The Plant Cell, Vol. 14, 321–332, February 2002, www.plantcell.org © 2002 American Society of Plant Biologists

Identification of the Carotenoid Isomerase Provides Insight into Carotenoid Biosynthesis, Prolamellar Body Formation, and Photomorphogenesis

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Carotenoids are essential photoprotective and antioxidant pigments synthesized by all photosynthetic organisms. Most carotenoid biosynthetic enzymes were thought to have evolved independently in bacteria and plants. For example, in bacteria, a single enzyme (CrtI) catalyzes the four desaturations leading from the colorless compound phytoene to the red compound lycopene, whereas plants require two desaturases (phytoene and ζ-carotene desaturases) that are unrelated to the bacterial enzyme. We have demonstrated that carotenoid desaturation in plants requires a third distinct enzyme activity, the carotenoid isomerase (CRTISO), which, unlike phytoene and ζ-carotene desaturases, apparently arose from a progenitor bacterial desaturase. The Arabidopsis *CRTISO* locus was identified by the partial inhibition of lutein synthesis in light-grown tissue and the accumulation of poly-*cis*-carotene precursors in dark-grown tissue of *crtISO* mutants. After positional cloning, enzymatic analysis of CRTISO expressed in *Escherichia coli* confirmed that the enzyme catalyzes the isomerization of poly-*cis*-carotenoids to all-*trans*-carotenoids. Etioplasts of dark-grown *crtISO* mutants accumulate acyclic poly-*cis*-carotenoids in place of cyclic all-*trans*-xanthophylls and also lack prolamellar bodies (PLBs), the lattice of tubular membranes that defines an etioplast. This demonstrates a requirement for carotenoid biosynthesis to form the PLB. The absence of PLBs in *crtISO* mutants demonstrates a function for this unique structure and carotenoids in facilitating chloroplast development during the first critical days of seedling germination and photomorphogenesis.

INTRODUCTION

Carotenoids are a large class of isoprenoid-derived compounds that are synthesized by plants, bacteria, fungi, and animals. In plants, carotenoids are essential components of the photosynthetic apparatus and are responsible for the red, orange, and yellow color of many flowers and fruit. In addition to their roles in plants, they contribute fundamentally to human health.

Our understanding of carotenoid biosynthesis has advanced dramatically in recent years (Hirschberg, 2001). The pathway involves a series of desaturations, cyclizations, hydroxylations, and epoxidations (Figure 1) commencing with the formation of phytoene. A subsequent series of desaturations is responsible for lycopene synthesis (Figure 1). After the desaturation reactions, the cyclization of lycopene is catalyzed by two enzymes, the β-cyclase and the ε-cyclase, leading to the formation of β-carotene (two β rings) and α-carotene (one β and one ε ring) (Cunningham et al., 1996; Pogson et al., 1996). Lutein is an α-carotene–derived xanthophyll, and zeaxanthin, violaxanthin, and neoxanthin are β-carotene–derived xanthophylls (Rissler and Pogson, 2001).

It is accepted generally that most carotenoid biosynthetic enzymes arose independently in oxygenic, photosynthetic organisms (cyanobacteria and plants) and anoxygenic or nonphotosynthetic bacteria, because little if any homology exists between orthologous enzymes in these organisms (Hirschberg et al., 1997). In particular, the four desaturation reactions shown in Figure 1 from phytoene to lycopene (or three reactions from phytoene to neurosporene) are performed by one desaturase (CrtI or CrtN, respectively) in bacteria and by the phytoene and ζ-carotene desaturases (PDS and ZDS) in cyanobacteria and plants, which are unrelated to the bacterial CrtI-like desaturases.

An additional difference between plant and bacterial carotenoid biosynthesis is carotenoid isomerization during desaturation. That is, the carotenes from phytoene to lycopene

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.010302.

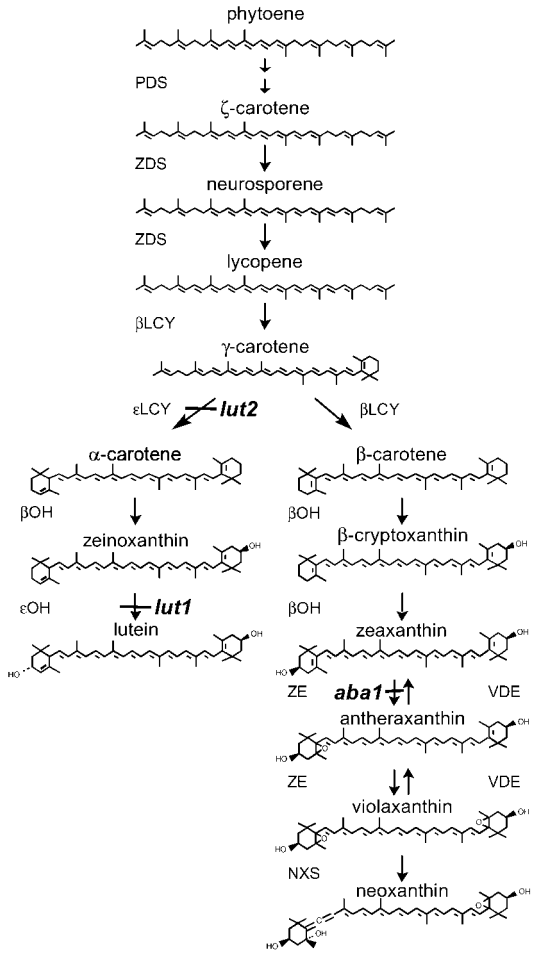

Figure 1. Carotenoid Biosynthetic Pathway.

The commonly held view of the carotenoid biosynthetic pathway in plants is a series of four desaturations to form all-*trans*-lycopene from phytoene. Lycopene is subject to two cyclization reactions to form α- or β-carotene, which are modified further to form the various xanthophylls. PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; βLCY, β-cyclase; εLCY, ε-cyclase; βOH, β-hydroxylase; εOH, ε-hydroxylase; ZE, zeaxanthin epoxidase, NXS, neoxanthin synthase; VDE, violaxanthin deepoxidase.

are all-*trans* in bacteria, but there is evidence of a much more complex set of carotene isomers in plants (Beyer et al., 1989; Britton, 1998). This was first suggested in the 1950s by the isolation of an orange-colored tomato mutant, *tangerine*, that accumulated a poly-*cis* form of lycopene, prolycopene, in its fruit (Tomes et al., 1953; Isaacson et al., 2002). Subsequently, mutations causing *cis*-carotenoid accumulation were reported in other plant and algal systems (Powls and Britton, 1977; Cunningham and Schiff, 1985; Ernst and Sandmann, 1988). More recently, in vitro expression of Arabidopsis PDS and ZDS in *Escherichia coli* has offered further evidence for the requirement of an isomerase during desaturation (Bartley et al., 1999). However, there is no consensus for the number of isomerizations or the biosynthetic step(s) at which they occur, and no carotenoid isomerase activity or protein has been identified (Beyer et al., 1989; Britton, 1998). Thus, despite elegant biochemical and genetic studies (Powls and Britton, 1977; Cunningham and Schiff, 1985; Beyer et al., 1989; Bartley et al., 1999), the carotenoid isomerase reaction(s) have remained enigmatic since first being postulated nearly 50 years ago.

