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Journal research area: Biochemical Processes and Macromolecular Structures
Maize Y9 encodes a product essential for 15-cis-ζ-carotene isomerization

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This research was supported by NIH (#S06-GM08225), PSC-CUNY, and New York State

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Abstract

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 (PDS and 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 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 PDS, 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, vp9. We also identify a locus in Euglena 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 that it 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.

Keywords: carotenoids, maize, ζ-carotene geometrical isomers, y9 mutant, photoisomerization
Introduction

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 in 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 15-15' 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, while 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 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 needed in plant carotenoid biosynthesis, especially since reports of cloning of “the carotene isomerase 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 chromoplasts (Beyer et al., 1989) suggested that further progression in the plant carotenoid biosynthetic pathway beyond ζ-carotene (e.g. ζ-carotene desaturation and onwards), required an isomerase activity and only the 15-cis position was recognized as an isomerase target. Expression of Arabidopsis PDS and ZDS in E. 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 prolycopene, unless cells were exposed to light (Bartley et al., 1999). In study of the coupled maize 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 tangerine locus, for which recessive alleles condition accumulation of prolycopene (poly cis lycopene), led to cloning of the corresponding gene that encoded what was thought to be the long sought-after “Carotene Isomerase,” and it was so named as 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 prolycopene to all-trans lycopene, but it did not isomerize the single 15-15' 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 prolycopene 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). The in vitro assays of CRTISO (Isaacson et al., 2004), no longer supported the possibility of a multi-substrate enzyme, but suggested that indeed a second isomerase might exist. While 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-carotene isomerase), 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 presence of the predicted Z-ISO geometrical isomer substrate that is distinguishable from the ZDS substrate. We identified the maize 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 carotene isomerase 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 cause lethality and manifest 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 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, while 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 affect 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) (Conrad, Brutnell, and Wurtzel, unpublished) indicated that y9 did not encode CRTISO, because y9 mapped to chromosome 10. While 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, while 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**

In order 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 absorbance at 297 nm and which could be photoisomerized to 9,9′-di-*cis*-ζ-carotene, which elutes slower and whose spectrum lacks absorbance at 297 nm (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 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 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 \( \zeta \)-carotene based on its retention time being 0.5 min earlier than that of 9,15,9'-tri-\( \text{cis} \)-\( \zeta \)-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 \( \zeta \)-carotene isomers accumulating in homozygous \( y9 \) and \( vp9 \) mutant endosperms (see Fig. 3 for chromatograms and Table 1 for corresponding peak quantifications). We compared spectral fine structure including % III/II peak ratio, presence of a \( \text{cis} \) 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-\( \text{cis} \)-\( \zeta \)-carotene as compared to 14.1% of 9,9'-di-\( \text{cis} \)-\( \zeta \)-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-\( \text{cis} \)-\( \zeta \)-carotene and 17.6% of 9,15,9'-tri-\( \text{cis} \)-\( \zeta \)-carotene. Taken together, these data show that \( y9 \) and \( vp9 \) accumulate different geometrical isomers of \( \zeta \)-carotene. The carotenoid biosynthetic pathway in \( y9 \) is blocked in the \( \text{cis} \) to \( \text{trans} \) conversion of the 15-\( \text{cis} \) bond in 9,15,9'-tri-\( \text{cis} \)-\( \zeta \)-carotene and therefore accumulates 9,15,9'-tri-\( \text{cis} \)-\( \zeta \)-carotene. In contrast, \( vp9 \) tissue has isomerase activity needed to convert 9,15,9'-tri-\( \text{cis} \)-\( \zeta \)-carotene to 9,9'-di-\( \text{cis} \)-\( \zeta \)-carotene, but accumulates 9,9'-di-\( \text{cis} \)-\( \zeta \)-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 photoconversion of 9,15,9'-tri-\( \text{cis} \)-\( \zeta \)-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).

**\( \zeta \)-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-\( \text{cis} \)-\( \zeta \)-carotene accumulation in dark grown plants, when light is not available to compensate for absence of the \( \text{cis}-\text{trans} \) conversion; \( y9 \) leaves of light-grown plants have been reported not to accumulate \( \zeta \)-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 the 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 hours with white light (50 μmol m⁻² 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, lower chromatogram trace). After 2 hours 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, upper 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 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, Capsicum, 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 re-examined earlier studies of mutants that exhibited blocks in ζ-carotene desaturation as such blocks might alternatively represent lesions in ZDS or Z-ISO (Table 2). Specific details on how Table 2 was developed are provided (see Methods). Nine mutants were examined, all of which exhibit significant accumulation of ζ-carotene (see % 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 non-responsive (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 nondormant-1 (nd-1); 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 /di-cis ζ-carotene isomers and accumulate 9,9'-di-cis-ζ-carotene, the ZDS substrate.

