

Maize Y9 Encodes a Product Essential for 15-cis- ζ -Carotene Isomerization¹[OA]

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Carotenoids are a diverse group of pigments found in plants, fungi, and bacteria. They serve essential functions in plants and provide health benefits for humans and animals. In plants, it was thought that conversion of the C40 carotenoid backbone, 15-cis-phytoene, to *all-trans*-lycopene, the geometrical isomer required by downstream enzymes, required two desaturases (phytoene desaturase and ζ -carotene desaturase [ZDS]) plus a carotene isomerase (CRTISO), in addition to light-mediated photoisomerization of the 15-cis-double bond; bacteria employ only a single enzyme, CRTI. Characterization of the maize (*Zea mays*) *pale yellow9* (*y9*) locus has brought to light a new isomerase required in plant carotenoid biosynthesis. We report that maize Y9 encodes a factor required for isomerase activity upstream of CRTISO, which we term Z-ISO, an activity that catalyzes the *cis*- to *trans*-conversion of the 15-cis-bond in 9,15,9'-tri-cis- ζ -carotene, the product of phytoene desaturase, to form 9,9'-di-cis- ζ -carotene, the substrate of ZDS. We show that recessive *y9* alleles condition accumulation of 9,15,9'-tri-cis- ζ -carotene in dark tissues, such as roots and etiolated leaves, in contrast to accumulation of 9,9'-di-cis- ζ -carotene in a ZDS mutant, *viviparous9*. We also identify a locus in *Euglena gracilis*, which is similarly required for Z-ISO activity. These data, taken together with the geometrical isomer substrate requirement of ZDS in evolutionarily distant plants, suggest that Z-ISO activity is not unique to maize, but will be found in all higher plants. Further analysis of this new gene-controlled step is critical to understanding regulation of this essential biosynthetic pathway.

Carotenoids are a diverse group of more than 750 naturally occurring pigments found in plants, fungi, and bacteria (Britton et al., 2004). In higher plants, carotenoids serve as accessory pigments in photosynthesis and as photoprotectors at high light intensities. Apocarotenoids, carotenoid degradative products, are signals in plant development and stress responses; their roles include extracellular rhizosphere signals that attract beneficial fungi and damaging parasitic plants that have opposite effects on plant yield (Milborrow, 2001; Bouvier et al., 2003; Booker et al., 2004; Schwartz et al., 2004; Simkin et al., 2004; Castillo et al., 2005; Matusova et al., 2005; Moise et al., 2005; Nambara and Marion-Poll, 2005). Increasing interest has also been placed on carotenoid content and composition of food crops because of the manifold importance of carotenoids in human health (Fraser and Bramley, 2004; Wurtzel, 2004; Quinlan et al., 2007).

In plant cells, carotenoids are synthesized in plastids from isoprenoid precursors through reactions catalyzed by nuclear-encoded enzymes (DellaPenna and Pogson, 2006). The first committed step, mediated by phytoene synthase, is condensation of two molecules of geranylgeranyl pyrophosphate to form 15-cis-phytoene, containing a central 15 to 15'-*cis*-double bond (Beyer et al., 1985; Dogbo et al., 1988; Misawa et al., 1994). A four-step enzymatic desaturation of 15-cis-phytoene to *all-trans*-lycopene also requires an electron transport chain (Mayer et al., 1990). Lycopene cyclases then catalyze ring formation at both ends of *all-trans*-lycopene to form carotenes, which can be further hydroxylated to produce xanthophylls (Kim and DellaPenna, 2006; Quinlan et al., 2007).

In bacteria, four desaturation steps from 15-cis-phytoene to *all-trans*-lycopene are mediated by a single enzyme, CrtI (Linden et al., 1991). In contrast, plants employ two desaturases, phytoene desaturase (PDS), which forms *trans*-double bonds at 11 and 11', concomitant with *cis*-bond formation at existing 9 and 9' double bonds, whereas ζ -carotene desaturase (ZDS) forms *cis*-double bonds at 7 and 7' (Breitenbach and Sandmann, 2005; Fig. 1). In addition, plant desaturation steps require supplementary isomerization reactions to produce acceptable geometrical isomer substrates for the desaturases and for the following lycopene cyclization steps (Beyer et al., 1989). Such differences in isomerization capacities of the plant and bacterial desaturases are important considerations for metabolic engineering of carotenoid content in food crops

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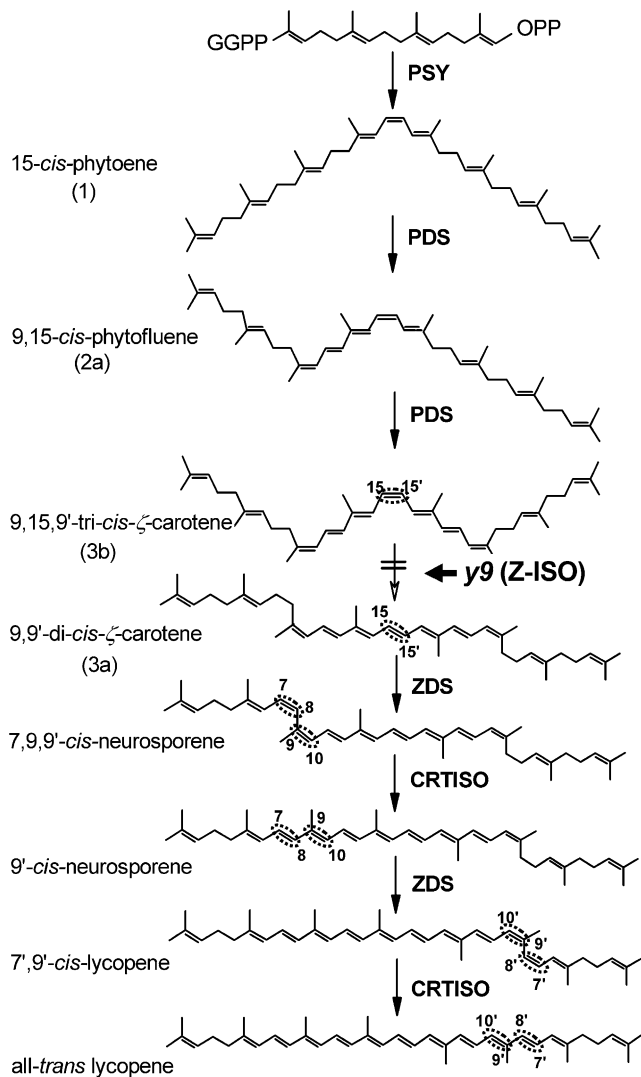


