One ring or two? Determination of ring number in carotenoids by lycopene ε-cyclases

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Carotenoids in the photosynthetic membranes of plants typically contain two β-rings (e.g., β-carotene and zeaxanthin) or one ε- and one β-ring (e.g., lutein). Carotenoids with two ε-rings are uncommon. We reported earlier that the *Arabidopsis thaliana* lycopene ε-cyclase (LCYe) adds one ε-ring to the symmetrical linear substrate lycopene, whereas the structurally related lycopene β-cyclase (LCYb) adds two β-rings. Here we describe a cDNA encoding LCYe in romaine lettuce (*Lactuca sativa* var. romaine), one of the few plant species known to accumulate substantial quantities of a carotenoid with two ε-rings: lactucaxanthin. The product of the lettuce cDNA, similar in sequence to the *Arabidopsis* LCYe (77% amino acid identity), efficiently converted lycopene into the bicyclic ε-carotene in a heterologous *Escherichia coli* system. Regions of the lettuce and *Arabidopsis* ε-cyclases involved in the determination of ring number were mapped by analysis of chimeric ε-cyclases constructed by using an inverse PCR approach. A single amino acid was found to act as a molecular switch: lettuce LCYe mutant H457L added only one ε-ring to lycopene, whereas the complementary *Arabidopsis* LCYe mutant, L448H, added two ε-rings. An R residue in this position also yields a bi-ε-cyclase for both the lettuce and *Arabidopsis* enzymes. Construction and analysis of chimera of related enzymes with differing catalytic activities provide an informative approach that may be of particular utility for studying membrane-associated enzymes that cannot easily be crystallized or modeled to existing crystal structures.

Carotenoids with cyclic end groups are invariably present in the photosynthetic reaction centers of plants, algae, and cyanobacteria (1, 2). These lipid-soluble isoprenoid pigments protect against photooxidation, harvest light for photosynthesis, and dissipate excess light energy absorbed by the antenna pigments (3–5). The cyclization of the linear pink carotenoid lycopene[†] (Fig. 1) is a pivotal branch point in the pathway of carotenoid biosynthesis in green plants. Two types of cyclic end groups (and derivatives thereof) are commonly found in carotenoids of plants: β- and ε-rings. These two end groups differ only in the position of the double bond within the cyclohexene ring (Fig. 1). Carotenoids with two β-rings are ubiquitous (1, 2) and include β-carotene and zeaxanthin, pigments thought to serve primarily in protecting against photooxidation and/or in dissipation of excess light energy. Carotenoids with one β- and one ε-ring are also common in plants and include lutein, the predominant carotenoid in the light-harvesting antenna of most green plants. Carotenoids with two ε-rings are not commonly found, other than in trace amounts, in plants and algae (1).

The enzymes that catalyze the formation of the β- and ε-rings are encoded by distantly related (36% identity for the deduced amino acid sequences) single-copy genes in the green plant *Arabidopsis thaliana* (7). The *Arabidopsis* lycopene β-cyclase (LCYb) efficiently adds two rings to the symmetrical lycopene to form the bicyclic β-carotene (Fig. 1; ref. 7). In conspicuous contrast, the lycopene ε-cyclase (LCYe) of *Arabidopsis* adds one ε-ring to form the monocyclic δ-carotene rather than the bicyclic ε-carotene (Fig. 1; ref. 7). These differing abilities of the two cyclases disclose a mechanism (i.e., adjustment of the relative activities of the two cyclases) by which plants can apportion substrate to either the β,β-carotenoids that are essential for photoprotection or the β,ε-carotenoids that serve primarily to capture light energy for photosynthesis, while at the same time avoiding formation of inappropriate carotenoids with two ε-rings (7–9).

We sought to understand the molecular basis for the inability of the *Arabidopsis* ε-cyclase to add more than one ε-ring to lycopene. To this end, we isolated an ε-cyclase cDNA from romaine lettuce, a plant known to accumulate substantial amounts of a carotenoid with two ε-rings (10, 11). Indeed, the product of the lettuce cDNA efficiently converted lycopene into the bicyclic ε-carotene in a strain of *Escherichia coli* engineered to accumulate lycopene. A region of the *Arabidopsis* and lettuce ε-cyclases integral to the determination of ring number was subsequently defined by construction and analysis of chimeric lettuce and *Arabidopsis* ε-cyclases. Site-directed mutagenesis in the delineated region identified a single amino acid that determines whether the monocyclic δ-carotene or the bicyclic ε-carotene is produced from lycopene through the action of the *Arabidopsis* and lettuce ε-cyclases.