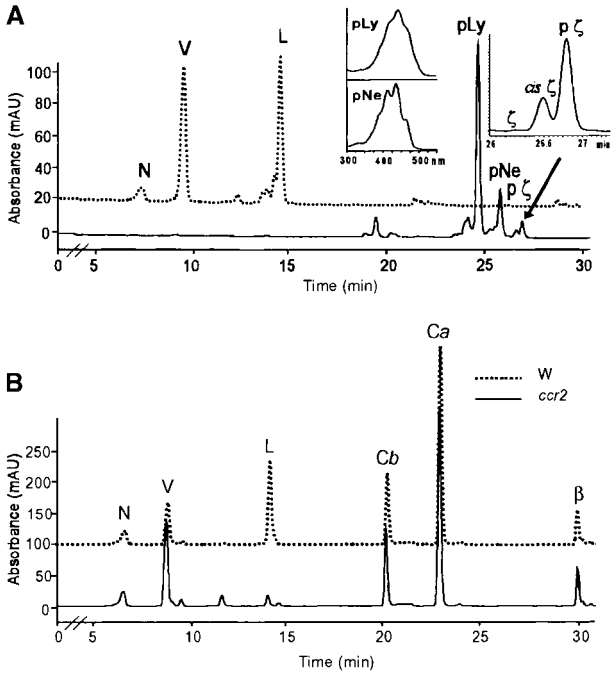

Figure 2. HPLC Analysis of *ccr2* Pigments.

(A) Extracts from etiolated *ccr2* tissue accumulated prolycopene, proneurosporene, and ζ-carotene, whereas etiolated wild-type tissues accumulated lutein and violaxanthin. Insets show the absorbance spectra of prolycopene and proneurosporene and an enlargement of the HPLC trace between 26 and 28 min.

(B) The amount of lutein in *ccr2* leaves ranged from ~10% after 4 days of illumination as shown here to ~40% of wild-type levels in mature leaves.

N, neoxanthin; V, violaxanthin; L, lutein; C*a*, chlorophyll *a*; C*b*, chlorophyll *b*; β, β-carotene; pLy, prolycopene (peak 3); pNe, proneurosporene (peak 4); mAU, milli-absorbance units. Three ζ-carotene peaks were identified and tentatively assigned as ζ-carotene (ζ; peak 5), *cis*-ζ-carotene (*cis*ζ; peak 6), and pro-ζ-carotene (pζ; peak 7) based on their retention times and spectral properties. Peaks 1 and 2 correspond to a monohydroxy xanthophyll (retention time, 19.1 min) and all-*trans*-lycopene (23.5 min), respectively. Absorbance was at 440 nm, which underestimates the proportion of ζ-carotene. See text and Table 3 for percentages of each carotenoid and further details on carotenoid identification.

Table 1. Pigment Content in Wild-Type, *ccr1*, and *ccr2* Mature Green 6-Week-Old Leaves

	Chlorophylls (μg/g fresh weight)		Carotenoids (mmol/mol chlorophyll)					
Plant Line	Total	Chl *a*/*b*	Neoxanthin	Violaxanthin	Antheraxanthin	Lutein	Zeaxanthin	β-Carotene
Wild type	1210 ± 70	3.30 ± 0.1	50 ± 1	45 ± 5	10 ± 1	185 ± 10	—	105 ± 5
ccr1	1040 ± 100	3.26 ± 0.1	50 ± 5	95 ± 10	15 ± 1	80 ± 10	5 ± 1	120 ± 20
ccr2	1150 ± 60	3.27 ± 0.2	45 ± 5	115 ± 5	15 ± 2	65 ± 5	5 ± 1	125 ± 10

Values shown are averages and standard deviations for three plants. Chl *a*/*b*, ratio of chlorophyll *a* to chlorophyll *b*.

Another elusive aspect of carotenoids is their role in photomorphogenesis. In light-grown plants, carotenoids are tightly associated with protein complexes of the photosystems, where they perform a variety of well-defined functions (Sundqvist and Dahlin, 1997). However, carotenoids, typically lutein and violaxanthin, also are present at significant levels in the etioplasts of dark-grown (etiolated) seedlings, where their function is unknown. The etioplast is defined by a uniformly curved lattice of tubular membranes called the prolamellar body, which contains several of the biochemical building blocks required in the chloroplast (Gunning and Jagoe, 1967). The prolamellar body (PLB) has a lipid composition similar to that of the thylakoids lutein and violaxanthin and the chlorophyll tetrapyrrole precursor protochlorophyllide (Pchlide), which is bound to its enzyme, protochlorophyllide oxidoreductase (POR) (Joyard et al., 1998). Both Pchlide and POR are essential for PLB formation (Nielsen and Gough, 1974; Armstrong et al., 1995; Lebedev et al., 1995; Sundqvist and Dahlin, 1997; Sperling et al., 1998). Upon illumination, the PLB disperses to form thylakoids, photosystems are assembled, and chlorophylls and the full complement of carotenoids are synthesized (Fankhauser and Chory, 1997).

The PLB, despite being the defining structure of an etioplast, has a function that is unknown. Its existence is confined mainly to the highly evolved angiosperms, and as a consequence, it has been suggested to provide an advantage to angiosperm seedlings during germination. One hypothesis is that it facilitates rapid chlorophyll synthesis and photosynthetic competence once the seedling emerges from the soil into the light (Sundqvist and Dahlin, 1997). A contrary view is that the existence of PLBs merely reflects the stable presence of the POR-Pchlide complex in plants that have lost the capacity to synthesize chlorophylls in the dark. It has been difficult to define any function of the PLB because previous mutations affecting the PLB have been linked to other factors that affect the greening process directly and dramatically, such as chlorophyll biosynthesis, POR content, and photomorphogenesis (Nielsen and Gough, 1974; Sundqvist and Dahlin, 1997; Sperling et al., 1998).

To elucidate the role carotenoids play in early plastid development, we have identified a novel class of mutations, carotenoid and chloroplast regulation (*ccr*), in Arabidopsis. *ccr* mutations disrupt carotenoid synthesis, resulting in the accumulation of acyclic carotene isomers in the etioplast and a reduction of lutein in the chloroplast. The cloning and analysis of the carotenoid isomerase gene disrupted in *ccr2* have provided new insight into carotenoid biosynthesis and indicate a role for carotenoids and the PLB in photomorphogenesis.