The second class of mutants have 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 ccr2 (Park et al., 2002), tomato tangerine (Isaacson et al., 2002), Synechocystis sp. PCC6803 sll003 (Breitenbach et al., 2001; Masamoto et
al., 2001), *Scenedesmus* 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 gracillis* 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 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 a 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.

The 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 \( \zeta \)-carotene accumulating mutants in multiple species. By applying a standard convention for naming the \( \zeta \)-carotene isomers, we showed that the mutants fell into three classes of \( \zeta \)-carotene accumulating mutants: ZDS, CRTISO and Z-ISO. The ZDS class contains two plant genes, the sunflower \( nd-1 \) 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 don’t 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-\( cis \)-\( \zeta \)-carotene.

We named the isomerase activity that is upstream of CRTISO, Z-ISO, to reflect isomerization of the 15-\( cis \) double bond in \( \zeta \)-carotene. We don’t know if Z-ISO will act on 15-\( cis \) bonds only in \( \zeta \)-carotene, or also on 15-\( cis \) phytoene, or the intermediate 9,15-\( cis \) phytofluene. It is unlikely that 15-\( cis \) phytoene is a substrate, as 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 as it is the tri-\( cis \)-\( \zeta \)-carotene isomer that predominates in accumulation and not 15-\( cis \) phytoene that would accumulate were it the preferred substrate. Therefore, perhaps Z-ISO activity requires the unique conformation of the 15-\( cis \) double bond in \( \zeta \)-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-\( cis \) \( \zeta \)-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 carotene isomerase. 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 \( \zeta \)-carotene desaturase 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 gerninated and grown in the dark for two weeks at 25 °C. Endosperms were collected at 20 days after pollination from field grown plants as described (Gallagher et al., 2004). The plasmid pACCRT-EBP, carrying bacterial \( \text{crtE, crtB} \) and maize \( \text{Pds} \) genes confers accumulation of \( \zeta \)-carotene isomers in \( \text{E. coli} \) and was used to produce \( \zeta \)-carotene standards for HPLC (Matthews et al., 2003); transformed cells were incubated for 48 hours 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 \( \mu \text{l} \) injected for HPLC analysis.

Photoisomerization of 9,15,9'-tri-cis-\( \zeta \)-carotene to 9,9'-cis \( \zeta \)-carotene
Carotenoids were extracted (Kurilich and Juvik, 1999) from ~ 6g underground roots taken from field-grown plants and dissolved in 1mL methanol. For in vitro photoisomerization of ζ-carotene isomers, samples were placed in a Petri dish and exposed for 2 hours to light (50 μmol m⁻² s⁻¹ photon flux) prior to HPLC analysis.

**Carotenoid Analysis by HPLC**

HPLC separation was carried out using a Waters (Millipore, Franklin, MA) HPLC system with a 2695 separation module, Empower 1 software (Waters), 996 photodiode array detector (Waters), and 717 autosampler. Gradient separation was conducted as described with slight modification for improved peak resolution using a Waters Spherisorb 5μm OSD1 analytical Column (5μ, 4.6 x 250 mm, Waters, MA) and Nucleosil C18 (5μ, 4 x 3.0 mm) guard column (Phenomenex, CA) (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 (Sigma, MO). Quantification was performed by integrating the peak areas using the 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 2 contains data derived from this study for vp9 and y9 plus the following mutants:
Sunflower *nondormant1* (*nd-1*) (Conti et al., 2004): Like maize *vp9* (Matthews et al., 2003), *nd-1* is a mutant with a known lesion in the *Zds* gene, both of which exhibit an albino phenotype regardless of light. For all of the other mutants, light reverses the mutant phenotype. The percentage of carotenoids in *nd-1* were calculated based on data from low light treated plants (1 μmole m\(^{-2}\) s\(^{-1}\)) as provided in Table 1 (Conti et al., 2004).

*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 paper. 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 2 (Isaacson et al., 2002).

*Synechocystis sll0033*: this mutation was shown to be in *crtH*, which is a homolog of the plant *CRTISO* gene (Breitenbach et al., 2001; Masamoto et al., 2001); we used the carotenoid composition from Table 1 (Masamoto et al., 2001). By identifying the photoisomerizable compounds described in the paper, 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 (Ernst and Sandmann, 1988): 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).