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Materials and Methods

Plant ε-Cyclase cDNAs. A *Lactuca sativa* var. romaine (romaine lettuce) leaf cDNA library was obtained from Harry Yamamoto (University of Hawaii, Honolulu) (12). An *Adonis aestivalis* var. palaestina flower cDNA library has been described (13). The libraries were screened for cDNAs encoding lycopene cyclases by functional "color" complementation in *E. coli*, as previously described (7). A *Solanum tuberosum* (potato) ε-cyclase cDNA (GenBank accession no. R27545) was obtained from Nicholas J. Provart, Institut für Genbiologische Forschung, Berlin. Nucleotide sequences of the various cDNAs and of chimera and mutants of these (see below) were determined by the DNA Sequencing Facility of the Center for Agricultural Biotechnology at the University of Maryland (College Park, MD).

Construction and Characterization of Chimeric ε-Cyclases. An inverse PCR strategy (Fig. 2) was developed and used to create chimera of the lettuce and *Arabidopsis* ε-cyclase cDNAs. Plasmids containing both cDNAs, in tandem and in the same orientation, were first constructed. Plasmid templates were linearized by digestion with an appropriate restriction enzyme (*Blp*I for plasmid pLse/AtE, see Fig. 2; *Nco*I for pAtE/LsE). PCR was performed in 100-μl tubes in an MJ Research (Cambridge, MA) PTC-150–25 MiniCycler with heated lid. The reaction volume of

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Abbreviations: LCYb, lycopene β-cyclase; LCYe, lycopene ε-cyclase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos.: *Adonis aestivalis LCYb*, AF321534; *A. aestivalis LCYe1*, AF321535; *A. aestivalis LCYe2*, AF321536; *Lactuca sativa LCYe*, AF321538; *Solanum tuberosum LCYe*, AF321537.

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[†]Common names of carotenoids are used in this manuscript. Systematic names (6) for carotenoids discussed in the text are: α-carotene, β,ε-carotene; β-carotene, β,β-carotene; δ-carotene, ε,ψ-carotene; ε -carotene, ε,ε-carotene; γ-carotene, β,ψ-carotene; lactucaxanthin, ε,ε-carotene-3,3′-diol; lycopene, ψ,ψ-carotene.

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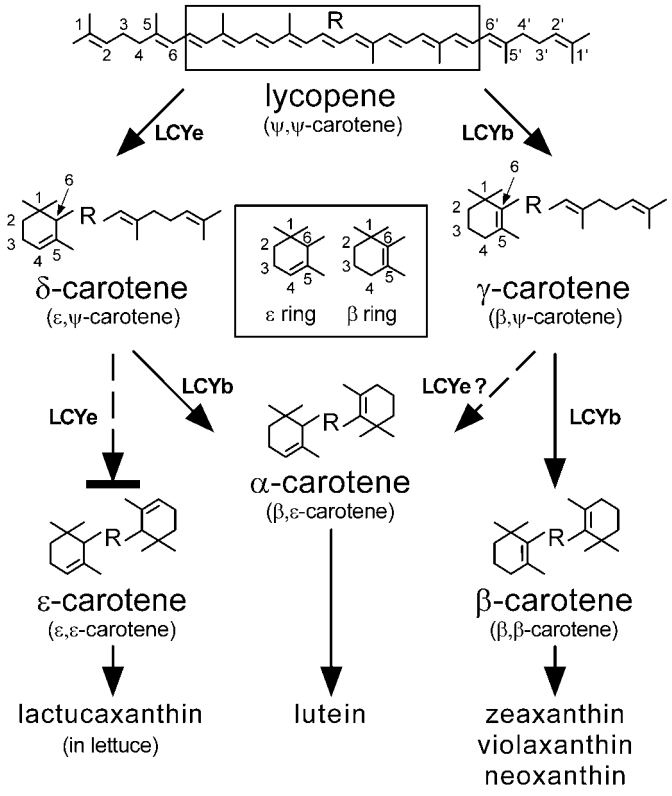

Fig. 1. Biosynthesis of cyclic carotenoids in plants. The symmetrical linear lycopene (ψ,ψ-carotene; ψ indicates a linear or acyclic end group) is converted to the bicyclic β-carotene (β, β-carotene) by the LCYb of *Arabidopsis*. LCYe of *Arabidopsis* adds only one ε-ring to lycopene to form the monocyclic δ-carotene (ε,ψ-carotene). Together, the two cyclases produce α-carotene (β,ε-carotene) and β-carotene. Both α- and β-carotene serve as precursors for production of carotenoids typically found in the photosynthetic apparatus of green plants, including zeaxanthin, violaxanthin, neoxanthin, and lutein. For those few plants that contain substantial amounts of carotenoids with two ε-rings (e.g., lactucaxanthin), it may be surmised that the endogenous LCYe converts lycopene to ε-carotene.

