The Plant Cell, Vol. 14, 333–342, February 2002, www.plantcell.org © 2002 American Society of Plant Biologists

Cloning of *tangerine* from Tomato Reveals a Carotenoid Isomerase Essential for the Production of β-Carotene and Xanthophylls in Plants

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Carotenoid biosynthesis in plants has been described at the molecular level for most of the biochemical steps in the pathway. However, the *cis-trans* isomerization of carotenoids, which is known to occur in vivo, has remained a mystery since its discovery five decades ago. To elucidate the molecular mechanism of carotenoid isomerization, we have taken a genetic map-based approach to clone the *tangerine* locus from tomato. Fruit of *tangerine* are orange and accumulate prolycopene (7Z,9Z,7′Z,9′Z-tetra-*cis*-lycopene) instead of the all-*trans*-lycopene, which normally is synthesized in the wild type. Our data indicate that the *tangerine* gene, designated *CRTISO*, encodes an authentic carotenoid isomerase that is required during carotenoid desaturation. CRTISO is a redox-type enzyme structurally related to the bacterial-type phytoene desaturase CRTI. Two alleles of tangerine have been investigated. In *tangerine*^{mic}, loss of function is attributable to a deletion mutation in *CRTISO*, and in *tangerine*^{3183}, expression of this gene is impaired. *CRTISO* from tomato is expressed in all green tissues but is upregulated during fruit ripening and in flowers. The function of carotene isomerase in plants presumably is to enable carotenoid biosynthesis to occur in the dark and in nonphotosynthetic tissues.

INTRODUCTION

Carotenoid pigments are essential components in all photosynthetic organisms. They assist in harvesting light energy and protect the photosynthetic apparatus against harmful reactive oxygen species that are produced by overexcitation of chlorophyll. They also furnish distinctive yellow, orange, and red colors to fruit and flowers to attract animals. Carotenoids are mainly 40-carbon isoprenoids, which consist of eight isoprene units. The polyene chain in carotenoids contains up to 15 conjugated double bonds, a feature that is responsible for their characteristic absorption spectra and specific photochemical properties (Goodwin, 1980; Britton et al., 1998). These double bonds enable the formation of *cis-trans* geometric isomers in various positions along the molecule. Indeed, although the bulk of carotenoids in higher plants occur in the all-*trans* configuration, different *cis*-isomers exist as well, but in small proportions.

In plants, carotenoids are synthesized within the plastids from the central isoprenoid pathway (reviewed by Hirschberg, 2001) (Figure 1). The first carotenoid in the committed pathway is phytoene, which is produced by the enzyme phytoene synthase through a condensation of two molecules of geranylgeranyl diphosphate. Four double bonds are introduced subsequently into phytoene by two enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), each catalyzing two symmetric dehydrogenation steps to yield ζ-carotene and lycopene, respectively. It is recognized that *cis-trans* isomerizations take place in vivo because phytoene is synthesized in the 15-*cis* configuration, whereas most of the other carotenoids are found in the all-*trans* form (Britton, 1988). Furthermore, a small proportion of *cis*-isomers exist in many carotenoid species, for example, 9-*cis*- and 13-*cis*-isomers of β-carotene, zeaxanthin, and violaxanthin. However, the process of carotenoid isomerization remains unexplained. The existence of a potential carotene isomerase enzyme could be expected from the phenotype of recessive mutations in tomato (Tomes et al., 1953) and *Scenedesmus* (Ernst and Sandmann, 1988), which accumulate prolycopene (7Z,9Z,7′Z,9′Z tetra-*cis*-lycopene) as well as poly-*cis*-isomers of phytofluene, ζ-carotene, and neurosporene. Coexpression of PDS and ZDS from *Arabidopsis thaliana* in *Escherichia coli* cells that synthesized phytoene produced mainly prolycopene, whereas all-*trans*-lycopene was produced in these cells by the bacterial PDS CRTI (Bartley et al., 1999). The authors concluded that an active

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.010303.

