sanchar, 1994).

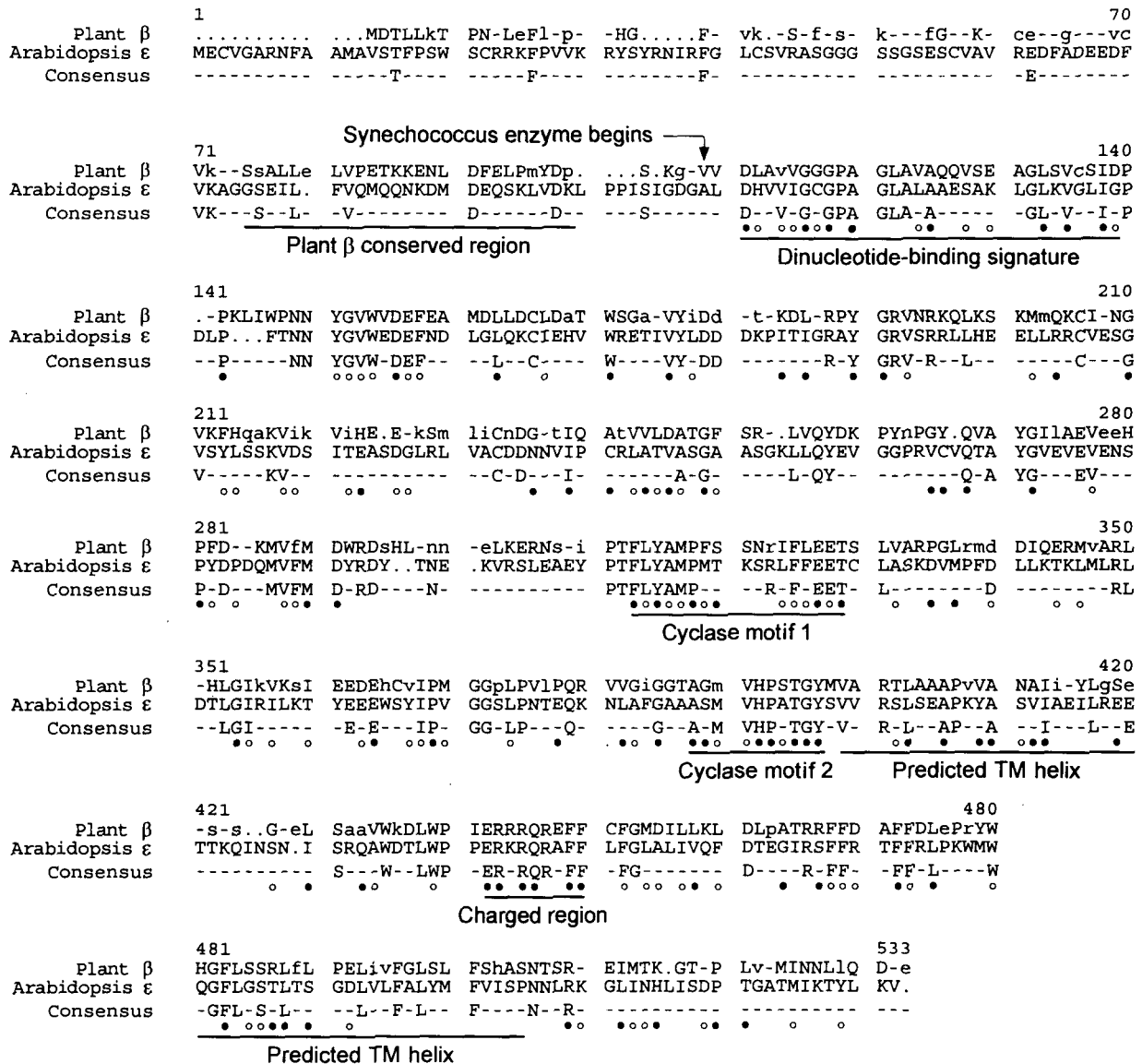


Figure 11. Amino Acid Sequence Alignment of Plant β and ε Cyclase Enzymes.