50 μl contained 100 ng of template DNA and 2.5 units of Platinum *Pfx* DNA polymerase (Life Technologies, Gaithersburg, MD). Concentrations of primers, dNTPs, magnesium, and buffer components were as suggested in the manufacturer's protocol. Oligonucleotide primer combinations used to construct the various chimera are listed in the supplemental material that accompanies this manuscript (www.pnas.org). Typical cycling parameters were: 94°C for 3 min, 15 cycles of 94/55/68°C for 20/60/360 sec, 10 cycles of 94/55/68°C for 20/60/360 + 15 additional sec each cycle, 68°C for 10 min, and hold at 4°C. PCR products were purified by gel electrophoresis (0.8% SeaKem GTG agarose; FMC) and recovered from gels by using the Geneclean kit (Bio 101). The ends of the recovered PCR products were phosphorylated with T4 polynucleotide kinase (New England BioLabs; 5 units of enzyme and one-half of the recovered PCR product in a final volume of 10 μl) with incubation at 37°C for 30 min, and the reactions were then cooled on ice. T4 DNA ligase (0.5 μl containing 200 NEB units; New England BioLabs) was then added, and samples were incubated for 12–16 h at 15°C. One microliter of each ligation mixture was used to transform chemically competent *E. coli* (25 μl of XL10 Gold cells; Stratagene), and the transformation mixture was plated on a single large (15 cm) LB agar (1.5%, wt/vol) plate containing 150 μg/ml ampicillin (sodium salt). The resulting colonies (typically several thousand) were collected and combined in 5–10 ml LB medium, and the plasmids they contained were purified and transformed into a pink-colored, lycopene-accumulating *E. coli* strain (14) for analysis (see below). Pigments were extracted and analyzed from several of the resulting yellow colonies (a yellow color is indicative of an active cyclase). Usually more than 50% of the colonies were yellow. The plasmid from one of the colonies was recovered, and the nucleotide sequence was determined to verify the construct.

An *Ava*II site located in the same relative position in the lettuce and potato ε-cyclase cDNAs was used to construct a chimeric lettuce/potato ε-cyclase in which the first 262 amino acids of the encoded polypeptide derived from the lettuce cDNA and the subsequent 272 were specified by the potato cDNA. The plasmid containing this chimeric cDNA is referred to as pLsE262/StE108. The product of this chimeric cDNA converted lycopene to δ-carotene in *E. coli* (data not shown), thereby indicating that the potato enzyme is a mono-ε-cyclase.

Site-Directed Mutagenesis. N-terminal truncations of cDNAs encoding the *Arabidopsis* and lettuce lycopene ε-cyclases were created by using convenient restriction sites or by using the Chameleon Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene) to introduce an *Nco*I site at the position desired for the initiation codon. The resulting product was excised and inserted into the *Nco*I site of plasmid vector pTrcHisA (Invitrogen), downstream of and in the appropriate context for inducible expression under control of the *Trc* promoter. C-terminal truncations were created by introducing termination codons at the desired positions. Various other site-specific mutations, usually accompanied by the introduction of a restriction site to facilitate the identification of mutants, were also created with the Chameleon kit. Primers used to introduce the various mutations are listed in the supplemental data (www.pnas.org). Mutations were confirmed by analysis of the nucleotide sequence.

Functional Analysis of ε-Cyclases. Plasmids containing individual ε-cyclase cDNAs, chimera, or site-directed mutants were transformed into lycopene-accumulating *E. coli* strain TOP10 (14). Cultures in 6-ml LB medium containing 150 μg/ml ampicillin and 30 μg/ml chloramphenicol were grown for 1 day with shaking in darkness at 28°C, as described previously (15). Cells

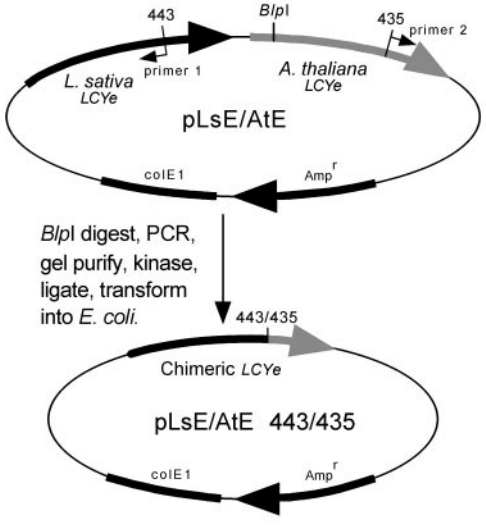

Fig. 2. A PCR strategy was used to construct chimera of lettuce and *Arabidopsis* lycopene ε-cyclase cDNAs. The cDNAs were inserted, in tandem, in the vector pBluescript SK(−), and various chimera were then created by inverse PCR by using the appropriate primers.