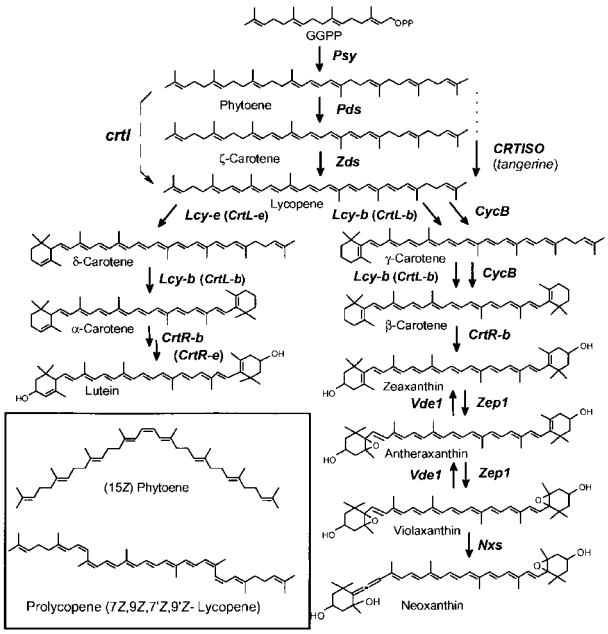

Figure 1. The Carotenoid Biosynthesis Pathway in Plants.

Enzymes are named according to the designation of their genes. *crtI*, phytoene desaturase (bacterial type); *Lcy-b* (*CrtL-b*), lycopene β-cyclase; *CrtL-e*, lycopene ε-cyclase; *CrtR-b*, β-ring hydroxylase, *CrtR-e*, ε-ring hydroxylase; *CycB*, chromoplast-specific lycopene; cyclase; GGPP, geranylgeranyl diphosphate; *Nxs*, neoxanthin synthase; *Pds*, phytoene desaturase; *Psy*, phytoene synthase; *Vde*, violaxanthin deepoxidase; *Zds*, ζ-carotene desaturase; *Zep*, zeaxanthin epoxidase.

isomerization function is required in conjunction with the plant-type carotene desaturation reactions to produce all-*trans*-lycopene.

To elucidate the mechanism of carotene isomerization, we have studied the tomato gene (*Lycopersicon esculentum*) that encodes the recessive mutation *tangerine* (Tomes, 1952). Fruit of *tangerine* are orange and accumulate prolycopene instead of all-*trans*-lycopene, which normally is synthesized in wild-type fruit (Figure 2). The structure of prolycopene isolated from the *tangerine* variety of tomato fruit has been elucidated by NMR (Clough and Pattenden, 1979). The phenotype of *tangerine*^{mic} is manifested also in yellowish young leaves and sometimes light green foliage and in pale flowers. Using map-based cloning, we identified the gene that encodes *tangerine*. Our data indicate that this gene, *CRTISO*, encodes a redox-type enzyme that is related structurally to the bacterial-type phytoene desaturase, CRTI. An ortholog of *CRTISO* also is found in Arabidopsis and cyanobacteria, where it plays a similar function in carotenoid isomerization.

RESULTS

Carotenoid Composition in the Wild Type and *tangerine*

Carotenoids accumulated in fruit and flowers of the wild type and *tangerine* mutants were extracted and analyzed by HPLC (Figure 3, Table 1). In the wild type, 7 days after the breaker stage, 75% of total carotenoids in ripe fruit consist of all-*trans*-lycopene and <15% are lycopene precursors (neurosporene, ζ-carotene, phytofluene, and phytoene). In fruit of the mutant *tangerine*^{3183}, the major carotenoid accumulated is prolycopene, whereas lycopene precursors, mostly in the *cis* configuration, constitute the rest of the carotenoids. Only a small fraction of <2% is all-*trans*-lycopene. In *tangerine*^{mic}, the phenotype is similar but more severe, with the main carotenoids being ζ-carotene (32%) and prolycopene (15%). The identification of geometric isomers of *tangerine* fruit carotenoids was based on comparison of the HPLC data with previously published analyses that also used NMR (Clough and Pattenden, 1979). Identification of some of the mono-*cis*- and di-*cis*-ζ-carotene, neurosporene, and lycopene isomers should be confirmed by the NMR method.

In wild-type flowers, the yellow xanthophylls neoxanthin, violaxanthin, and lutein make up 95% of total carotenoids. In contrast, the fraction of xanthophylls is ~50% of total carotenoids in flowers of *tangerine*^{3183} and <10% in *tangerine*^{mic}. In both mutants, the flowers accumulate large quantities of prolycopene and its precursors.