Figure 1. Proposed pathway of carotenoid biosynthesis in plants. PSY, Phytoene synthase. Bonds isomerized by CRTISO and Z-ISO are circled. In maize *y9* mutant tissues, the step from tri-cis- ζ -carotene to di-cis- ζ -carotene is blocked as indicated by a crosshatched and white arrow. Numbers in parentheses indicate HPLC peaks identified in the subsequent figures.

and in influencing biological activities of plant-derived geometrical isomers, including intestinal absorption and localization (Krinsky et al., 1990; Osterlie et al., 1999; Bjerkeng and Berge, 2000; Holloway et al., 2000; Patrick, 2000).

There has been confusion in the literature regarding the required number of carotene isomerases (CRTISO) needed in plant carotenoid biosynthesis, especially since reports of cloning of the CRTISO gene encoding CRTISO from cyanobacteria and plants (Breitenbach et al., 2001; Masamoto et al., 2001; Isaacson et al., 2002; Park et al., 2002). Early *in vitro* studies using daffodil (*Narcissus pseudonarcissus*) chromoplasts (Beyer et al., 1989) suggested that further progression in the plant carotenoid biosynthetic pathway beyond ζ -carotene (e.g. ζ -carotene desaturation and onward) required

isomerase activity and only the 15-cis position was recognized as an isomerase target. Expression of *Arabidopsis thaliana* PDS and ZDS in *Escherichia coli* also revealed a missing plant factor required for ζ -carotene desaturation that could be replaced by photoisomerization; ζ -carotene accumulated instead of the predicted polycopene, unless cells were exposed to light (Bartley et al., 1999). In a study of coupled maize (*Zea mays*) desaturases, it was proposed that there was a need for either two companion isomerases or a single isomerase having multiple substrates (Matthews et al., 2003). In an effort to identify the missing isomerase gene, research groups looked at a number of pathway mutants that exhibited accumulation of atypical geometrical isomers. The tomato (*Lycopersicon esculentum*) *tangerine* locus, for which recessive alleles condition accumulation of polycopene (poly-cis-lycopene), led to cloning of the corresponding gene that encoded what was thought to be the long-sought-after CRTISO (Isaacson et al., 2002). Homologs were also found in *Arabidopsis* and cyanobacteria (Breitenbach et al., 2001; Masamoto et al., 2001; Park et al., 2002).

When CRTISO (Isaacson et al., 2004) was assayed, it was found that isomerase activity followed, rather than preceded, ζ -carotene desaturation, which was inconsistent with earlier biochemical data indicating isomerization was needed prior to ζ -carotene desaturation; CRTISO was shown to be specific for adjacent double bonds at 7,9 and 7',9' positions needed to convert polycopene to *all-trans*-lycopene, but it did not isomerize the single 15 to 15'-cis-double bond that was required for producing the proper substrate for ZDS (Beyer et al., 1989; Bartley et al., 1999; Matthews et al., 2003). Later, it was unequivocally demonstrated that in biosynthesis of polycopene from phytoene, the product of PDS was 9,15,9'-tri-cis- ζ -carotene, whereas the substrate for the following desaturase, ZDS, was 9,9'-di-cis- ζ -carotene. Therefore, isomerization of the 15-cis position of ζ -carotene was clearly required to convert the PDS product to a suitable ZDS substrate (Breitenbach and Sandmann, 2005). *In vitro* assays of CRTISO (Isaacson et al., 2004) no longer supported the possibility of a multisubstrate enzyme, but suggested that, indeed, a second isomerase might exist. Whereas light might compensate for this requirement in light-exposed tissue, there was still the question of whether any genetic locus was responsible for such activity in the dark or in dark-grown tissue. In considering the phenotype associated with a lesion affecting such a second isomerase, which we will call Z-ISO (15-cis- ζ -CRTISO), it seemed that mutants would be predicted to accumulate the PDS product (and Z-ISO substrate), 9,15,9'-tri-cis- ζ -carotene, which could be shown to photoisomerize to 9,9'-di-cis- ζ -carotene. In comparison, ZDS mutants should accumulate 9,9'-di-cis- ζ -carotene in a genetic background conditioning functional Z-ISO. Unlike ZDS mutants that are lethal because they accumulate ζ -carotene under both light and dark conditions, Z-ISO mutants should be nonlethal because the mutant phenotype only manifests in the dark

and not in light-grown tissues. Therefore, ZDS and Z-ISO mutants should both condition accumulation of ζ -carotene in the dark, but only ZDS mutants will show accumulation in light-grown tissues and exhibit an albino phenotype. We therefore searched for ζ -carotene-accumulating mutants in maize (Wurtzel, 2004) to test for the presence of the predicted Z-ISO geometrical isomer substrate that is distinguishable from the ZDS substrate. We identified the maize *pale yellow9* (*y9*) locus as a candidate and demonstrated that recessive alleles condition accumulation of 9,15,9'-tri-cis- ζ -carotene in dark tissues. This finding supports the role of an additional genetic locus in plants that controls an upstream CRTISO activity, Z-ISO, which is independent of CRTISO. Comparison of the *y9* phenotype with that of ζ -carotene-accumulating mutants in other species shows that Z-ISO activity is not limited to maize.