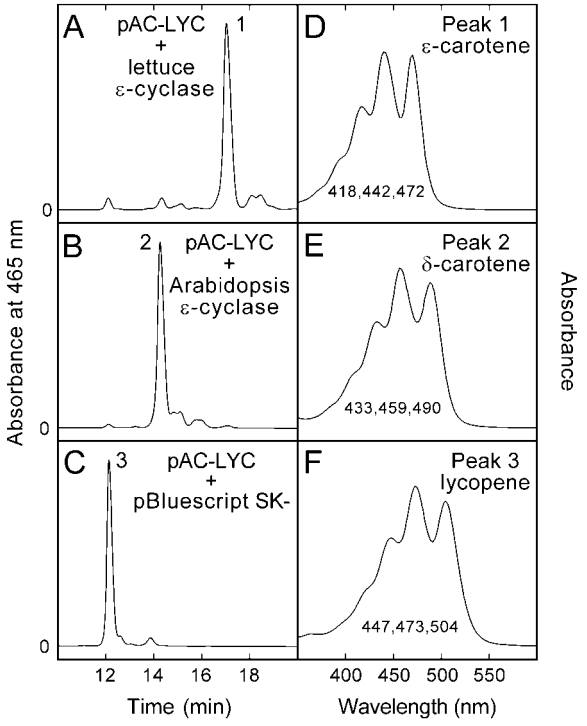

Fig. 3. HPLC elution profiles and absorption spectra of carotenoids produced in a lycopene-accumulating *E. coli* strain (14) in the presence of cDNAs encoding a lettuce lycopene ε-cyclase (*A*) and an *Arabidopsis* lycopene ε-cyclase (*B*). The elution profile of a control culture, containing the empty cloning vector [pBluescript SK(−)], is shown in *C*. Absorption spectra in *D–F* are for the indicated peaks in the adjacent elution profiles. Absorption maxima (for the pigments in the HPLC mobile phase; see *Materials and Methods*) are listed for each spectrum. Elution times were ≈12.2 min for lycopene (ψ,ψ-carotene), 14.3 min for δ-carotene (ε,ψ-carotene), and 17.1 min for ε-carotene (ε,ε-carotene).

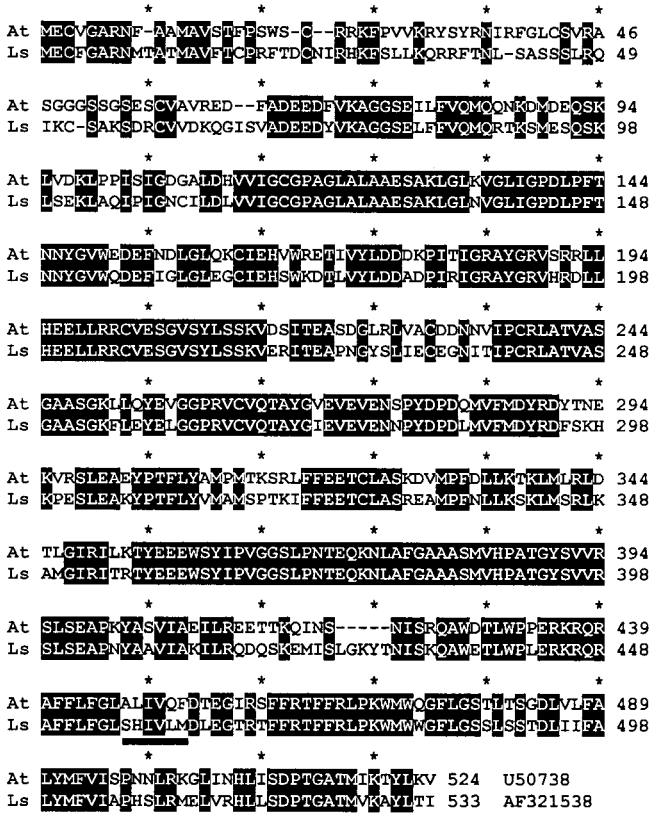

Fig. 4. Alignment of deduced amino acid sequences of *Arabidopsis* (At) and lettuce (Ls) lycopene ε-cyclases. Residues identical for both sequences in a given position are in white text on a black background. A region of interest is underlined. The alignment was created by using CLUSTAL X Ver. 1.8 (17). GenBank accession nos. are listed at the ends of the sequences. Asterisks above the alignment are spaced every 10 residues. Numbers to the right denote the number of the amino acid residue that ends the row.

were harvested by centrifugation, and pigments were extracted and analyzed by HPLC essentially as described previously (7, 16), except that an isocratic mobile phase of 40% B was used for the analysis. Pigments were identified on the basis of absorption spectra and HPLC retention times relative to standard compounds.