A consensus sequence for the four available plant β cyclase enzymes (Plant β) is aligned with the predicted amino acid sequence of the Arabidopsis ε cyclase (Arabidopsis ε). A capital letter in the Plant β consensus is used where all four β cyclase cDNAs predict the same amino acid residue in this position. A lowercase letter indicates that an identical residue was found in three of the four. Dashes indicate that the amino acid residue was not conserved, and dots in the sequence denote a gap. A consensus for the aligned sequences is given in capital letters below the alignment where the β and ε cyclase have the same amino acid residue. Below this consensus, a filled circle indicates that the predicted amino acid sequences for at least eight of the nine known bacterial, cyanobacterial, and plant β cyclase genes (four plant, four bacterial, and one cyanobacterial gene; see below) have an amino acid residue identical to that of the Plant β consensus. An open circle indicates an identical residue for at least six of the nine extant β cyclases, with the additional requirement that at least eight of the nine residues at this position are similar (i.e., substitutions are conservative; see below). Several regions of interest are indicated below the alignment and are underlined. Amino acid equivalences used to define conservative substitutions are as follows: S, A, and G; S and T; A and V; T and V; Q, N, E, and D; H, K, and R; L, I, V, M, F, and W; F, Y, W, and H. GenBank accession numbers are as follows: Arabidopsis ε, U50738; Arabidopsis β, U50739 (see also L40176); tobacco β, X81787; tomato β, X86452; pepper β, X86221; *Synechococcus* sp strain PCC7942 β, X74599; *E. herbicola* Eho10 β, M87280; *E. herbicola* Eho13 β, M90698; *E. uredoovora* β, D90087; *Agrobacterium aurianticum* β, D58420. TM, transmembrane.

The initial methionine residue in the *Synechococcus* sp strain PCC7942 β cyclase (Cunningham et al., 1994; not displayed) corresponds to position 109 in the alignment of Figure 11, and the initial residues of the bacterial β cyclase enzymes also align at or near this position. Some of the amino acids preceding position 109 in the plant cyclases are presumably involved in targeting the polypeptides to the chloroplasts and may subsequently be removed to produce the mature polypeptides. We deleted the portion of the gene encoding this region in the β cyclase of tobacco (the remainder was fused to the initiation codon downstream of a strong bacterial promoter; the polypeptide encoded by this truncated gene begins with MVDLA) and found that it is not required for catalytic activity (F.X. Cunningham, Jr., and E. Gantt, unpublished data). However, the marked sequence conservation in the plant β cyclases between positions 75 and 99 (Figure 11, "Plant β conserved region") implies an important function for this region of the polypeptide. It has been suggested that the presence of an N-terminal extension in the mature plant β cyclase that has no counterpart in the cyanobacterial or bacterial enzymes might provide for specific interaction of the cyclases with components of the membrane (Hugueney et al., 1995).

The substrates and products of the ϵ and β cyclase enzymes are hydrophobic and are localized within the membranes of plant and algal chloroplasts. The enzymes themselves are also tightly associated with plastid membranes (Bramley, 1985; Camara and Dogbo, 1986; Beyer, 1987). The predicted amino acid sequences of the Arabidopsis ϵ and β cyclases are not particularly hydrophobic, with an average hydrophobic index for each of approximately -0.18 and with 29% of the amino acids charged ones. Plots of the hydropathic index of the amino acid sequences of the Arabidopsis β and ϵ cyclases (not shown) are similar, and both contain several hydrophobic regions that could correspond to membrane-spanning helical domains. However, none of these regions reaches a magnitude of hydrophobicity (a value >1.6 for a window of 19 amino acid residues) that would indicate a high probability of forming a transmembrane helix, according to Kyte and Doolittle (1982).

The Kyte and Doolittle threshold is a very exclusionary one, and many transmembrane helical segments known from crystal structures do not meet it. Other, empirically based analyses, employing data bases of known helical membrane-spanning domains and making use of the additional information available in multiple sequence alignments, were also used to analyze the cyclase enzymes (see Methods). From the results of these analyses, we conclude that two regions of the plant cyclases are likely to form transmembrane helices. These are marked below the alignment in Figure 11. The outer boundaries or limits of the predicted transmembrane helical regions are approximate, and we do not mean to imply that these two regions are certain to form transmembrane helices or that they are the only regions that may do so.