RESULTS

Identification of a Maize Candidate Z-ISO Mutant

Numerous mutations affecting maize carotenoid biosynthesis, including phytoene desaturation, have been reported (Wurtzel, 2004). Endosperm phenotypes include white endosperm (compared to the yellow) and vivipary caused by an absence of abscisic acid, a carotenoid cleavage product promoting seed dormancy. Absence of leaf carotenoids causes lethality and manifests as albino tissue due to pleiotropic effects on chloroplast biogenesis. Pathway blocks are also associated with accumulation of pathway intermediates. Accumulation of ζ -carotene was predicted for mutations in genes controlling either ZDS or a putative Z-ISO; such accumulation was found for maize *viviparous9* (*vp9*) and *y9* mutant endosperms (Robertson, 1975a). These mutants had otherwise dissimilar phenotypes; *y9* homozygous mutants were nonlethal recessives affecting only endosperm and leaves remained green, whereas *vp9* mutants were lethal recessives affecting both endosperm and leaf tissues and plants were albino. When the maize ZDS gene was isolated (Matthews et al., 2003), it was mapped to *vp9* on chromosome 7 and not to *y9* on chromosome 10 (Robertson, 1975b); also, *y9* had no effect on ZDS transcript accumulation, indicating that *y9* did not encode ZDS or control ZDS transcript levels. Given that a block in isomerase activity would be light complemented, we predicted that a carotenoid isomerase mutant should have an almost normal leaf phenotype, as seen for *y9*, and not an albino phenotype, as in *vp9*. Therefore, it was conceivable that *y9* might encode CRTISO, although it did not accumulate prolycopene as did other CRTISO mutants (Isaacson et al., 2002). Furthermore, mapping of CRTISO to chromosomes 2 and 4 (Wurtzel, 2004; L. Conrad, T. Brutnell, and E.T. Wurtzel, unpublished data) indicated that *y9* did not encode CRTISO because *y9* mapped to chromosome 10. Whereas *y9* was not associated with ZDS or CRTISO, it remained a good

candidate to consider for a possible genetic locus that might encode a factor necessary for putative Z-ISO activity. To validate that *y9* was necessary for Z-ISO activity (and to further prove that light was insufficient and that a genetic locus was involved), it was necessary to demonstrate that *y9* conditioned accumulation specifically of 9,15,9'-tri-cis- ζ -carotene in contrast to accumulation of 9,9'-di-cis- ζ -carotene in a ZDS mutant, such as *vp9*. Furthermore, whereas prior reports suggested that *y9* had no leaf phenotype (Janick-Buckner et al., 2001), we predicted that light was photoisomerizing the accumulated 9,15,9'-tri-cis- ζ -carotene intermediate to relieve the pathway block such that plants appeared green. Therefore, it was necessary to demonstrate that, in etiolated leaf tissue, ζ -carotene accumulated because of the absence of photoisomerization. In contrast, photoisomerization would have no effect on the phenotype of a ZDS mutant.

HPLC Assay and Standards for ζ -Carotene Geometrical Isomers

To distinguish the ζ -carotene isomers appearing in *y9* and *vp9* endosperm tissues, ζ -carotene isomers were extracted from *E. coli* cells containing pACCRT-EBP for use as HPLC standards (Matthews et al., 2003). It had been previously shown that expression of a plant PDS resulted in accumulation of 9,15,9'-tri-cis- ζ -carotene, which has a characteristic spectrum, including A_{297} , and which could be photoisomerized to 9,9'-di-cis- ζ -carotene, which elutes slower, and whose spectrum lacks A_{297} (Bartley et al., 1999; Isaacson et al., 2004; Breitenbach and Sandmann, 2005). Following HPLC conditions used earlier (Isaacson et al., 2004), in combination with photoisomerization to demonstrate isomer conversion, we were able to replicate separation and spectral identification of the tri-cis and di-cis isomers as follows. Based on spectrum and retention times, the carotenoids extracted from dark-grown *E. coli* cells contained peaks 3a and 3b, ζ -carotene isomers, as well as a small amount of phytofluene (peak 2) and phytoene (peak 1; Fig. 2), which was consistent with earlier reports (Bartley et al., 1999; Matthews et al., 2003; Isaacson et al., 2004; Breitenbach and Sandmann, 2005). Of the three ζ -carotene peaks, only peak 3b showed absorption at 297 nm (see spectra; Fig. 2, top), consistent with 9,15,9'-tri-cis- ζ -carotene (Bartley et al., 1999; Isaacson et al., 2004). When cells were exposed to light (as described in "Materials and Methods"), the ratio of peak 3b to 3a changed from 3:1 in dark-grown cultures (Fig. 2, dashed trace) to 2:5 in light-exposed cultures (Fig. 2, solid trace), as previously seen for photoisomerization of 9,15,9'-tri-cis- ζ -carotene to 9,9'-di-cis- ζ -carotene (Bartley et al., 1999; Isaacson et al., 2004). The ζ -carotene isomer, peak 3b, was converted to the ζ -carotene isomer, peak 3a, due to photoisomerization of the 15-cis-double bond in tri-cis- ζ -carotene to form di-cis- ζ -carotene. In addition to matching spectra and photoisomerization-induced changes in peak heights, relative retention times also