RESULTS

Identification and Phenotypic Characterization of *ccr* Mutations

The *ccr* class of mutations is so defined because its members disrupt both pigment biosynthesis and aspects of plastid development. The first two *ccr* mutations, *ccr1-1* and *ccr2-1*, were identified in an HPLC-based screen of ethyl

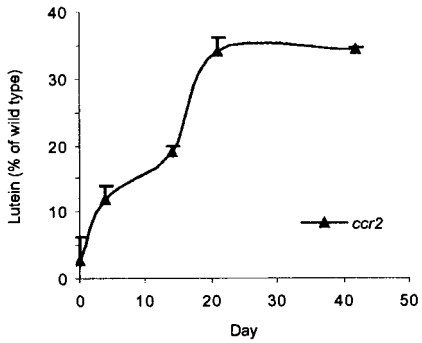

Figure 3. Lutein Accumulation in *ccr2*.

Lutein content of *ccr2* leaves increased during development to a maximum of 30 to 40% of wild-type levels in mature leaves. Standard deviations are shown.

Table 2. Genetic Analysis of *ccr* Loci

Cross	Wild Type	Lutein Reduced
ccr2-1 × *ccr1-1*	18	0
ccr2-1 × *lut1*	29	0
ccr2-1 × *lut2*	48	0
ccr1-1 × *lut1*	13	0
ccr1-1 × *lut2*	13	0
ccr2-1 × *ccr2-3*	0	28
ccr2-1 × *ccr2-4*	0	20
ccr2-1 × *ccr2-5*	0	14

F1 progeny from pairwise reciprocal crosses were analyzed for reduced lutein by HPLC.

methanesulfonate mutants to identify genes required for lutein biosynthesis in Arabidopsis (Pogson et al., 1996). Two lutein-deficient mutations, *lut2* and *lut1* (Figure 1), were shown to disrupt the two enzyme activities required for lutein synthesis, ε-cyclase and ε-hydroxylase, respectively (Pogson et al., 1996; Pogson and Rissler, 2000). Like *lut* mutations, *ccr* mutations also resulted in very low levels of lutein early in development, with a compensatory increase in other xanthophylls (Figure 2B, Table 1). However, unlike the *lut* mutants, the percentage of lutein increased during development, from nearly zero to 30 to 40% of wild-type levels for *ccr2* and up to 60 to 80% for *ccr1* (Figure 3 and results not shown, respectively). Complementation tests and genetic mapping confirmed that *ccr1* and *ccr2* define two recessive, nucleus-encoded genes that map to the proximal and distal ends, respectively, of chromosome 1 (Table 2, Figure 4). Furthermore, the *ccr* mutations did not map to *lut1*, *lut2*, or any known carotenoid biosynthetic loci or Lhcb gene.

Developmental studies revealed that the primary effects of *ccr2* are on carotenoid biosynthesis, whereas *ccr1* is epistatic to *ccr2*. In brief, although the carotenoid profile of *ccr1* is similar to that of *ccr2*, *ccr1* is much more complex in that it exhibits delayed germination, an altered lipid profile, and impaired fertility. Consequently, the focus of this report is on the *ccr2* locus.

We analyzed the carotenoid profile of *ccr2* during development to determine if the disruption to lutein accumulation was observed in nonphotosynthetic tissues. That is, did the phenotype reflect biosynthetic perturbations or impaired incorporation of lutein into the photosystems? Analyses of etiolated tissues revealed the primary phenotype of *ccr2*: instead of lutein and violaxanthin, as in wild-type etioplasts, *ccr2* etioplasts accumulated acyclic, poly-*cis*-carotene precursors, namely prolycopene (7,9,9′,7′-tetra-*cis*-lycopene), proneurosporene (7,9,9′-tri-*cis*-neurosporene), and a mixture of ζ-carotene isomers (Table 3, Figure 2A; see Figure 5 for structures). The major carotenoids present and their percentage of the total in etiolated *ccr2*, in order of retention time, are cryptoxanthin (4.9%), all-*trans*- and *cis*-lycopene (5.3%), prolycopene (54.3%), all-*trans*-neurosporene (1.1%), proneurosporene (14.3%), and three ζ-carotene isoforms (23.1%) (Table 3). All-*trans*-, *cis*-, and pro-ζ-carotene have similar spectra but different elution times (Sandmann, 1991). On the basis of the data of Sandmann (1991) and the retention time of ζ-carotene isoforms produced by bacterial enzymes in *E. coli* that would lack pro-ζ-carotene and the *tangerine* mutant of tomato, we tentatively assign all-*trans*-ζ-carotene (0.2%) as eluting 0.5 min in advance of *cis*-ζ-carotene (5.5%) and pro-ζ-carotene (14.4%) as eluting 0.3 min afterward. The *cis*-carotenes had spectra that appeared to be 15*Z*. Unequivocal determination requires further analysis by NMR.

The accumulation of these lycopene isomers resulted in a distinctive orange-yellow coloration in the leaf-like cotyledons, which is in striking contrast to the yellow cotyledons of wild-type seedlings (Figures 6A and 6B). Upon exposure to light, there was a rapid decrease in the poly-*cis*-carotenes and an increase in the photosynthetic pigments, except for lutein (Figure 2B, Table 1). At 2.5 weeks, plants were placed in complete darkness for 24 hr, and a small additional peak, identified as prolycopene, was observed in *ccr2* but not in wild-type leaves (data not shown).

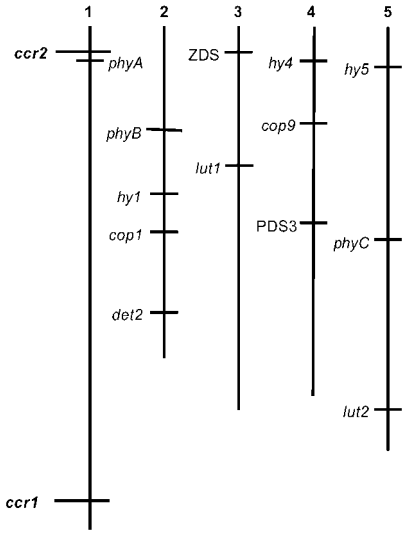

Figure 4. Genetic Map of *ccr1* and *ccr2*.

Genomic DNA from recombinant inbred lines produced by crossing *ccr1* and *ccr2* with Landsberg *erecta* were used for genetic mapping. *ccr1* mapped near the distal end and *ccr2* mapped near the proximal end of chromosome 1. The map locations of a range of carotenoid biosynthetic genes (PDS3, phytoene desaturase; ZDS, ζ-carotene desaturase; *lut2*, ε-cyclase; *lut1*, ε-hydroxylase) and photomorphogenic loci (*phyA*, *phyB*, *phyC*, *hy1*, *hy4*, *hy5*, *cop1*, *cop9*, and *det2*) are shown.