Results

The Romaine Lettuce LCYe Is a Bi-ε-Cyclase. Carotenoids with two ε-rings are uncommon in plants. A notable exception is romaine lettuce, where lactucaxanthin (ε,ε-carotene-3,3′-diol) comprises as much as 21% (mol/mol) of the total carotenoid pigment in the leaves (10, 11). We selected 26 prospective lycopene ε-cyclase cDNAs in a screen of a romaine lettuce leaf cDNA library. Other than in length, the cDNAs appeared to be identical. The complete nucleotide sequence of the longest cDNA was ascertained, and a plasmid containing it, pDY4, was introduced into a lycopene-accumulating strain of *E. coli* for analysis of the activity of the encoded enzyme.

In accord with the accumulation of carotenoids with two ε-rings in lettuce *in vivo* (10, 11), introduction of the lettuce ε-cyclase cDNA into the lycopene-producing *E. coli* yielded predominantly (≥90%) ε-carotene, as indicated by the HPLC retention time (Fig. 3*A*) and absorption spectrum (Fig. 3*D*) of the major product. In marked contrast, introduction of the *Arabidopsis* LCYe into lycopene-accumulating *E. coli* yielded, as we earlier reported (7), almost exclusively (≈98% of the total carotenoid) the monocyclic δ-carotene (Fig. 3*B*; absorption spectrum in *E*). The HPLC elution profile of a lycopene-accumulating control culture (Fig. 3*C*) and the absorption spectrum of the major compound in this strain (lycopene; Fig. 3*F*) are also displayed for comparison.

Mapping Regions of LCYe That Specify Ring Number. The *Arabidopsis* and lettuce lycopene ε-cyclases are substantially similar in their deduced amino acid sequences (≈77% identity overall; Fig. 4) and closely resemble other known LCYe (see supplementary data, www.pnas.org). We postulated that specific amino acid differences at the N terminus of the ε-cyclases might be involved in determination of ring number. The lettuce and *Arabidopsis* ε-cyclase cDNAs were therefore modified to produce polypeptides truncated at the N terminus. Substantial portions of the N termini of the lettuce and *Arabidopsis* ε-cyclases were found not to be essential to catalytic function (Fig. 5), but all truncations that yielded an active enzyme did not alter the mixture of products produced from lycopene in *E. coli* (Fig. 5). C-terminal truncations were also constructed, but those that eliminated even a relatively small portion of the polypeptide were found to completely eliminate ε-cyclase enzyme activity in *E. coli* (Fig. 5).

The results of the truncation experiments led us to consider that sequence determinants of ring number might reside in internal portions of the polypeptides. To identify such regions, we constructed a series of chimeric cDNAs encoding portions of both the lettuce and *Arabidopsis* LCYe. To facilitate construction of these chimera, we developed and used an inverse PCR-based method (Fig. 2) that minimizes constraints on the choice of the chimera junction. Construction of the chimera and