*Scenedesmus obliquus* PG1 (Britton and Powls, 1977; Britton et al., 1977): 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 paper from the same laboratory, the isomers are quantified (Britton and Powls, 1977); ζ-carotene II (15-cis-ζ-carotene) 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 (Cunningham and Schiff, 1985): 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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Figure legends

Figure 1. Proposed pathway of carotenoid biosynthesis in plants. CRTISO, Carotene isomerase; PDS, phytoene desaturase; PSY, Phytoene synthase; ZDS, ζ-carotene desaturase; Z-ISO, 15-cis-ζ-carotene isomerase. 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 cross-hatched and open arrow. Numbers in parentheses indicate HPLC peaks identified in the subsequent figures.

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 panel: Spectra of peaks identified in chromatogram shown in bottom panel. Bottom panel: HPLC chromatograms of carotenoids extracted from dark-grown (lower, dashed trace) or illuminated E. coli cells containing pACCRT-EBP plasmid (upper, 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). Peak1, 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.

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. Peak1. 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.

Figure 4. Carotenoid extracts from field-grown underground roots of y9 plants before (lower, dashed trace) and after illumination (upper, 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.
Table 1. Carotenoid composition of y9 and vp9 mutant tissues (μg g⁻¹)

|                | y9                | vp9                | 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            |
| all-trans 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            |

[^a]Online absorbance spectrum taken during HPLC separation; underlined peak is highest peak (II); parentheses indicates additional cis peak. *in the case of phytoene, “peaks” I and III are actually shoulders.

[^b]Fine 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.

Values shown are averages and standard deviation for 3 samples.
Table 2. Geometrical isomers accumulating in dark-grown tissues of \( \zeta \)-carotene accumulation mutants (% total carotenoids)

| Organism      | mutation | tissue | Phytoene | Phytofluene | Tri-cis-\( \zeta \)-carotene | Di-cis-\( \zeta \)-carotene | All-cis \( \zeta \)-carotene isomers | Pro-neurosporene | Prolycopene | Mutated gene | % \( \zeta \)-carotene isomers | LIGHT responsive | RATIO tri-cis/di-cis \( \zeta \)-carotene isomers |
|---------------|----------|--------|----------|-------------|-----------------------------|-----------------------------|-------------------------------------|------------------|-------------|--------------|-----------------------------|-----------------|--------------------------------|
| **ZDS**       |          |        |          |             |                             |                             |                                     |                  |             |              |                             |                  |                                |
| Sunflower     | nd-1\(^a\) | d      | 4.8      | 6.7         | n.a.                        | n.a.                        | 88.6                                | -                | -           | Zds          | 88.6                        | NO              | n.a.                           |
| Corn          | vp9      | b      | 17.2     | 9.4         | 17.6                        | 55.8                        | -                                   | -                | -           | Zds          | 73.4                        | NO              | 0.3                            |
| **CRTISO**    |          |        |          |             |                             |                             |                                     |                  |             |              |                             |                  |                                |
| Arabidopsis   | ccr2     | b      | -        | -           | 5.5                         | 14.4                        | -                                   | 14.3             | 54.3        | CRTISO       | 19.9                        | YES             | 0.4                            |
| Tomato        | tangerine| b      | 17.8     | 7.7         | n.a.                        | n.a.                        | 24.2                                | 15.1             | 33.6        | CRTISO       | 24.2                        | YES             | n.a.                           |
| Synechocystis | sll0033  | c      | 2        | 2           | <1                          | 10                          | -                                   | 15               | 60          | crtH         | 10.0                        | YES             | <0.1                           |
| Scenedesmus   | C-6D     | c      | 14       | 8           | 12                          | 44                          | -                                   | 12               | 10          | CRTISO       | 66.7                        | YES             | 0.3                            |
| Scenedesmus   | PG1\(^a\) | c      | 20.9     | 3.2         | 4.3                         | 62.4                        | -                                   | -                | -           | CRTISO       | 66.7                        | YES             | 0.1                            |
| **Z-ISO**     |          |        |          |             |                             |                             |                                     |                  |             |              |                             |                  |                                |
| Euglena       | W3BUL    | c      | -        | 7.6         | 24.7                        | 9.8                         | -                                   | -                | -           | unknown      | 34.5                        | YES             | 3.0                            |
| Corn          | y9       | b      | 37.6     | 16.0        | 46.3                        | -                           | -                                   | -                | -           | unknown      | 46.3                        | YES             | >50                            |

\(^a\) Percentage of total carotenoids calculated based on data provided
\(^b\) Etiolated leaf
\(^c\) Dark-grown cells
\(^d\) Leaf tissue from low-light grown plants

n.a. = not available