Table 3. Identification of Carotenoids That Accumulated in Etiolated *ccr2* Seedlings

Peak[a]	Retention Time (min)	Mass	Wavelength	Peak III/II Ratio (%)
Monohydroxy xanthophyll				
Peak 1	19.1	552.4	438, 462, 488	10
β-Cryptoxanthin	21	552	425, 449, 476	25
Prolycopene				
Peak 3	24.9	536.1	424, 442, 464	5–10
Standard[b]	24.9	536	424, 442, 464	10
Proneurosporene				
Peak 5	25.8	538.2	412, 436, 462	5–10
Standard[b]	25.8	538	412, 434, 462	10
Pro-ζ-carotene				
Peak 7	26.8	540.6	380, 404, 428	107
Standard[b]	26.8	540	382, 402, 428	98

Values for mass, peak III/II ratio, and some spectra for the standards are from published data.

[a] Peak numbers correspond to those given in Figure 4. Peak 1 is a monohydroxy cyclic carotenoid; however, low concentrations made definitive identification difficult.

[b] Standards purified from *E. coli* overexpressing ζ-carotene, neurosporene, and lycopene were used for all-*trans*-carotenoid standards, and pigments purified from the *tangerine* mutant of tomato were used for poly-*cis*-carotene standards.

Significantly, *ccr2* seedlings greened at approximately half the rate of wild-type seedlings during a 3-day period (Figure 6C). As stated above, green *ccr2* seedlings were fully as viable as the wild type except for reduced lutein (Table 1). The delayed greening in *ccr2* is not caused by the lutein deficiency, because lutein-deficient *lut2* plants produce chlorophyll at wild-type rates during photomorphogenesis (Pogson et al., 1998). Additionally, *ccr2* does not map to any of the known loci that regulate photomorphogenesis, and its phenotype is not consistent with a lesion in any of the phytochrome or cryptochrome receptors or the *cop/det/fus* loci (Figure 4) (Kreunen, 2000).

Positional Cloning and Identification of *CCR2* (*CRTISO*)

Additional *ccr2* alleles were identified by a delayed greening screen (Kreunen, 2000). All three *ccr2* alleles exhibited very similar phenotypes, confirming the link between the observed phenotypes and the *ccr2* locus. A positional cloning strategy was implemented to identify the gene by fine mapping of *ccr2* (Kreunen, 2000). The mutation was tightly linked to a 100-kb genomic fragment cloned into the bacterial artificial chromosome F4H5. Analysis of the open reading frames of F4H5 revealed a candidate gene, F4H5.10 (Figure 7A). F4H5.10 contains 12 introns and encodes a mRNA of 1800 bp. The ethyl methanesulfonate–generated allele *ccr2-1* contains a point mutation (G to A at position 2206) at the start of intron 9, resulting in missplicing such that the F4H5.10 mRNA in *ccr2-1* is 200 bp longer than that in the wild type (results not shown). *ccr2-3* is an untagged allele from a T-DNA population and has a 42-bp deletion between nucleotides 349 and 386. T-DNA integration results in a 29- to 37-bp deletion of genomic DNA (Koncz et al., 1992), so *ccr2-3* is consistent with an aborted integration event or splicing out of the T-DNA. *ccr2-5* is from a fast neutron–treated population, a mutagenic agent known to cause large deletions and chromosomal rearrangements (Redei and Koncz, 1992). The 5′ half of the gene failed to amplify in *ccr2-5* as a result of a deletion, whereas the 3′ end and polymerase chain reaction products 0.3 and 1.3 kb upstream of the coding region did amplify. Thus, the molecular basis of the three alleles of *ccr2* unequivocally confirmed F4H5.10 as *CCR2* (Figure 7A). We named the F4H5.10 gene *CRTISO* because it encodes a carotenoid isomerase (see below).

CRTISO Orthologs and Isomerase Activity in Vitro

CRTISO is highly homologous with expressed sequence tags from a range of plant species, such as tomato, and with an open reading frame from the cyanobacterium *Synechocystis* sp PCC6803 (Figure 7B). A second open reading frame in Arabidopsis, AAG12117, also is related significantly to *CRTISO* and is under investigation; however, its function is unknown at present. Importantly, the *CRTISO* gene appears to belong to the bacterial desaturase gene family. *CRTISO* shows 20 to 30% identity to bacterial carotenoid desaturases (crtN and crtI), including a number of conserved motifs, such as the dinucleotide binding domain. In fact, *CRTISO* is related more closely to the bacterial desaturases than to the plant desaturases, for which the level of identity

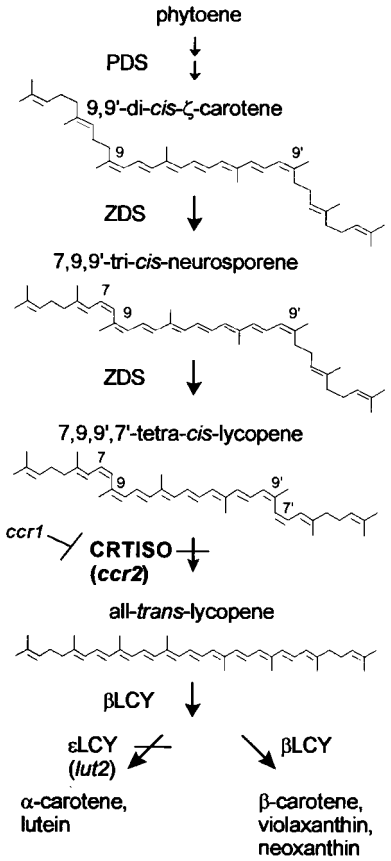

Figure 5. CRTISO Functions as an Isomerase in the Carotenoid Biosynthetic Pathway in Higher Plants.

As opposed to one desaturase enzyme in the all-*trans* pathway of bacteria, higher plants require two desaturases and CRTISO, which can catalyze the isomerization of poly-*cis*-carotenes to all-*trans*-carotenes. See Figure 1 for abbreviations.

(14%) was not significant. The crtN class of desaturases act on C30 carotenoids as opposed to the related crtI C40-specific bacterial desaturases (Wieland et al., 1994). Despite this sequence similarity, *CRTISO* lacked desaturase or cyclase activity when expressed in *E. coli* with all possible carotenoid desaturase substrates (phytoene, phytofluene, neurosporene, ζ-carotene, or lycopene; data not shown).