The *tangerine* mutation also affects carotenoid biosynthesis in chloroplasts, as is evident by the yellow color that appears in the newly developed leaves (Figure 2). Leaves of etiolated seedlings of *tangerine*^{mic}, but not *tangerine*^{3183} or the wild type, accumulate prolycopene and its precursors and do not contain any xanthophylls (Table 2). In green leaves of light-grown seedlings of *tangerine*^{mic}, the proportion of β-xanthophylls increases at the expense of lutein. Green fruit of *tangerine* contain the normal composition of carotenoids in the outer pericarp, but the inner pericarp and the placental tissue are orange (Figure 2) and contain high levels of prolycopene (data not shown).

All of the data described above indicate that the locus *tangerine* is involved in carotenoid isomerization that is essential for the biosynthesis of cyclized carotenes and xanthophylls.

Map-Based Cloning of *tangerine*

The recessive mutation *tangerine* was mapped to the long arm of chromosome 10, 4 centimorgans away from the locus *l2*. This locus is located in a region that overlaps IL10-2. Because none of the known carotenoid biosynthesis genes maps near this locus (data not shown), it has been predicted that *tangerine* is determined by a new gene. To further map *tangerine*, we crossed it to IL10-2 and analyzed 1045 F2

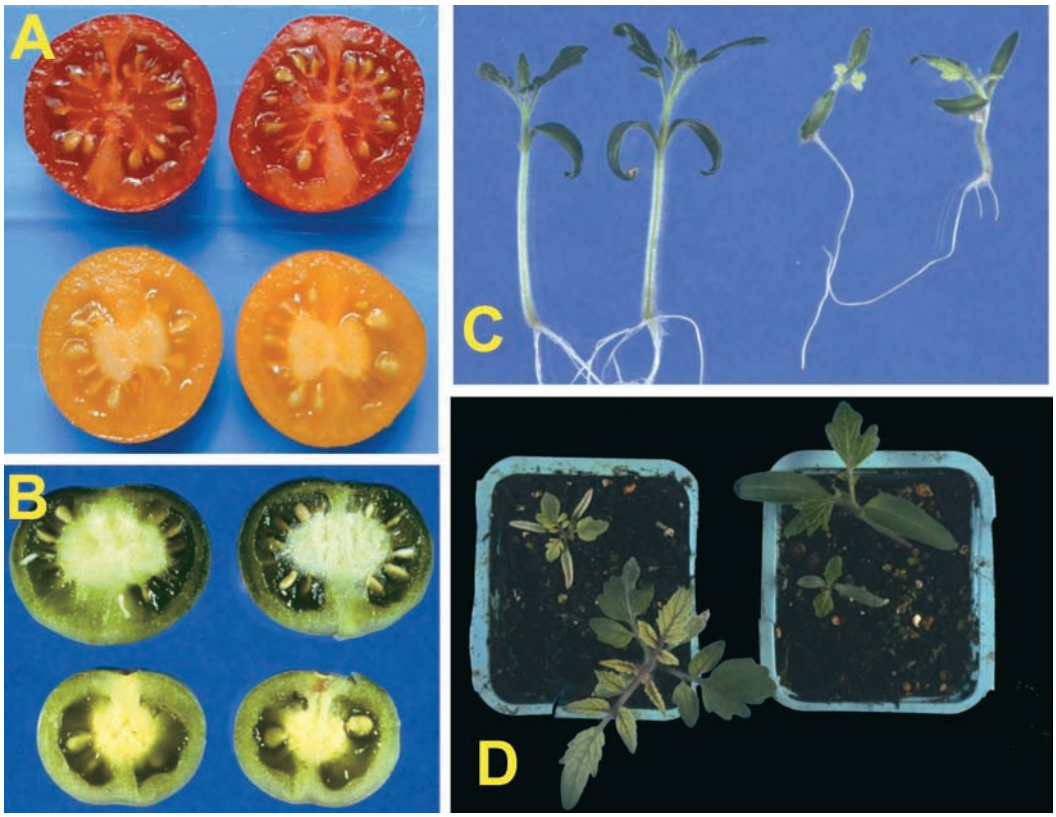

Figure 2. Fruits and Seedlings of Tangerine Mutants.