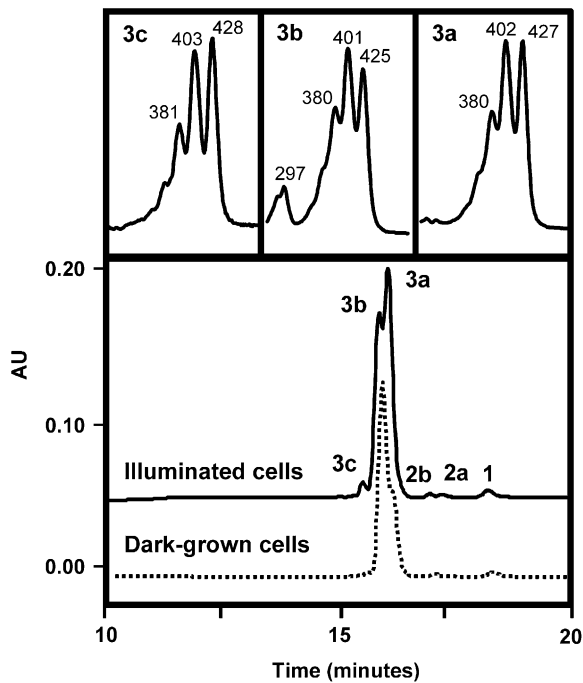


Figure 2. HPLC analysis of PDS enzymatic product, 9,15,9'-tri-cis- ζ -carotene, which is photoisomerized to 9,9'-di-cis- ζ -carotene. Top, Spectra of peaks identified in chromatogram shown on the bottom. Bottom, HPLC chromatograms of carotenoids extracted from dark-grown (bottom, dashed trace) or illuminated *E. coli* cells containing pACCRT-EBP plasmid (top, solid trace). With light exposure, 9,15,9'-tri-cis- ζ -carotene (3b) was converted into 9,9'-di-cis- ζ -carotene (3a), as well as trace amounts of *all-trans*- ζ -carotene (3c). Peak 1, phytoene; peak 2a and 2b, phytofluene isomers; peak 3a, 9,9'-di-cis- ζ -carotene; peak 3b, 9,15,9'-tri-cis- ζ -carotene; peak 3c, *all-trans*- ζ -carotene.

matched earlier reports using the same HPLC separation system (Isaacson et al., 2004). Based on this replication, we therefore identified peak 3a as 9,9'-di-cis- ζ -carotene and peak 3b as 9,15,9'-tri-cis- ζ -carotene. A third ζ -carotene isomer, peak 3c, was also identified in the carotenoid extract of light-exposed cultures and tentatively identified as *all-trans*- ζ -carotene based on its retention time being 0.5 min earlier than that of 9,15,9'-tri-cis- ζ -carotene (Isaacson et al., 2004).

Carotenoid Compositions in Endosperm Tissue of *y9* and *vp9* Mutants

Bacterial standards described above were used to identify the specific ζ -carotene isomers accumulating in homozygous *y9* and *vp9* mutant endosperms (Fig. 3, chromatograms; Table I, corresponding peak quantifications). We compared spectral fine structure, including percent III/II peak ratio, presence of a cis-peak at 297 nm, and retention time with those of the standards and prior values (Isaacson et al., 2004). In *y9* endosperm, the major carotenoids were 40.4% of 9,15,9'-tri-cis- ζ -carotene as compared to 14.1% of 9,9'-di-cis- ζ -carotene, and 15% phytoene, 13.2% lutein, 11.8% phytofluene, and 3.0% zeaxanthin. In comparison, endosperms of the *zds*

mutant, *vp9*, showed the reverse geometrical isomer composition with 55.7% of 9,9'-di-cis- ζ -carotene and 17.6% of 9,15,9'-tri-cis- ζ -carotene. Taken together, these data show that *y9* and *vp9* accumulate different geometrical isomers of ζ -carotene. The carotenoid biosynthetic pathway in *y9* is blocked in the cis- to trans-conversion of the 15-cis-bond in 9,15,9'-tri-cis- ζ -carotene and therefore accumulates 9,15,9'-tri-cis- ζ -carotene. In contrast, *vp9* tissue has isomerase activity needed to convert 9,15,9'-tri-cis- ζ -carotene to 9,9'-di-cis- ζ -carotene, but accumulates 9,9'-di-cis- ζ -carotene, which indicates it is blocked in desaturation of this compound by ZDS. The accumulation of lutein and other downstream carotenoids in *y9* endosperms might be due to photo-conversion of 9,15,9'-tri-cis- ζ -carotene in endosperms of field-grown plants. In contrast to *y9*, *vp9* endosperms did not accumulate any detectable xanthophylls as expected for a mutation in a desaturase as compared to an isomerase (Fig. 3).

ζ -Carotene Isomers in Etiolated Leaf and Root Tissues of *y9* and *vp9* Mutants

If the *y9* locus indeed controls expression of a new isomerase, then a lesion in the gene should manifest as 9,15,9'-tri-cis- ζ -carotene accumulation in dark-grown

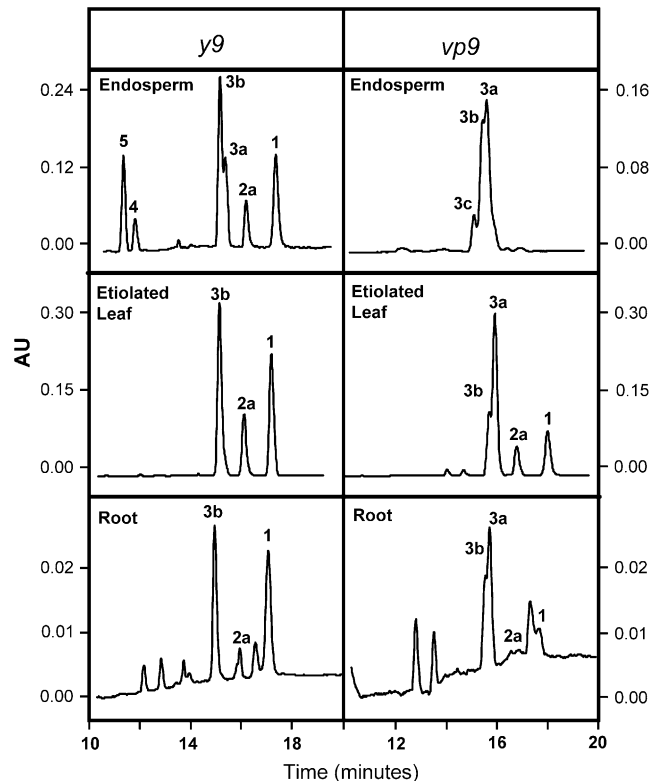


Figure 3. HPLC analysis of carotenoids extracted from endosperms, etiolated leaves, or roots of *y9* or *vp9* mutants shows mutant-specific geometrical isomers. Etiolated leaves and roots were samples from dark-grown plants. Peak 1, Phytoene; peak 2, phytofluene; peak 3a, 9,9'-di-cis- ζ -carotene; peak 3b, 9,15,9'-tri-cis- ζ -carotene; peak 3c, *all-trans*- ζ -carotene; peak 4, zeaxanthin; peak 5, lutein.