The absence of desaturase activity and the accumulation of poly-*cis*-carotenes in the etioplasts led us to postulate that the CRTISO enzyme functions to isomerize the poly-*cis*-carotenes to an all-*trans* configuration. Earlier studies (Beyer et al., 1989; Bartley et al., 1999) have proposed that the default desaturation pathway in plants is the production of the poly-*cis* intermediates pro-ζ-carotene, proneurosporene, and prolycopene, with the latter being the substrate for a combined cyclization and isomerization reaction to all-*trans*-α- or β-carotene. We demonstrated in vitro carotenoid isomerase activity for the CRTISO enzyme by combining dark-grown *ccr2* tissue extracts (as substrates) with *E. coli* extracts expressing the CRTISO protein. CRTISO consistently catalyzed an increase in all-*trans*-lycopene, neurosporene, and ζ-carotene isoforms, with a corresponding decrease in their poly-*cis* isoforms (Table 4). The incomplete isomerization may reflect the well-established lability of carotenoid enzymes in vitro or limiting cofactors (Cunningham and Gantt, 1998).

ccr2 Etioplasts and Photomorphogenesis

In addition to lacking lutein and violaxanthin, dark-grown knockout *ccr2* alleles lacked the PLB (Figure 8D) seen in all wild-type etioplasts (Figure 8A). The majority of etioplasts examined (29 of 34) had a few prothylakoid membranes; however, five of 34 contained an amorphous swirl of membranes, but this structure was not organized and did not resemble a PLB (Figure 8E). We grew etiolated wild-type seedlings in the presence of the herbicide norflurazon, which

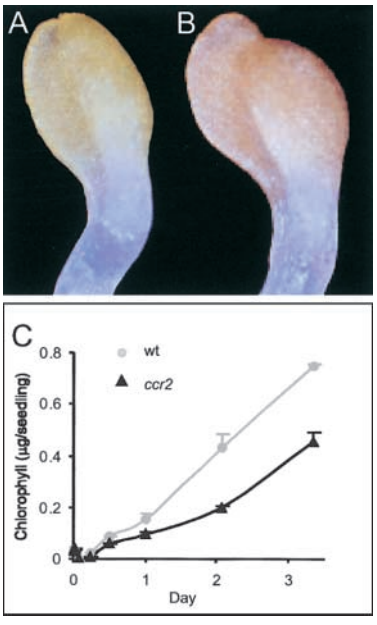

Figure 6. Seedling Development and Chlorophyll Accumulation.

(A) Wild-type etiolated seedlings were yellow from lutein and violaxanthin.

(B) *ccr2* seedlings were an orange-yellow color as a result of the presence of lycopene isomers.

(C) In the absence of the PLB in *ccr2*, chlorophyll accumulation during photomorphogenesis was delayed markedly. wt, wild type.

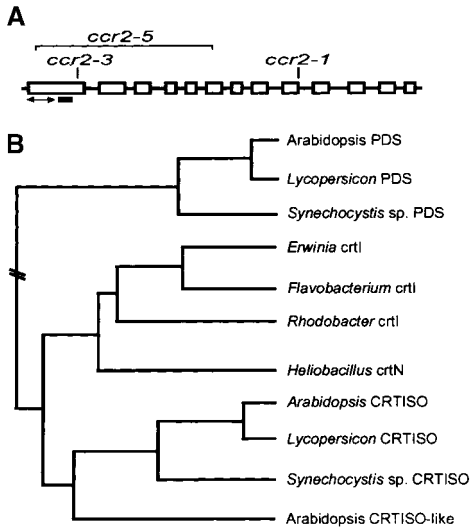

Figure 7. Diagram of the *CRTISO* Gene and a Phylogenetic Tree.

(A) The candidate open reading frame, F4H5.10, was identified based on its linkage to *ccr2*. It contains a predicted chloroplast targeting sequence (arrow), a dinucleotide binding domain (closed box), and other characteristics of carotene biosynthetic enzymes. The sites of the mutations/deletions caused by the three *ccr2* alleles are shown (see text for details).

(B) Phylogenetic tree of isomerase genes (*CRTISO*) and desaturase genes from bacteria (crtI, crtN) and plants (PDS). The identity between plant and bacterial desaturases is not statistically significant.

inhibits phytoene desaturation. These etioplasts accumulated the acyclic carotenoid phytoene and still formed a PLB (data not shown), as has been reported in other species (Axelsson et al., 1982).

The loss of the PLB in *ccr2* is unlike any previously described finding in PLB-deficient plants in that POR levels were unaffected in five replicate protein gel blots and Pchlide levels were reduced only slightly (Figure 9). In *ccr2*, total Pchlide decreased by 15% and the ratio of "active" ("phototransformable"; λmax at 665 nm) to "inactive" (λmax at 625 nm) shifted from 6:4 in the wild type to 4:6 in *ccr2*. This shift in ratios probably is a result of the loss of the PLB, which is the location of the majority of active Pchlide (Sundqvist and Dahlin, 1997).

Significantly, not all *ccr* mutations lacked a PLB, and this seemed to influence photomorphogenesis. The less severe knockdown allele of the other locus, *ccr1*, contained 30 to 40% of wild-type lutein levels, retained a PLB, and greening occurred at a wild-type rate (data not shown). However, a severe allele of *ccr1* appeared to lack a PLB, and its carotenoid content and rate of greening more closely resembled those of the knockout mutations of *ccr2*.

DISCUSSION

Carotenoid Isomerization

The identification of lesions in three alleles of the *CRTISO* locus unequivocally confirms it as the gene whose disruption causes poly-*cis*-carotene accumulation in dark-grown *ccr2* and reduced lutein in light-grown *ccr2*. Furthermore, *CRTISO* is structurally similar to carotene biosynthetic enzymes and has an isomerase activity against poly-*cis*-carotenes in vitro. Therefore, we conclude that *CRTISO* encodes the elusive carotenoid isomerase and that plants actually require three enzymes (PDS, ZDS, and CRTISO) to complete the synthesis of lycopene from phytoene, not two as thought previously or one as observed in bacteria (Figures 1 and 5). This study also confirms that, as has been suggested by biochemical studies (Beyer et al., 1989, 1994), the synthesis of prolycopene is the default pathway in plants, as opposed to the synthesis of all-*trans*-lycopene in bacteria. The four *cis* bonds of prolycopene possibly are introduced either during the desaturation reactions or by geranylgeranyl pyrophosphate synthase and the desaturation reactions (Britton, 1998).