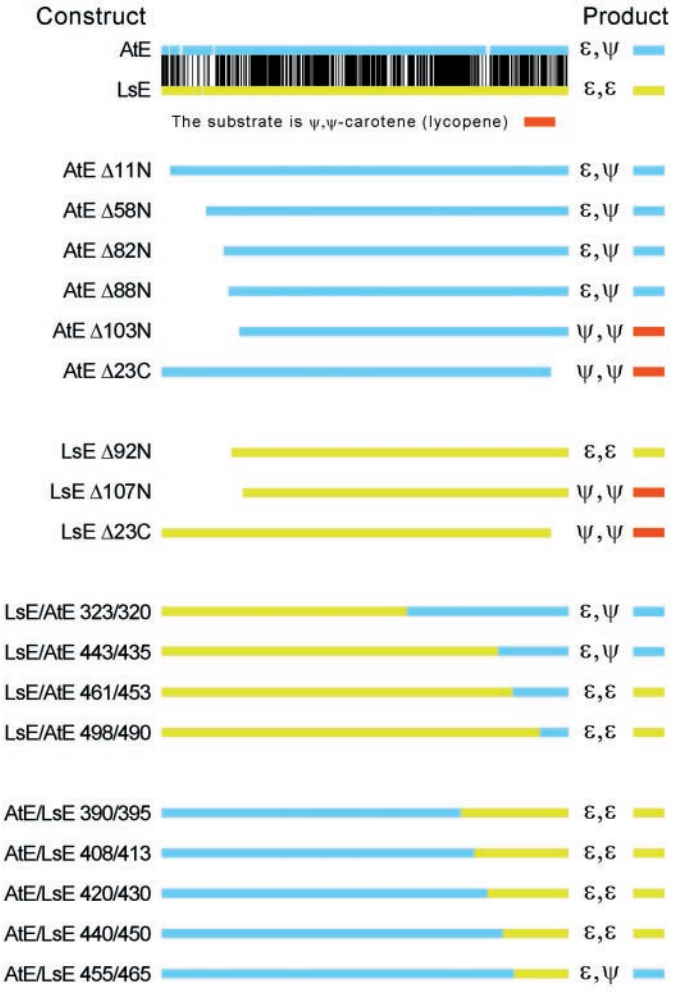

Fig. 5. Schematic illustration of truncated and chimeric lycopene ε-cyclase cDNAs. Carotenoids that accumulate in an *E. coli* strain that contains the indicated cDNA [in plasmid vector pBluescript SK(−)], and that otherwise accumulates only lycopene (ψ,ψ-carotene), are indicated to the right. Only the predominant carotenoid (>90% of the total in all cases) is listed. Solid black vertical lines connecting the *Arabidopsis* and lettuce cyclases (*Top*) indicate identically conserved amino acid residues. LsE/AtE 323/320 defines a chimera consisting of the 5′ portion of the lettuce ε-cyclase cDNA up to and including nucleotide bases specifying amino acid residue 323 and the 3′ portion of the *Arabidopsis* ε-cyclase cDNA beginning with nucleotide bases that encode amino acid residue 320 and proceeding to the end of the cDNA.

the function of their polypeptide products are presented in Fig. 5, first for constructs with the lettuce bi-ε-cyclase for the N-terminal portion and the *Arabidopsis* bi-ε-cyclase for the C-terminal portion of the chimeric cDNA. Characterization of these chimeric lettuce/*Arabidopsis* ε-cyclases defined a region of six amino acids (underlined in the alignment of Fig. 4) that is involved in ring-number determination. These initial experiments did not rule out that other amino acids elsewhere in the polypeptides also influence the ring number. However, analysis of a second series of chimera, this time with the *Arabidopsis* cDNA providing the N terminus and the lettuce cDNA contributing the C terminus, identified the same 6-aa window involved in ring-number determination (Fig. 5). Therefore, it can be concluded that amino acids within this region alone, within the context of the entire sequences of the lettuce or *Arabidopsis* ε-cyclases, are able to determine whether the enzyme adds one or two rings to lycopene.

cDNA	Deduced AA Sequence			Product
Arabidopsis *LCYe*	FFLFGL	ALIVQF	DTEGIR 458	ε,ψ-carotene
Potato *LCYe*	FFLFGL	ALILQL	DIEGIR 312	ε,ψ-carotene
Tomato *LCYe*	FFLFGL	ALILQL	DIEGIR 460	ε,ψ-carotene
Marigold *LCYe*	FFLFGL	ALIVQM	DIEGTR 450	ε,ψ-carotene
Lettuce *LCYe*	FFLFGL	SHIVLM	DLEGTR 467	ε,ε-carotene
Adonis *LCYe1*	FFLFGL	BLIVQL	DIEATR 463	ε,ψ- and ε,ε-
Arabidopsis *LCYb*	FFCFGM	DILLKL	DLDATR 435	β,β-carotene

Fig. 6. A determinant of the number of ε-rings added to lycopene by *Arabidopsis* and lettuce lycopene ε-cyclases was mapped to a 6-aa region defined by the residues ALIVQF in the *Arabidopsis* ε-cyclase and SHIVLM in the lettuce ε-cyclase (see Figs. 4 and 5). Deduced amino acid sequences of lycopene mono-ε-cyclases from tomato (9), marigold (18), and potato (this work) are also displayed for this region. Similarly conserved residues are shown in black text on a gray background. Three amino acid residues in the lettuce bi-ε-cyclase that differ significantly from those in the known mono-ε-cyclases are in white text on a black background. Sequences of an *Arabidopsis* LCYb (a bicyclase) and an *Adonis* LCYe of mixed function are displayed below the lettuce LCYe with two residues of interest shown in white text on a black background. Similarity was defined according to the BLOSUM 45 scoring matrix (19): DE, NH, ST, QKR, FYW, LIVM. GenBank accession nos.: *Adonis LCYe*1, AF321535; *Arabidopsis LCYb*, U50739; *Arabidopsis LCYe*, U50738; lettuce *LCYe*, AF321538; marigold *LCYe*, AF251016; potato *LCYe*, AF321537; tomato *LCYe*, Y14387.

A Single Amino Acid Residue in LCYe Determines Ring Number. The 6-aa segment implicated in determination of ring number is displayed in Fig. 6 for the lettuce and *Arabidopsis* LCYe. For comparison, sequences in this region for other known mono-ε-cyclases are also displayed. The nucleotides that specify these amino acids in the *Arabidopsis* ε-cyclase (ALIVQF) were replaced with those that specify the amino acids of the lettuce ε-cyclase (SHIVLM). The enzyme produced by this cDNA (mutant AtE ALIVQF447–52SHIVLM) functions much as the lettuce ε-cyclase (Table 1), thereby confirming that determination of ring number is influenced by one or more of the amino acids in this small region of the polypeptide.