Table 1. Carotenoid composition of *y9* and *vp9* mutant tissues ($\mu\text{g g}^{-1}$)

Values shown are averages and SD for three samples.

	<i>y9</i>			<i>vp9</i>			Wavelength ^a	% III/II ^b
	Endosperm	Etiolated Leaf	Root	Endosperm	Etiolated Leaf	Root		
Phytoene	2.27 ± 0.14	13.0 ± 0.52	0.28 ± 0.01	–	4.06 ± 0.19	0.04 ± 0.00	276 286 298	0
Phytofluene	1.78 ± 0.07	5.54 ± 0.14	0.13 ± 0.01	0.06 ± 0.01	2.22 ± 0.05	0.04 ± 0.00	333 349 369	58
Tri-cis- ζ -carotene	6.07 ± 0.24	15.98 ± 0.38	0.30 ± 0.04	2.44 ± 0.06	4.17 ± 0.27	0.12 ± 0.01	(297) 380 401 425	75
Di-cis- ζ -carotene	2.12 ± 0.18	–	–	4.09 ± 0.13	13.18 ± 0.51	0.17 ± 0.01	380 402 427	98
<i>all-trans</i> - ζ -Carotene	–	–	–	0.61 ± 0.01	–	–	381 403 428	112
Lutein	1.98 ± 0.13	–	–	–	–	–	420 446 474	56
Zeaxanthin	0.45 ± 0.06	–	–	–	–	–	427 452 479	15

^aOnline absorbance spectrum taken during HPLC separation; underlined peak is highest peak (II); parentheses indicate additional cis-peak. In the case of phytoene, peaks I and III are actually shoulders. ^bFine structure of absorbance spectra expressed as relative height of longest wavelength peak (III) compared to middle peak (II). Value of zero indicates absence of peak III.

plants, when light is not available to compensate for the absence of the cis-trans-conversion; *y9* leaves of light-grown plants have been reported not to accumulate ζ -carotene (Janick-Buckner et al., 2001). Therefore, we germinated plants in the dark and collected etiolated leaves and roots, extracted carotenoids, and analyzed by HPLC (Fig. 3; Table 1). In both etiolated leaves and roots of homozygous *y9* plants, 9,15,9'-tri-cis- ζ -carotene accumulated; no 9,9'-di-cis- ζ -carotene was detected nor were there any other downstream xanthophylls. This suggests that the carotenoid biosynthetic pathway in dark-grown *y9* is completely blocked at this step, thereby preventing downstream synthesis leading to xanthophylls typically found in either light-grown *y9* or in dark-grown maize seedlings carrying the dominant *y9* allele (data not shown). In comparison, homozygous *vp9* roots and etiolated leaves accumulated mainly 9,9'-di-cis- ζ -carotene. No xanthophylls were observed in carotenoid extracts from etiolated leaves or roots of either mutant as compared to the profile in *y9* endosperm. The absence of downstream carotenoids in dark-grown tissues further supports the hypothesis that the xanthophylls detected in *y9* endosperm were due to some photoconversion in field-grown plants. As predicted for a Z-ISO lesion, 9,15,9'-tri-cis- ζ -carotene was found to accumulate in dark-grown tissues of *y9* plants. These results establish that *y9* does interfere with carotenoid biosynthesis in the absence of light in leaves and roots; the only reason that *y9* plants are normally green is that light compensates for the lesion.

In Vitro Photoisomerization of *y9* Carotenoids

To further confirm identification of the ζ -carotene isomers accumulating in dark-grown *y9* roots, in vitro photoisomerization was used to demonstrate conversion of 9,15,9'-tri-cis- ζ -carotene to 9,9'-di-cis- ζ -carotene using carotenoid extracts from underground roots of field-grown homozygous *y9* plants. Carotenoid extracts were illuminated for 2 h with white light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux) and the carotenoid composition before and after illumination was examined by HPLC (Fig. 4). Before illumination, the ratio of

9,15,9'-tri-cis- ζ -carotene (peak 3b) to 9,9'-di-cis- ζ -carotene (peak 3a) was 3:2. (Fig. 4, bottom chromatogram trace). After 2 h of illumination, the ratio of 9,15,9'-tri-cis- ζ -carotene (peak 3b) to 9,9'-di-cis- ζ -carotene (peak 3a) changed to 1:3 (Fig. 4, top chromatogram trace). The photoconversion of peak 3b to 3a is consistent with identification of peak 3b as 9,15,9'-tri-cis- ζ -carotene and peak 3a as 9,9'-di-cis- ζ -carotene. In summary, we demonstrated that roots accumulate 9,15,9'-tri-cis- ζ -carotene, which was shown to be photoisomerizable to the 9,9'-di-cis- ζ -carotene isomer.

Identification of Other Putative Genetic Loci Needed for Z-ISO Activity

It is unlikely that Z-ISO activity is only required for maize carotenoid biosynthesis, but it is likely

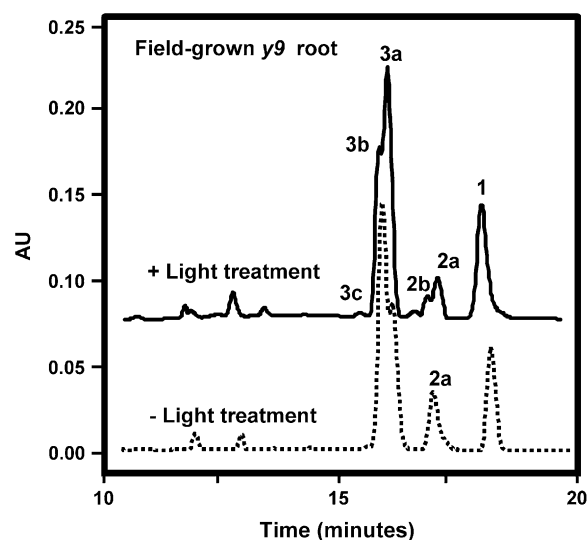


Figure 4. Carotenoid extracts from field-grown underground roots of *y9* plants before (bottom, dashed trace) and after illumination (top, solid trace). Light exposure converted 9,15,9'-tri-cis- ζ -carotene (3b) into 9,9'-di-cis- ζ -carotene (3a), as well as trace amount of *all-trans*- ζ -carotene (3c). Peaks are numbered as in Figure 2.