(A) Ripe fruit of the wild type (top) and mutant *tangerine*^{mic} (bottom).
(B) Immature green fruit of *tangerine*^{3183} (top) and mutant *tangerine*^{mic} (bottom).
(C) Light-grown seedlings of the wild type (left) and mutant *tangerine*^{mic} (right).
(D) Light-grown seedlings of mutant *tangerine*^{mic} (left) and the wild type (right).

plants using the markers TG408 and TG241 that flank *tangerine*. A total of 218 recombinant plants were obtained, and these individuals were selfed to determine their genotypes with respect to the recessive mutation *tangerine*. The recombinant plants were probed with additional markers, and CT57 was found to cosegregate with *tangerine* (Figure 4). A genomic library of tomato in bacterial artificial chromosomes (BACs) (Budiman et al., 2000) was screened with CT57, and BAC 21O12 was identified. Sequences at the ends of the insert of BAC 21O12 were amplified by polymerase chain reaction (PCR) and used as probes in genomic DNA hybridization of the 218 recombinant plants. The results indicated that BAC 21O12 contained the entire region of the *tangerine* locus because both BAC ends revealed recombinations with the target gene (Figure 4).

The entire insert of BAC 21O12 was sequenced. An open reading frame sequence with similarity to the bacterial gene for PDS, *crtI*, was found to cosegregate with the *tangerine* phenotype. The cDNA clone of this gene, designated *CRTISO*, was obtained by reverse transcriptase–mediated PCR using primers that were designed according to data from the tomato expressed sequence tag database. Comparison between the genomic and cDNA sequences revealed that the gene is composed of 13 exons and 12 introns (Figure 5). DNA gel blot hybridization with total genomic DNA indicated that *CRTISO* exists as a single copy in the tomato genome (data not shown).

Sequence Analysis of *CRTISO* in Wild-Type and *tangerine* Alleles

The cDNA of *CRTISO* contains an open reading frame of 615 codons that encodes a polypeptide with a calculated molecular mass of 67.5 kD. No differences in amino acid sequence were found between CRTISO from the wild type of

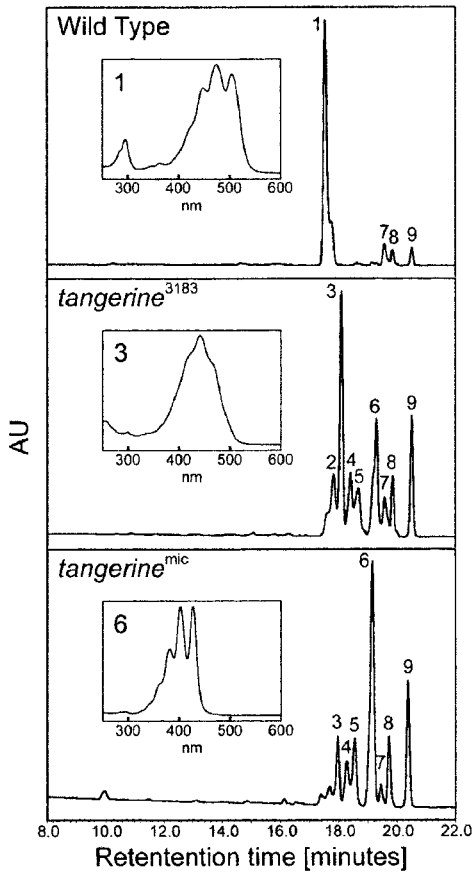

Figure 3. Analysis by HPLC of Carotenoids in Tomato Fruit of Wild Type and Two Alleles of *tangerine* Mutants.

Peak 1, all-*trans*-lycopene; peak 2, di-*cis*-lycopene; peak 3, prolycopene; peak 4, neurosporene; peak 5, tri-*cis*-neurosporene; peak 6, ζ-carotene; peak 7, β-carotene; peak 8, phytofluene; peak 9, phytoene. Absorption spectra of peaks are presented in the insets.

cv M82, Ailsa Craig, and Micro-Tom and the polypeptide in *tangerine*^{3183}. In contrast, analysis of both cDNA and genomic sequences of *CRTISO* from *tangerine*^{mic} indicated that this allele contains a deletion of 282 bp that encompasses 24 bp of the first exon and 258 bp of the first intron (Figure 5). As a result of this deletion, a splicing site is eliminated and the abnormal mRNA that is produced contains an early stop codon that aborts the synthesis of functional CRTISO. A deletion of 348 bp was discovered in the promoter region of *CRTISO* of *tangerine*^{3183} (Figure 5).