Because of the similarity of the reactions, we expect that polypeptide regions involved in catalysis and substrate binding will be alike in the ϵ and β cyclases. Among the several

well-conserved regions that can be seen in the alignment of Figure 11, those labeled "cyclase motif 1," "cyclase motif 2," and "charged region" are also highly or moderately conserved in the cyanobacterial and in the distantly related bacterial β cyclase enzymes. A search of the data bases for proteins containing amino acid sequence patterns resembling these three conserved regions did not find any meaningful matches other than the already known carotenoid cyclase enzymes. These three regions, we think, are good candidates for domains that are involved in the binding of substrate and/or in catalysis.

With but a single ϵ cyclase to examine, it is not possible to determine from the sequence alone which few of the many differences in the amino acid sequences of the ϵ and β cyclases are important in differentiating their respective activities. A search of the sequence data bases has yielded three expressed sequence tags from *Brassica napus* (H07750), *B. campestris* (L46452), and potato (R27545) that probably encode ϵ cyclase enzymes. We have begun to sequence additional ϵ cyclase cDNAs and have undertaken other experiments that should enable us to identify regions and residues essential to cyclase enzyme function and to ascertain which amino acid residues define and differentiate the ϵ and β cyclases.

METHODS

Plasmid Construction

Construction of plasmids pAC-LYC, pAC-NEUR, and pAC-ZETA has been described in Cunningham et al. (1994). In brief, the appropriate carotenoid biosynthetic genes from *Erwinia herbicola*, *Rhodobacter capsulatus*, and *Synechococcus* sp strain PCC7942 were cloned in the plasmid vector pACYC184 (New England BioLabs, Beverly, MA). Cultures of *Escherichia coli* containing the plasmids pAC-ZETA, pAC-NEUR, and pAC-LYC accumulate ζ -carotene, neurosporene, and lycopenene, respectively.

The plasmid pAC-BETA was constructed as follows. An 8.6-kb BglIII fragment containing the carotenoid biosynthetic genes of *E. herbicola* (GenBank accession number M87280; Hundle et al., 1991) was obtained after partial digestion of plasmid pPL376 (Perry et al., 1986; Tuveson et al., 1986) and cloned in the BamHI site of pACYC184 to give the plasmid pAC-EHER (not shown). Deletion of the adjacent 0.8- and 1.1-kb BamHI-BamHI fragments (deletion Z in Cunningham et al. [1994]) and of a 1.1-kb Sa1I-Sa1I fragment (deletion X) served to remove most of the coding regions for the *E. herbicola* β -carotene hydroxylase (*crtZ* gene) and zeaxanthin glucosyltransferase (*crtX* gene), respectively. The resulting plasmid, pAC-BETA, shown in Figure 12, retains functional genes for geranylgeranyl pyrophosphate synthase (*crtE*), phytoene synthase (*crtB*), phytoene desaturase (*crtI*), and lycopenene cyclase (*crtY*). Cells of *E. coli* containing this plasmid accumulate β -carotene and form yellow colonies. A plasmid containing both the β and ϵ cyclase cDNAs of Arabidopsis was constructed by excising the ϵ cyclase in plasmid y2 as a PvuII-PvuII fragment and ligating this piece in the SnaBI site of a plasmid (pZL1; Gibco BRL, Gaithersburg, MD) that already contained the cDNA encoding the β cyclase.

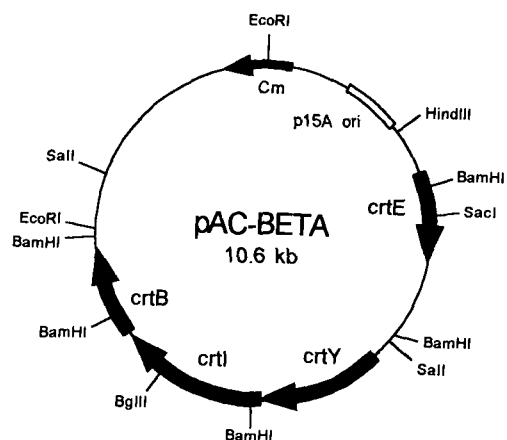


Figure 12. Schematic Diagram and Restriction Map of Plasmid pAC-BETA.