ubiquitous in all plants and cyanobacteria given that both dicot and monocot, as well as cyanobacterial PDS enzymes, produce 9,15,9'-tri-cis- ζ -carotene, which is not the isomeric substrate of ZDS, as shown here for maize and previously reported for Arabidopsis, *Cap-sicum annuum*, and *Synechococcus* (Bartley et al., 1999; Isaacson et al., 2004; Breitenbach and Sandmann, 2005). To identify genetic loci in other photosynthetic organisms that may be similarly required for Z-ISO activity, we reexamined earlier studies of mutants that exhibited blocks in ζ -carotene desaturation because such blocks might alternatively represent lesions in ZDS or Z-ISO (Table II). Specific details on how Table II was developed are provided (see "Materials and Methods"). Nine mutants were examined, all of which exhibit significant accumulation of ζ -carotene (see percent ζ -carotene isomers), and some of which have already been associated with a structural locus for ZDS or CRTISO. To distinguish between ZDS and isomerase mutants, we grouped the mutants into two classes, light nonresponsive (ZDS mutants), where light has no effect on the phenotype, or light responsive (isomerase mutants), where light can reverse the phenotype by compensation for a genetic lesion.

The first class included maize *vp9* and sunflower (*Helianthus annuus*) *nondormant1 (nd1)*; mutants in this class have been shown to be light nonresponders and therefore albino, which is typical for ZDS lesions based on phenotype and, in these cases, supported by gene analysis (Matthews et al., 2003; Conti et al., 2004). Members of this group show a low ratio of tri-cis- to di-cis- ζ -carotene isomers and accumulate 9,9'-di-cis- ζ -carotene, the ZDS substrate.

The second class of mutants has been shown to be light responders; photoisomerization releases a pathway block and hence normal carotenoid accumulation in the light can be observed (e.g. in the case of plants, they are green and viable). This class could alternatively represent either CRTISO or Z-ISO isomerase mutants. To distinguish between the two isomerase mutant types, we predicted that dark-grown CRTISO mutants should accumulate prolycopene or proneurosporene because there is no block in tri-cis- to di-cis-

isomerization, as evidenced by a lower tri-cis- to di-cis- ζ -carotene isomer ratio. On the other hand, dark-grown Z-ISO mutants will not accumulate prolycopene (or proneurosporene), but instead accumulate a high ratio of tri-cis- to di-cis- ζ -carotene isomers due to the block in the cis- to trans-conversion, which is otherwise released by photoisomerization.

The CRTISO class included Arabidopsis *carotenoid and chloroplast regulation2 (ccr2)* (Park et al., 2002), tomato *tangerine* (Isaacson et al., 2002), *Synechocystis* sp. PCC6803 *sll003* (Breitenbach et al., 2001; Masamoto et al., 2001), *Scenedesmus obliquus* C-6D (Ernst and Sandmann, 1988), and *Scenedesmus* PG1 (Britton and Powls, 1977; Britton et al., 1977). Among them, *ccr2*, *tangerine*, and *sll003* have all been shown to represent mutations in CRTISO or *crtH* genes. With the exception of PG1, these mutants all accumulated prolycopene and proneurosporene, which is expected for blocks in CRTISO activity as reported (Isaacson et al., 2002). These mutants, including PG1, all share a low tri-cis- to di-cis- ζ -carotene isomer ratio as predicted for a CRTISO mutant that has functional Z-ISO activity. PG1 is included in this class because it has a close to normal pigment composition in light-grown cells, indicating it carries a mutation in an isomerase because it can be light complemented; were it a lesion in ZDS, it would exhibit ζ -carotene accumulation even in light-grown cells. The low tri-cis- to di-cis- ζ -carotene isomer ratio found in dark-grown PG1 cells also suggests that the mutation blocks CRTISO and not Z-ISO activity, which would otherwise have given a high ratio.

The Z-ISO class of light responders included the *Euglena gracilis* mutant *W₃BUL* (Cunningham and Schiff, 1985) and maize *y9*. As expected for a block in Z-ISO activity, neither mutant accumulated prolycopene nor proneurosporene; both mutants exhibited a high ratio of tri-cis- to di-cis- ζ -carotene isomers, for which the tri-cis isomer was found to be photoconvertible to the di-cis isomer. This biochemical evidence suggests that both *W₃BUL* and *y9* loci affect Z-ISO and not CRTISO activity and that Z-ISO activity is not

Table II. Geometrical isomers accumulating in dark-grown tissues of ζ -carotene accumulation mutants (% total carotenoids)

n.a., Not available.

Enzyme	Organism	Mutation	Tissue	Phytoene	Phytofluene	Tri-cis- ζ -Carotene	Di-cis- ζ -Carotene	Total cis- ζ -Carotene Isomers	Proneurosporene	Prolycopene	Mutated Gene	% ζ -Carotene Isomers	Light Responsive	Ratio Tri-cis- to Di-cis- ζ -Carotene Isomers
ZDS	Sunflower	<i>nd1</i> ^a	^b	4.8	6.7	n.a.	n.a.	88.6	–	–	ZDS	88.6	No	n.a.
	Maize	<i>vp9</i>	^c	17.2	9.4	17.6	55.8	–	–	–	ZDS	73.4	No	0.3
CRTISO	Arabidopsis	<i>ccr2</i>	^c	–	–	5.5	14.4	–	14.3	54.3	CRTISO	19.9	Yes	0.4
	Tomato	<i>tangerine</i>	^c	17.8	7.7	n.a.	n.a.	24.2	15.1	33.6	CRTISO	24.2	Yes	n.a.
	<i>Synechocystis</i>	<i>sll0033</i>	^d	2	2	<1	10	–	15	60	<i>crtH</i>	10.0	Yes	<0.1
	<i>Scenedesmus</i>	C-6D	^d	14	8	12	44	–	12	10	Unknown	56.0	Yes	0.3
	<i>Scenedesmus</i>	PG1 ^a	^d	20.9	3.2	4.3	62.4	–	–	–	Unknown	66.7	Yes	0.1
Z-ISO	<i>Euglena</i>	<i>W₃BUL</i>	^d	–	7.6	24.7	9.8	–	–	–	Unknown	34.5	Yes	3.0
	Maize	<i>y9</i>	^c	37.6	16.0	46.3	–	–	–	–	Unknown	46.3	Yes	>50

^aPercentage of total carotenoids calculated based on data provided.