Although much remains to be learned about the mechanism of the CRTISO enzyme, several insights have been obtained to date. First, the *CRTISO* gene is related most closely to a group of bacterial desaturases, crtN, that acts on C30 carotenoids (Wieland et al., 1994). The C40 plant carotenoids encompass the C30 compound, and the CRTISO-targeted *cis* bonds all are located within this region. The incorporation of such an atypical desaturase into an ancestral cyanobacterium may have facilitated the coordinated evolution of desaturation and isomerization reactions before the endosymbiotic event that is believed to have given rise to plant chloroplasts. Second, this identity to desaturases includes the conserved dinucleotide binding site, and this is

Table 4. CRTISO Enzyme Assay

Carotenoid	Etiolated *ccr2* + *E. coli*	Etiolated *ccr2* + CRTISO	Proportional Change[a]
All-*trans*-lycopene	5.0 ± 0.7	9.5 ± 1.8	1.89 ↑
Prolycopene	31.9 ± 3.1	26.3 ± 4.7	0.82 ↓
Neurosporene	3.1 ± 0.01	4.8 ± 0.5	1.53 ↑
Proneurosporene	9.9 ± 1.9	7.9 ± 2.5	0.79 ↓
All-*trans*-ζ-carotene	1.6 ± 0.47	2.2 ± 0.16	1.41 ↑
Mono-*cis*-ζ-carotene	7.8 ± 0.4	8.2 ± 0.7	1.05 —
Pro-ζ-carotene	10.6 ± 0.8	8.9 ± 1.3	0.84 ↓

[a] Incubation of recombinant CRTISO protein with *ccr2* etiolated tissue resulted in a decrease in prolycopene, proneurosporene, and pro-ζ-carotene with a similar increase in the respective all-*trans* isoforms. Averages and standard deviations for four experiments are provided.

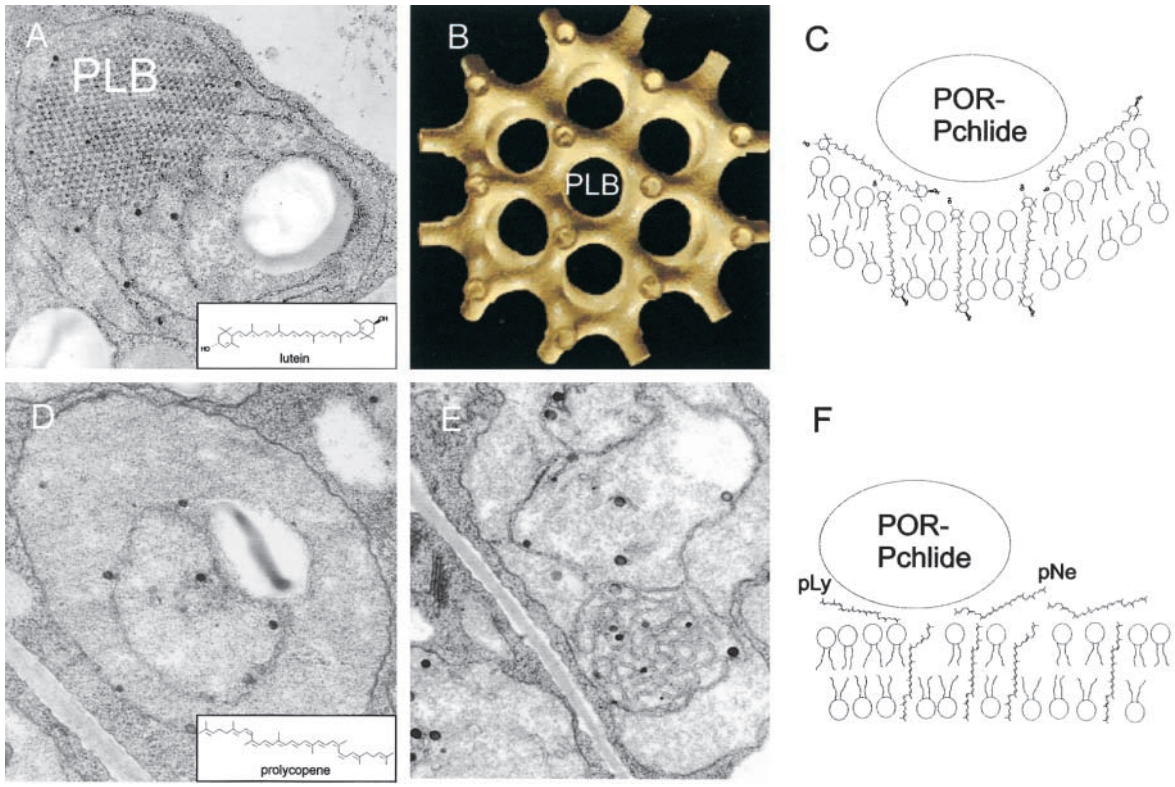

Figure 8. CRTISO and PLB Formation.

(A) Wild-type etioplasts contained a PLB.
(B) PLB diagram is based on the "wurtzite" PLB structure observed in Arabidopsis and many other species. The diagram was provided by Dr. Brian Gunning (Australian National University).
(C) Model of the possible interactions between membranes, POR:Pchlide, and carotenoids in facilitating PLB formation. In vitro studies have shown that some lutein lies parallel with the membrane surface and other lutein molecules span the bilayer in a manner analogous to cholesterol (Sujak et al., 1999). It is not known which of these orientations would facilitate PLB formation.
(D) All *ccr2* etioplasts examined lacked a PLB, with most (29 of 34) having just a few prothylakoid membranes.
(E) Some *ccr2* etioplasts (15%) contained an amorphous prothylakoid aggregate.
(F) A model of how the stepped structure of poly-*cis*-carotenes could perturb membrane curvature by increasing the spacing between fatty acids and/or by disrupting the association between the membranes and POR:Pchlide. pLy, prolycopene; pNe, proneurosporene.

consistent with the temporary "holding" of carotene electrons while *cis* double bonds are broken and then reformed in an all-*trans* conformation.

Third, CRTISO is a *cis*-*trans*-isomerase (Beyer et al., 1994). This conclusion is based on the poly-*cis* to all-*trans* isomerization activity of CRTISO in vitro, the presence of poly-*cis*-carotenoids in dark-grown *ccr2* mutants, including the accumulation and subsequent photoisomerization of prolycopene before xanthophyll synthesis, and the reformation of prolycopene in the dark in *ccr2* plants (Britton, 1998). Although the sequence of reactions shown in Figure 5 is not confirmed, it seems likely that either prolycopene is the substrate for a simultaneous cyclization/isomerization reaction using CRTISO and the cyclases (Beyer et al., 1994) or CRTISO carries out the *cis*-to-*trans* isomerization of the poly-*cis*-carotenes before cyclization. Further enzymatic studies, including examination of the substrate specificity of CRTISO, are required.