The appropriate genes of *E. herbicola* were cloned in the vector pACYC184 such that cells of *E. coli* containing this plasmid manufacture and accumulate β -carotene. Cm, chloramphenicol resistance gene from Tn9; p15A ori, origin of replication from plasmid p15A.

Organisms and Growth Conditions

E. coli strains TOP10 and TOP10 F' (Invitrogen Corporation, San Diego, CA) and XL1-Blue (Stratagene, La Jolla, CA) were grown in Luria-Bertani (LB) medium (Sambrook et al., 1989) at 37°C in darkness on a platform shaker at 225 cycles per min. Media components were from Fisher Scientific (Difco yeast extract and Difco tryptone; Pittsburgh, PA) and Sigma. Ampicillin (150 μ g/mL) and/or chloramphenicol (50 μ g/mL) (United States Biochemical Corporation) were used, as appropriate, for selection and maintenance of plasmids.

Mass Excision and Color Complementation Screening of an Arabidopsis cDNA Library

A size-fractionated 1- to 2-kb cDNA library of Arabidopsis in λ ZAPII (Kieber et al., 1993) was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus; stock no. CD4-14). Other size-fractionated libraries were also screened (stock nos. CD4-13, CD4-15, and CD4-16). An aliquot of each library was treated to cause a mass excision of the cDNAs, thereby producing a phagemid library, according to the instructions provided by the supplier of the cloning vector (Stratagene; we used *E. coli* XL1-Blue and the helper phage R408).

The titers of the excised phagemid libraries were determined, and the libraries were introduced into a lycopene-accumulating strain of *E. coli* TOP10 F' (this strain contained the plasmid pAC-LYC) by incubation with the *E. coli* cells for 15 min at 37°C. Cells had been grown overnight at 30°C in LB medium supplemented with 2% (w/v) maltose and 10 mM MgSO₄ (final concentrations) and harvested in 1.5-mL microcentrifuge tubes at a setting of 3 in an Eppendorf microcentrifuge (model 5415C; Brinkman Instruments, Inc., Westbury, NY) for 10 min. The pellets were resuspended in 10 mM MgSO₄ to a volume equal

to one-half that of the initial culture volume before addition of the phagemid. Transformants were spread on large (150-mm diameter) LB agar petri plates containing antibiotics to provide for selection of cDNA library plasmids (ampicillin) and maintenance of pAC-LYC (chloramphenicol). Approximately 10,000 colony-forming units were spread on each plate. The petri plates were incubated at 37°C for 16 hr and then at room temperature for 2 to 7 days to allow maximal color development. Plates were screened visually with the aid of an illuminated 3 \times magnifier and a low power stage-dissecting microscope for the rare, pale pinkish yellow to deep yellow colonies that could be observed in the background of pink colonies. A colony color of yellow or pinkish yellow was taken as presumptive evidence of cyclization activity.

The yellow colonies were collected with sterile toothpicks and used to inoculate 3 mL of LB medium in culture tubes, which were incubated overnight at 37°C with shaking at 225 cycles per min. Cultures were split into two aliquots in microcentrifuge tubes and harvested by centrifugation at a setting of 5 in an Eppendorf 5415C microcentrifuge. After the liquid was discarded, one pellet was frozen for later purification of plasmid DNA. To the second pellet was added 1.5 mL of ethanol; the pellet was resuspended by vortex mixing, and extraction was allowed to proceed in the dark for 15 to 30 min with occasional remixing. Insoluble materials were pelleted by centrifugation at maximum speed for 10 min in the microcentrifuge. Absorption spectra of the supernatant fluids were recorded from 350 to 550 nm with a lambda six spectrophotometer (Perkin-Elmer, Norwalk, CT).