^bLeaf tissue from low light-grown plants.

^cEtiolated leaf.

^dDark-grown cells.

limited to plants, but is also present in a photosynthetic protist.

DISCUSSION

We demonstrated that isomerization of 9,15,9'-tri-cis- ζ -carotene to 9,9'-di-cis- ζ -carotene is not simply light mediated, but that it requires the product of a nuclear-encoded gene. We showed that, in the absence of light, the 9,15,9'-tri-cis- ζ -carotene isomer accumulates in etiolated leaves and roots of maize plants carrying the recessive *y9* allele in comparison to normal carotenoid composition in light-exposed *y9* leaves (Janick-Buckner et al., 2001). Therefore, an activity, which we termed Z-ISO, is essential in dark-exposed tissues. However, even in light-exposed *y9* tissues, there is evidence that Z-ISO activity is needed. Some striping seen in light-exposed homozygous *y9* plants did lead to reduced carotenoids in pale-green regions (Janick-Buckner et al., 2001), a phenotype exaggerated by cold treatment (Robertson, 1975b) or temperature fluctuations (Janick-Buckner et al., 2001). This suggests that photoisomerization is not entirely efficient in overcoming the pathway lesion associated with the recessive *y9* allele and that Z-ISO activity may be required in photosynthetic tissues when plants are subjected to abiotic stress.

Z-ISO activity, which we demonstrated to be genetically controlled in maize, is not unique to this species, as we deduced by examining a collection of ζ -carotene-accumulating mutants in multiple species. By applying a standard convention for naming the ζ -carotene isomers, we showed that the mutants fell into three classes of ζ -carotene-accumulating mutants: ZDS, CRTISO, and Z-ISO. The ZDS class contains two plant genes, the sunflower *nd1* and maize *vp9* loci; CRTISO includes mutants from plants, a cyanobacterium, and a green alga; the Z-ISO mutants include those in maize (*y9*) and *Euglena*. Therefore, unlike bacteria that encode one four-step desaturase, plants and other photosynthetic organisms possess two desaturases (PDS and ZDS) and two isomerases (Z-ISO and CRTISO), which have alternating functions in the biosynthetic pathway.

Z-ISO functions upstream of CRTISO and we ruled out *y9* as encoding CRTISO, although we do not as yet know whether *y9* encodes Z-ISO or regulates its expression. It is unlikely that *y9* encodes a factor that alters CRTISO activity because, if it did, then CRTISO mutants would not accumulate prolycopene, but instead would accumulate 9,15,9'-tri-cis- ζ -carotene.

We named the isomerase activity that is upstream of CRTISO, Z-ISO, to reflect isomerization of the 15-cis-double bond in ζ -carotene. We do not know whether Z-ISO will act on 15-cis-bonds only in ζ -carotene or also on 15-cis-phytoene or the intermediate 9,15-cis-phytofluene. It is unlikely that 15-cis-phytoene is a substrate because the primary phytoene isomer that was detected in plants is the 15-cis isomer and not trans (Jungalwalab and Porter, 1965). The *y9* mutant

phenotype also does not support 15-cis-phytoene as being a substrate because it is the tri-cis- ζ -carotene isomer that predominates in accumulation and not 15-cis-phytoene, which would accumulate were it the preferred substrate. Therefore, perhaps Z-ISO activity requires the unique conformation of the 15-cis-double bond in ζ -carotene, which also has the 9,9'-cis-double bonds uniquely produced during PDS desaturation. If one looks at the structure of 9,15,9'-tri-cis- ζ -carotene, its conformation is quite distinct from that of 15-cis-phytoene, which may be significant in substrate binding and recognition of the 15-cis-double bond.

The naming of CRTISO was optimistic at the time of its discovery and suggested that it catalyzed all carotene isomerizations, which we know now not to be the case. If one looks further at the isomerase substrates, it becomes clear why CRTISO was not the sole CRTISO. CRTISO isomerizes adjacent cis-double bonds (Isaacson et al., 2004), whereas the Z-ISO substrate is a single cis-double bond, making for a very different substrate structure. Therefore, it is likely that Z-ISO is different from CRTISO in terms of protein structure, which may explain why there has been no success in showing Z-ISO activity for any of the plant CRTISO homologs.

Characterization of the maize *y9* locus has brought to light a new factor required in plant carotenoid biosynthesis. Future characterization of this locus will lead to isolation of all of the components needed for plant desaturation/isomerization. Biogenesis and regulation of the complex plant desaturase/isomerase metabolon is an important and future problem to address; elucidation will have direct impact on metabolic engineering of this pathway in plants.

MATERIALS AND METHODS

Plant and Bacterial Materials

Maize (*Zea mays*) ZDS mutant *vp9-Bot100* (Maize Co-op 706B) and mutant *y9* (Maize Co-op X07C) were used in this study. For etiolated leaves and roots, seeds were germinated and grown in the dark for 2 weeks at 25°C. Endosperms were collected at 20 d after pollination from field-grown plants as described (Gallagher et al., 2004). The plasmid pACCRT-EBP, carrying bacterial *crte*, *crtB*, and maize *PDS* genes, confers accumulation of ζ -carotene isomers in *Escherichia coli* and was used to produce ζ -carotene standards for HPLC (Matthews et al., 2003); transformed cells were incubated for 48 h at 37°C under darkness or in the light (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux) prior to pigment extraction as described below.