In the presence of light, CRTISO activity appears partially redundant, because most wild-type carotenoids, with the exception of lutein, are made efficiently in *ccr2* (Figure 2B, Table 1). Photoisomerization has been observed in vitro and is believed to occur in vivo (Cunningham and Schiff, 1985; Sandmann, 1991). Certainly, we observed a marked increase in all-*trans*-lycopene after 30 min of light. Moreover, in the *tangerine* mutant of tomato, which also is a lesion in *crtISO* (Isaacson et al., 2002), light-induced isomerization occurs in chloroplasts but not in chromoplasts of fruit and flowers, which may reflect the crystalline nature of carotenes in tomato chromoplasts (Isaacson et al., 2002). Thus, the nature of light-induced isomerization in plastids remains

enigmatic. The reduction in lutein in light-grown *ccr2* tissue suggests that the β- and ε-cyclases differ in their requirement for CRTISO activity or protein. There are at least two alternatives. The first is that CRTISO interacts directly with the cyclases, perhaps in a multimeric complex, and its absence preferentially destabilizes the hypothesized β,ε-cyclase complexes but not β,β-cyclase complexes (Cunningham and Gantt, 1998). Another explanation is that photoisomerization in vivo produces lycopene isomers that are poor substrates for the ε-cyclase.

Carotenoids, PLB Formation, and Photomorphogenesis

The *ccr* mutations, which lack a PLB, provide the first evidence that specific carotenoids are essential for PLB formation. In yellow wild-type seedlings, lutein and violaxanthin are associated with the PLB seen in Figure 8A (Selstam and Sandelius, 1984). However, in the orange-yellow *ccr2* seedlings, various lycopene and carotene isomers accumulate (Figure 2A), the PLB is absent (Figures 8D and 8E), and unlike any previously described PLB-deficient plants (Sundqvist and Dahlin, 1997), POR levels are unaffected and Pchlide levels are reduced only slightly (Figure 9) (Axelsson et al., 1982; Sundqvist and Dahlin, 1997). Thus, the absence of a PLB in *ccr* plants is quite unexpected and is not attributable to the loss of cyclic end groups because it will form in the presence of all-*trans*- (or 15*Z*)-phytoene (data not shown) (Axelsson et al., 1982).

The absence of the PLB in *ccr2* is caused by either the altered carotenoid composition or the absence of the enzyme itself. Coomassie blue– and silver-stained gels of total PLB protein have shown repeatedly that POR is the most abundant polypeptide (Lindsten et al., 1988; Minkov et al., 1988), yet immunoblots suggest substantial levels of carotenoid enzymes, particularly phytoene synthase, relative to POR (Welsch et al., 2000). This difference, which may simply reflect affinity differences in phytoene synthase and POR antisera, needs to be resolved, because given the fact that POR content regulates PLB size (Franck et al., 2000), a degree of stoichiometry between the carotenoid enzymes and POR would be expected if they were to affect PLB formation directly.

Alternatively, it is quite probable that the poly-*cis*-carotenoids could disrupt PLB formation. POR clearly facilitates the curvature in vivo (Hyde et al., 1997; Sundqvist and Dahlin, 1997) such that PLBs (Figure 8B) will not form spontaneously in vitro; however, certain lipid classes in an aqueous phase in vitro will form similar but much smaller structures (Hyde et al., 1997). This includes monogalactosyl diacylglycerol, which is enriched in the PLB (Selstam and Sandelius, 1984; Sundqvist and Dahlin, 1997), and it has been noted that terpenes, such as cholesterol and carotenoids, regulate membrane fluidity, which could stabilize such curved membranes (Hyde et al., 1997). We propose that the role of wild-type carotenoids in PLB assembly may be to stabilize or facilitate the curved membranes that form as a result of interactions with POR:Pchlide (Figure 8C). The stepped shape of the poly-*cis*-carotenoids may destabilize membrane curvature by altering membrane fluidity (Figure 8F) (Hyde et al., 1997; Sundqvist and Dahlin, 1997). This thesis requires further investigation.

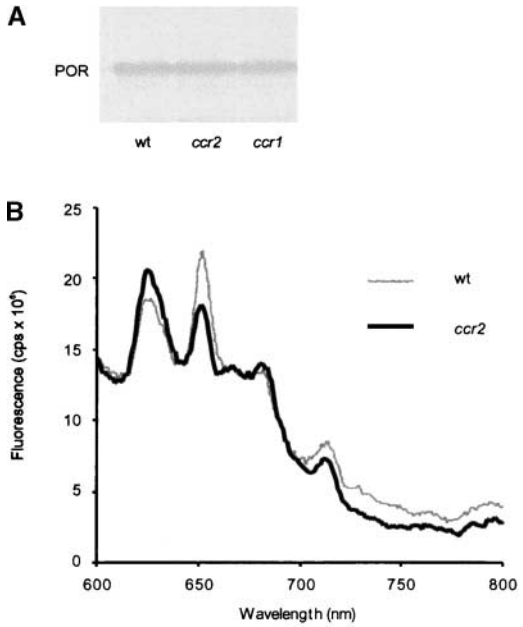

Figure 9. Content of POR and Pchlide in the Wild Type (wt) and *ccr2*.

(A) Immunoblot of POR. POR protein levels were identical in tissue from etiolated wild type, *ccr1*, and *ccr2*.

(B) Low temperature (77K) fluorescence spectra of etiolated seedlings. Fluorescence of the etiolated seedlings was measured from 600 to 700 nm with excitation at 436 nm. The total amount of Pchlide was determined by summation of peaks 650 to 657 nm and 628 to 623 nm and was slightly lower in *ccr2* than in wild-type seedlings. Wild-type seedlings contained more phototransformable Pchlide (650 to 657 nm) than did nontransformable Pchlide (628 to 633 nm), whereas *ccr2* had the reverse. The spectrum was normalized at 600 nm to zero. cps, counts per second.

Finally, and significantly, the *ccr* mutants allow direct testing of the hypothesis that PLB confers a fitness advantage. In dark-grown knockout *ccr* alleles, lutein, violaxanthin, and PLBs are absent, and in apparent consequence, chlorophyll is produced or accumulated more slowly and greening is delayed by several days (Figure 6C). However, in a knockdown *ccr1* allele with a mixture of prolycopene and some all-*trans*-xanthophylls, a PLB forms and greening occurs at a wild-type rate. The altered rate of greening appears to correlate better with the presence or absence of a PLB than with the carotenoid composition, suggesting that the PLB does in fact confer an enhancement of photomorphogenesis.

For more than 40 years since the isolation of the first prolycopene-accumulating mutant in tomato, the existence of a

carotenoid isomerase in plants has been postulated but never proven. Likewise, the existence of PLBs was established more than 30 years ago, but a function could not be demonstrated unequivocally. The *ccr* mutants have allowed physical isolation and characterization of the elusive carotenoid isomerase, demonstrating a requirement for carotenoid biosynthesis to form a PLB, which apparently accelerates photomorphogenesis. Thus, there is a key role for carotenoids in dark-grown tissues in addition to their well-established role in green, photosynthetic tissues.