Within the 6-aa region mapped by the chimeric lettuce and *Arabidopsis* ε-cyclases (Fig. 5), only four residues differ between the two sequences (Fig. 6). Of these four differences, the M461

Table 1. Activity of lycopene ε-cyclases and site-directed mutants with lycopene (Ψ,Ψ-carotene) as substrate in *E. coli*

ε-Cyclase cDNA	Mutation	*Carotenoids lyc : del : eps
(None)	—	100 : 0 : 0
Arabidopsis (AtE)	Wild type (y2)	1 : 98 : 1
AtE	ALIVQF447-52SHIVLM	0 : 2 : 98
AtE	L448H	0 : 8 : 92
AtE	L448R	0 : 8 : 92
AtE	L448D	37 : 56 : 8
AtE	A447D	1 : 98 : 1
Lettuce (LsE)	Wild type (DY4)	3 : 8 : 90
LsE	H457R	3 : 6 : 91
LsE	H457D	22 : 18 : 60
LsE	H457L	17 : 73 : 10
Adonis (AaE1)	Wild type (Ad3)	0 : 44 : 56

*Carotenoids accumulated in a strain of *E. coli* that also contains the plasmid pAC-LYC (14) and thereby normally accumulates lycopene. lyc, lycopene; del, δ-carotene; eps, ε-carotene. Data given as mol : mol : mol.

of lettuce vs. the F452 of *Arabidopsis* is likely unimportant, because it can be considered a conservative replacement and because the marigold mono-ε-cyclase (18) also has an M in this position (Fig. 6). The lettuce H457 (vs. L448 in *Arabidopsis*) and L460 (vs. Q451 in *Arabidopsis*) are the most conspicuous differences relative to the sequence of the *Arabidopsis* LCYe. The activity of a lettuce L460Q mutant did not differ significantly from that provided by the wild-type lettuce cDNA (Table 1). The lettuce H457L mutant, in contrast, exhibited an activity comparable to that of the *Arabidopsis* enzyme: δ-carotene was the predominant product accumulated in *E. coli* (Table 1). Conversely, the corresponding *Arabidopsis* mutant, L448H, gained the ability to produce ε-carotene as the predominant product (Table 1). Thus, the identity of the amino acid residue within this single position of the lettuce and *Arabidopsis* sequences specifies whether a monocyclase or bicyclase activity results.

The *Arabidopsis* L448 and lettuce H457 were also changed to D and R residues to gain some insight as to what properties of the residue in this position influence the determination of ring number. For both the *Arabidopsis* and lettuce ε-cyclases, conversion to an R, like H a positively charged residue, gave results essentially identical to those obtained with an H codon at this position (see AtE L448R and LsE H457R; Table 1). Conversion to D (AtE L447D and LsE H457D), a negatively charged residue, greatly impaired the overall activity of the enzymes (i.e., a substantial proportion of the substrate lycopene remained) and reduced, although did not eliminate, formation of ε-carotene (Table 1).

Discussion

Alternative Solutions to Obtaining a Bicyclase. We have shown that the change of a single amino acid in the polypeptide sequences of the *Arabidopsis* and lettuce lycopene ε-cyclases has a profound influence on the ability of these enzymes to add a second ε-ring to the symmetrical substrate lycopene. The gain of function engendered in the *Arabidopsis* LCYe mutants L448H and L448R and the importance of this specific amino acid residue are made all the more compelling by the contrasting loss of function in the lettuce LCYe mutant H457L. This single amino acid can therefore be said to constitute a molecular switch for ring-number determination by lycopene ε-cyclases.

The lycopene ε-cyclases are members of an extended family of carotenoid modifying enzymes (Fig. 7) that includes capsanthin–capsorubin synthase (CCS; ref. 21) and the recently identified neoxanthin synthase (NSY; refs. 22 and 23), as well as LCYb (7, 18, 20, 26). LCYb and CCS each act at both ends of their respective symmetrical substrates, whereas NSY acts at only one end of the symmetrical violaxanthin. The known plant LCYb do not contain a basic residue in the position corresponding to H457 of the lettuce LCYe; instead, they contain the nonpolar I (Fig. 6 and supplementary data). The ability of LCYb to add two β-rings to lycopene must, therefore, derive from an alternative solution to that which confers a bicyclase activity to the lettuce LCYe.

A more complete conversion to ε-carotene by *Arabidopsis* LCYe mutant ALIVQF447–42SHIVLM (98% ε-carotene) compared with what was obtained with mutant L448H (92% ε-carotene; Table 1) led us to consider that the properties of the preceding amino acid residue (a nonpolar A447 in *Arabidopsis* vs. a polar S456 of lettuce) might also have some influence on determination of ring number. The known plant β-cyclases contain an acidic residue (a D) in this position. More pertinent, two closely related *Adonis aestivalis* ε-cyclase cDNAs (Fig. 6) also specify an acidic amino acid (an E) in this position but do not otherwise differ significantly from mono-ε-cyclases in this region (Fig. 6). The *Adonis* LCYe produce a preponderance of ε-carotene in lycopene-accumulating *E. coli* (Table 1). However, conversion of the A447 residue of the *Arabidopsis* LCYe to a D did not yield a bicyclase (Table 1), indicating that the identity of this residue does not, by itself, determine ring number.