Carotenoid Extraction

Carotenoids were extracted from approximately 100-mL bacterial culture or 1-g endosperm, etiolated leaf, or root tissues taken from dark-grown plants as described (Kurilich and Juvik, 1999), dissolved in 1-mL methanol, and 50- μL injected for HPLC analysis.

Photoisomerization of 9,15,9'-Tri-cis- ζ -Carotene to 9,9'-cis- ζ -Carotene

Carotenoids were extracted (Kurilich and Juvik, 1999) from approximately 6-g underground roots taken from field-grown plants and dissolved in 1-mL methanol. For in vitro photoisomerization of ζ -carotene isomers, samples

were placed in a petri dish and exposed for 2 h to light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux) prior to HPLC analysis.

Carotenoid Analysis by HPLC

HPLC separation was carried out using a Waters (Millipore) HPLC system with a 2,695 separation module, Empower I software (Waters), a 996 photodiode array detector (Waters), and a 717 autosampler. Gradient separation was conducted as described, with slight modification for improved peak resolution using a Waters Spherisorb 5- μm OSD1 analytical column ($5 \mu\text{m}$, $4.6 \times 250 \text{ mm}$; Waters) and Nucleosil C18 ($5 \mu\text{m}$, $4 \times 3.0 \text{ mm}$) guard column (Phenomenex; Isaacson et al., 2004). The mobile phase consisted of acetonitrile:water (9:1; A) and ethylacetate (B), at a constant flow rate of 1.6 mL/min with the following gradient: 100% to 80% A for 8 min; 80% to 65% A for 4 min; 65% to 45% A for 28 min; and a final segment at 100% B. Identification of ζ -carotene isomers was based on comparison with spectra and elution time of authentic ζ -carotene isomers produced with expression of pACCRT-EBP and from published data (Beyer et al., 1989; Sandmann, 1991; Isaacson et al., 2004). Lutein and zeaxanthin were identified based on comparison with standards purchased from Sigma. Quantification was performed by integrating the peak areas using Empower I software (Waters) with β -carotene as an internal control. All chromatograms are shown with each peak at its λ_{max} (Maxplot).

Geometrical Isomer Profiles of ζ -Carotene-Accumulating Mutants

To compare the various ζ -carotene mutants in the literature, we converted the previously reported data to a standard format. Earlier reports named the same isomers by different naming conventions. Therefore, we used several criteria to identify 9,15,9'-tri-cis- ζ -carotene and 9,9'-di-cis- ζ -carotene based on the following properties: spectral fine structure, including presence or absence of the 297-nm cis-peak characteristic of 9,15,9'-tri-cis- ζ -carotene, potential for photoisomerization by light to the alternate isomer, and relative retention time. Table II contains data derived from this study for *vp9* and *y9* plus the following mutants:

Sunflower (*Helianthus annuus*) *nd1* (Conti et al., 2004): Like maize *vp9* (Matthews et al., 2003), *nd1* is a mutant with a known lesion in the *ZDS* gene, both of which exhibit an albino phenotype regardless of light. For all other mutants, light reverses the mutant phenotype. The percentage of carotenoids in *nd1* was calculated based on data from low light-treated plants ($1 \mu\text{mol m}^{-2} \text{s}^{-1}$) as provided in Table I (Conti et al., 2004).

Arabidopsis* (*Arabidopsis thaliana*) *ccr2 (Park et al., 2002): This mutation was demonstrated to be in the *CRTISO* gene. The carotenoid composition data of etiolated *ccr2* leaf was taken directly from the article. Included are pro- ζ -carotene, which elutes slower than cis- ζ -carotene, which they describe as having a spectrum appearing to be 15Z (cis), indicating that these compounds represent 9,9'-di-cis- ζ -carotene and 9,15,9'-tri-cis- ζ -carotene, respectively.

Tomato (*Lycopersicon esculentum*) *tangerine*: This mutation was demonstrated to be in the *CRTISO* gene; we used the carotenoid composition from dark-grown seedlings as presented in Table II (Isaacson et al., 2002).

Synechocystis* *sl10033: This mutation was shown to be in *crhH*, which is a homolog of the plant *CRTISO* gene (Breitenbach et al., 2001; Masamoto et al., 2001); we used the carotenoid composition from Table I (Masamoto et al., 2001). By identifying the photoisomerizable compounds described in the article, we interpreted cis- ζ -carotene (which contained a 15-cis-peak at 297 nm) to be 9,15,9'-tri-cis ζ -carotene and *all-trans*- ζ -carotene (which lacked the 15-cis-peak) to be 9,9'-di-cis- ζ -carotene.

***Scenedesmus obliquus* C-6D**: The percentage of each carotenoid in *Scenedesmus* mutant C-6D was calculated based on data presented in Table I (Ernst and Sandmann, 1988). The isomer 9,9'-di-cis- ζ -carotene is alternatively named pro- ζ -carotene; the isomer cis- ζ -carotene II (?Z) was later identified as 9,15,9'-tri-cis- ζ -carotene (Breitenbach and Sandmann, 2005).

***S. obliquus* PG1**: The percentage of each carotenoid in PG1 is calculated based on column A of Table I, which lists the total amount of ζ -carotene without specific isomers quantified (Britton et al., 1977). In Table I of a second article from the same laboratory, the isomers are quantified (Britton and Pows, 1977); ζ -carotene II (15-cis- ζ -isomere) and ζ -carotene-III (*all-trans*; lacking a cis-peak and most prevalent of the ζ -carotene isomer) represent 9,15,9'-tri-cis- ζ -carotene and 9,9'-di-cis- ζ -carotene, respectively.

***Euglena gracilis* var. *bacillaris* W₃BUL**: The carotenoid composition of W₃BUL was obtained from Table I (Cunningham and Schiff, 1985). Light

was shown to convert cis- ζ -carotene I (which contains a 15-cis-bond) to trans- ζ -carotene (which lacks the cis-spectral peak), indicating that these compounds represent 9,15,9'-tri-cis- ζ -carotene and 9,9'-di-cis- ζ -carotene, respectively.

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