DNA Sequence Analysis and Cloning Techniques

Nested deletions were created at both ends of the cDNAs in plasmids y2 and y8, using an Erase-A-Base kit (Promega, Madison, WI). Double-stranded DNA minipreps were made according to the procedure of Del Sal et al. (1988), and DNA sequence was determined by using the Sequenase 2 kit of United States Biochemical Corporation. Both strands were sequenced completely, and 7-deaza-2'-deoxyguanosine-5'-triphosphate (7-deaza-dGTP) was routinely used to minimize compression artifacts. Other procedures or methods were performed according to manufacturer's protocols or standard methodologies (Sambrook et al., 1989) or have been described previously (Cunningham et al., 1993, 1994).

DNA sequence analysis and searching of GenBank, SwissProt, and PROSITE data bases were done using the sequence analysis software package, version 8.0-UNIX (September, 1994), of the Genetics Computer Group (Madison, WI). The Hitachi MacDNASIS program, version 3.0, was also used (Hitachi Software Engineering Co., San Bruno, CA). The programs ENTREZ and BLAST were used via the National Center for Biotechnology Information (NCBI) e-mail server (<http://www.ncbi.nlm.nih.gov/>). The sequences reported in this paper have the GenBank accession numbers U50738 (for the ϵ cyclase cDNA in plasmid y2) and U50739 (for the β cyclase cDNA in plasmid y8).

Analysis of Amino Acid Sequences

Amino acid sequence alignments were created by using the Genetics Computer Group sequence analysis program and the MaxHom program (Sander and Schneider, 1991) available via the EMBL-Heidelberg server (<http://www.embl-heidelberg.de/>). Transmembrane helices were predicted with the TMAP (Persson and Argos, 1994) and PHDhtm (Rost et al., 1995) programs available at the EMBL-Heidelberg server and the program TMpred available at the ISREC server in Switzerland

(http://ulrec3.unil.ch/software/TMPRED_form.html). The Hitachi Mac-DNAsis program, version 3.0 (Hitachi Software Engineering Co.), was used for analyses of hydrophobicity.

Carotenoid Pigment Analysis

Insofar as possible, all procedures were performed under dim room lights. A 0.5-mL aliquot of an overnight culture was used to inoculate 60 mL of LB medium in a 250-mL Erlenmeyer flask. Cultures were grown for 48 hr in darkness at 25°C with shaking at 225 cycles per min and then harvested by low-speed centrifugation in 50-mL disposable conical centrifuge tubes. The pellets were resuspended with ~0.5 mL of water, and 4.5 mL of 6% KOH in methanol was then added. The air over the solution was replaced with nitrogen gas; the tubes were tightly capped and stored in darkness at 37°C for 2 hr. After this saponification step, insoluble material was pelleted by centrifugation, and the pellets were extracted with methanol-diethyl ether (1:1 [v/v]), and then with diethyl ether until the pellets were colorless. The extracts were combined, a few milliliters of aqueous 5 M NaCl was added to the extract, and the carotenoid pigments were transferred to diethyl ether and washed free of alkali, according to standard procedures (Jensen and Jensen, 1971; Davies, 1976). The diethyl ether extracts were evaporated to dryness under a stream of nitrogen and resolubilized in 50 μ L of ethyl acetate. A detailed description of the HPLC analysis is given in Norris et al. (1995). On-line spectra of unknown peaks were compared with those of authentic compounds and with absorption maxima listed in Britton et al. (1995). Mass spectra were obtained for selected chromatographic peaks in HPLC elution profiles, as described previously (Norris et al., 1995). Interpretation of the mass spectra was aided by the review of Enzell and Back (1995).

The common names of carotenoids are used in the text of this paper. The more descriptive systematic or semisystematic names (Weedon and Moss, 1995) are given in parentheses at first use in the text.

ACKNOWLEDGMENTS

We thank George Britton (University of Liverpool, England) for advice on mass spectral analysis of carotenoids and Jim Culver (University of Maryland, College Park) for generously providing the use of his facilities for some of the DNA sequencing. This work was supported by grants to E.G. and F.X.C., Jr., from the U.S. Department of Agriculture (Project MD-9302172) and to E.G. from the Binational Agricultural Research and Development Fund (Project US-2006-9112). This manuscript is scientific article No. A7854 and contribution No. 9184 of the Maryland Agricultural Experiment Station.

Received April 11, 1996; accepted June 7, 1996.

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