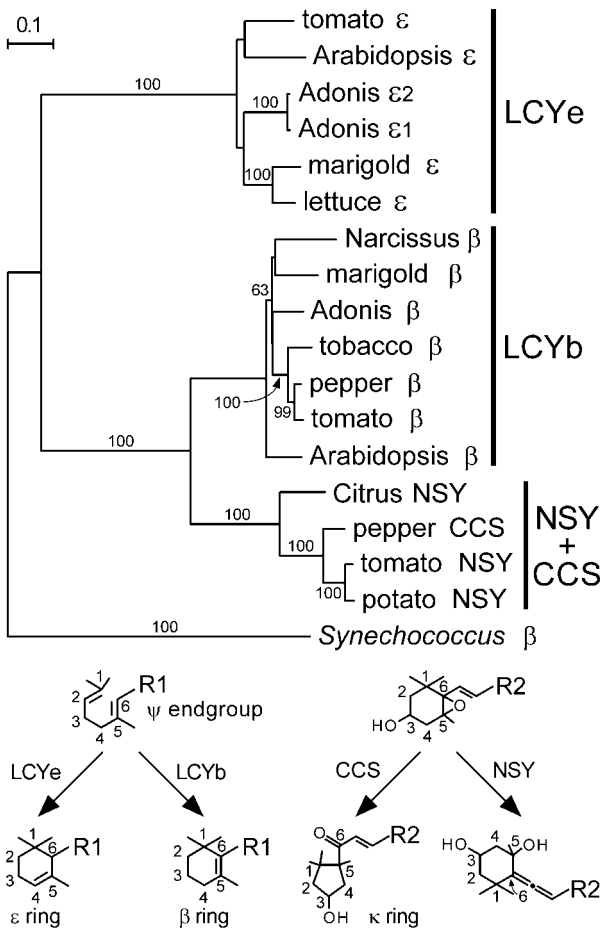

Fig. 7. Neighbor-joining tree for deduced amino acid sequences of plant LCYb and LCYe (7, 9, 18) and of the related plant enzymes capsanthin–capsorubin synthase (21) and neoxanthin synthase (NSY; refs. 22, 23). Reactions catalyzed by the various enzymes are illustrated below the tree. A lycopene β-cyclase from the cyanobacterium *Synechococcus* PCC7942 (14) was used as the outgroup. Branch lengths are drawn to scale. Bootstrap values greater than 50% (for 10,000 replicates with a seed value of 111) are indicated. The analysis encompassed 398 positions, beginning with the initiating Met of the *Synechococcus* cyclase, and excluded those positions with gaps in the alignment. The amino acid sequence alignment and GenBank accession nos. for the nucleotide sequences are included in supplemental data (www.pnas.org). The method of Saitou and Nei (24) was used to construct the tree. Distances were corrected for multiple substitutions (25).

Ramifications of Substrate Orientation in the Plane of the Membrane. Lycopene is a symmetrical nonpolar C_{40} hydrocarbon (Fig. 1) that is insoluble in aqueous solutions and will accumulate in membranes and oil bodies of plant cells. There is considerable uncertainty regarding the orientation of lycopene and other carotenoids in the plane of the membrane (see ref. 27 for a discussion) and also the position of the cyclase enzyme within or on the surface of the membrane (7, 26). If lycopene spans (i.e., is perpendicular to the plane of the membrane) or partially penetrates the membrane, then the two ends of the molecule will almost certainly not be equally accessible to the cyclase. There is experimental evidence that the two ends of β-carotene differ in accessibility to the hydroxylase enzyme that converts this compound to zeaxanthin (β,β-carotene-3,3′-diol; ref. 28). As was suggested for the hydroxylase enzyme (26, 28), the addition of

two rings to lycopene may depend on an ability of the cyclase to form dimers, whereby binding of the more accessible end of the substrate by one of the subunits would serve to bring the other end of the carotenoid molecule into proximity of the cognate subunit, where catalysis could then proceed. The region encompassing the L447 residue of the *Arabidopsis* LCYe might then constitute an interfacial surface that mediates subunit interaction.

Concluding Comments. We capitalized on the relatedness of two carotenoid cyclase enzymes of differing functionality to identify an amino acid residue that determines the distinctive properties of each. The success of this work demonstrates the utility of a domain-swapping approach (see also refs. 29 and 30) for identification of regions and residues of importance in the catalytic properties of a low-abundance membrane-associated enzyme. This approach holds promise for identifying regions and residues integral to the functioning of other members of the carotenoid cyclase family (Fig. 7) as well as members of other families that include closely related enzymes of differing function.

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