METHODS

Plant Growth Conditions

Arabidopsis thaliana seed were sterilized with 70% ethanol for 3 min and 20% bleach for 5 min and then washed thoroughly with sterilized water before sowing. Plants for etiolation and greening experiments were grown on Murashige and Skoog (1962) basal salt mixture with 2% sucrose. After vernalization for 3 days (4°C, dark), plants were grown in the dark for 4 days at 21°C and transferred to continuous light at 70 to 80 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ for photomorphogenesis. For experiments involving mature, green tissue (3 to 6 weeks old), plants were grown in soil under 16 hr of light per day at an intensity of 100 to 150 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ at 21°C as a standard condition (Pogson et al., 1996).

Mapping and Genetic Analysis

For mapping, lines of *ccr2* (Columbia ecotype) were crossed with wild-type plants (Landsberg *erecta* ecotype). Homozygous *ccr* F2 generation plants were identified by HPLC analysis, and genomic DNA from the plants was extracted from 20-day-old plants using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Published and newly designed markers were used for mapping and included simple sequence length polymorphisms, cleaved amplified polymorphic sequences, and derived cleaved amplified polymorphic sequence (Konieczny and Ausubel, 1993; Bell and Ecker, 1994; Michaels and Amasino, 1998). To identify additional alleles, ethyl methanesulfonate–mutagenized Arabidopsis lines, fast neutron–mutagenized lines (M2 seed from Lehle Seed, Round Rock, TX), and T-DNA lines (Arabidopsis Biological Resource Center, Columbus, OH) were etiolated, exposed to intermittent light, selected by a delayed greening phenotype, and transferred into soil. After the emergence of true leaves, pigments were analyzed by HPLC for a reduction in lutein content. To test for allelism, reciprocal crosses between homozygous mutants were performed as described by Pogson et al. (1996).

Pigment Analysis and Quantification

Pigment extraction, analysis, and quantification were performed as described (Pogson et al., 1996, 1998), except that carotenes were expressed as a peak area at the maximal wavelength for each pigment. All extractions and analyses on etiolated seedlings were performed under dim light, and the catalysis of protochlorophyllide, which is extremely sensitive to light, was used to determine the effective absence of light. Total chlorophyll and carotenoid contents were measured as described (Porra et al., 1989) with the following modifications: pigments were extracted in 80% acetone, and absorbance was measured at 663.2, 646.8, and 470 nm.

Peak identification was based on comparison with standards purified from the *tangerine* mutant of tomato and from bacteria expressing carotenoid biosynthetic genes (Cunningham and Gantt, 1998). In particular, retention time, UV/visible light spectra, and mass spectrometry determinations were undertaken and compared with standards and published data (Beyer et al., 1989; Sandmann, 1991; Wieland et al., 1994).

For CRTISO assays, etiolated *ccr2* plants were grown in the dark for 5 to 10 days, homogenized in a detergent buffer (Fernandez-Gonzalez et al., 1997), and then filtered through two layers of sterile cheesecloth. The *CRTISO* cDNA was expressed in *Escherichia coli* T-Easy vector (Promega), and the bacteria were ultrasonicated for 5 min and then centrifuged for 5 min. The bacterial supernatant was incubated with the plant extract for 6 hr at room temperature in the dark. An equal volume of acetone/ethyl acetate (6:4) was added followed by a 10-min centrifugation and then analysis by HPLC as described (Pogson et al., 1996, 1998). Endogenous plant carotenoid enzymes in the extract are labile and have negligible activity in vitro under the assay conditions used. The control samples were subjected to the same treatment with untransformed *E. coli*. The experiment was repeated four times, and averages and standard deviations are shown.

Mass Spectrometry

Unknown peaks from etiolated seedlings were analyzed by mass spectrometry. Each compound was collected during HPLC and dried under nitrogen gas. The pigment was resuspended in 50 μL of acetonitrile and embedded in an elemental sulfur matrix (Brune, 1999). Mass was recorded by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (HX100; JEOL).

Low-Temperature (77K) Fluorescence Spectroscopy

Low-temperature fluorescence of etiolated seedlings was measured as described (Hoober et al., 1994) with slight modifications. Measurements were conducted under a green safelight. Etiolated seedlings were ground under liquid nitrogen and then resuspended in 60% glycerol. The sample suspension then was loaded into NMR tubes and placed in the dark at –80°C for 20 min. The samples were submerged in liquid nitrogen, and fluorescence emission spectra were measured between 600 and 700 nm with excitation at 436 nm to excite protochlorophyllide (Hoober et al., 1994). For each measurement, three plants were pooled together and five replicates were conducted.

Immunoblots

Proteins were resolved by 12% SDS-PAGE, and immunoblotting was performed as described (Park and Hoober, 1997; Rissler and Pogson, 2001). Total proteins were extracted from etiolated seedlings or leaves from 20-day-old light-grown plants, and 10 μg of the extracted protein was loaded equally onto the gel. Five replicate protein gel blots were made using protochlorophyllide oxidoreductase

antisera kindly provided by Dr. G. Armstrong (Swiss Federal Institute of Technology, Zurich, Switzerland).

Transmission Electron Microscopy

For prolamellar body examination, seed were vernalized with or without norflurazon (10 mM) for 3 days in the dark at 4°C. After vernalization, plants were etiolated for 5 days, and the leaf tissue was fixed as described (Park et al., 1999). Briefly, the leaf was fixed with 2% glutaraldehyde for 1 hr at room temperature and then postfixed with 1% OsO_4. Samples were dehydrated in a gradient of ethanol and acetone and infiltrated gradually with Spurr's hard resin. Ultrathin sections were stained with 2% uranyl acetate and 2.6% lead citrate and examined with a Philips (Eindhoven, The Netherlands) CM12 transmission electron microscope.

Accession Numbers

The GenBank accession numbers for the sequences shown in Figure 7 are AAF63149 (F4H5.10), Q07356 (Arabidopsis PDS), A45381 (*Lycopersicon* PDS), S74886 (*Synechocystis* sp PDS), AAA24820 (*Erwinia* crtI), AAC44850 (*Flavobacterium* crtI), AAA50313 (*Rhodobacter* crtI), AAC84034 (*Heliobacillus* crtN), AAF63149 (Arabidopsis *CRTISO*), AI778443 and BE436865 (*Lycopersicon CRTISO*), BAA10798 (*Synechocystis CRTISO*), and AAG12117 (Arabidopsis *CRTISO*-like).

ACKNOWLEDGMENTS

We thank Raymund Beirne and Dahlia Blunt for their technical assistance, Dan Brune for assistance with mass spectrometry, and our colleagues who provided advice and materials. Additional thanks to Dr. J. Kenneth Hoober (Arizona State University) and Dr. Brian Gunning (Australian National University) for helpful discussions. We thank the Australian National University, Arizona State University, and the Australian Research Council for financial support.

Received July 23, 2001; accepted October 30, 2001.

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