Functional Expression of *CrtISO* in *E. coli*

E. coli cells of the strain XL1-Blue, carrying plasmids pGB-Ipi and pAC-Zeta, accumulate mainly ζ-carotene. The latter plasmid contains the genes *CrtE* and *CrtB*, which encode geranylgeranyl pyrophosphate synthase and phytoene synthase, respectively, from *Erwinia herbicola*, and *crtP* from *Synechococcus* PCC7942, which encodes PDS. When cotransformed with plasmid pT-Zds, which encodes ZDS from tomato, the cells also produced prolycopene. A similar result has been reported (Bartley et al., 1999). Expressing both *Zds* and *CRTISO* from plasmid pCRTISO-TZds resulted in significant accumulation of lycopene (Table 3). The differences between dark-grown and light-grown bacteria probably are caused by the photoisomerization of 15-*cis*-ζ-carotene, which releases a barrier in the pathway. These results indicate that the polypeptide encoded by *CRTISO* is an authentic carotenoid isomerase that is able to convert *cis*-carotenoids to all-*trans*-carotenoids in *E. coli*.

Expression of *CRTISO* during Fruit Ripening

To determine the pattern of expression of *CRTISO*, we measured its mRNA level in different stages of fruit development (Figure 6). In wild-type fruit, the mRNA levels of *CRTISO* increased 10-fold during the breaker stage of fruit ripening, similar to the mode of expression of the genes *Psy* and *Pds*. In this analysis, no mRNA of *CRTISO* could be detected in fruit of *tangerine*^{3183} during fruit ripening. However, low amounts of mRNA, estimated to be ~1 to 2% of the wild-type level, were measured after 33 cycles of PCR amplification (data not shown). Expression of *CRTISO* in fruit of the mutant *tangerine*^{mic} was similar to that in the wild type (data not shown).

DISCUSSION

CRTISO Carotenoid Isomerase

Several lines of evidence designate CRTISO as a genuine carotenoid isomerase, which serves the indispensable function of carotenoid biosynthesis in oxygenic photosynthetic organisms. In *tangerine* tomato mutants, *CRTISO* cosegregates with the phenotype of prolycopene accumulation in fruit; a deletion mutation in *CRTISO*, which nullifies its function, was discovered in the allele *tangerine*^{mic} that exhibits a typical *tangerine* phenotype, and a deletion of 348 bp in the promoter of *CRTISO* abolishes its expression in fruit of *tangerine*^{3183}. The definite identification of CRTISO as an isomerase was confirmed by its functional expression in *E. coli*, in which it demonstrated activity of *cis*-to-*trans* isomerization of carotenes, especially prolycopene. The discovery of CRTISO solves a long-standing question that has puzzled investigators of carotenoid biosynthesis since the early 1940s.

In *E. coli*, the two desaturases from *A. thaliana*, PDS and ZDS, produce prolycopene from phytoene (Bartley et al., 1999). Our results confirm this finding in a combination of

Table 1. Carotenoid Composition in Fruit and Flowers of Wild-Type and *tangerine* Mutants

	M82 (Wild Type)	*tangerine*^{3183}	Micro-Tom	*tangerine*^{mic}
Fruits				
Phytoene	5.2 ± 2.1	15.3 ± 2.6	6.9 ± 2.1	16.0 ± 1.2
Phytofluene	3.6 ± 0.8	8.7 ± 1.53	5.9 ± 1.0	9.8 ± 1.8
ζ-Carotene	1.5 ± 2.0	23.6 ± 6.3	1.1 ± 0.4	31.7 ± 8.4
Cis-neurosporene		8.0 ± 4.1		11.4 ± 0.6
Neurosporene	0.2 ± 0.3	6.0 ± 3.1	0.4 ± 0.4	6.2 ± 1.7
Di-*cis*-lycopene		6.4 ± 3.2		3.6 ± 0.3
Prolycopene	0.4 ± 0.7	25.4 ± 7.7		15.2 ± 7.8
Lycopene	75.2 ± 10.2	0.6 ± 1.2	78.0 ± 6.0	
β-Carotene	5.9 ± 3.1	2.8 ± 3.3	1.2 ± 0.4	2.4 ± 1.0
Others/unidentified	8.0	3.2	6.5	3.7
Total carotenoids (μg g fresh weight)	77.0 ± 5	60.0 ± 17	104.0 ± 33	53.0 ± 8
Flowers				
Phytoene[a]		11.5 ± 3.4	0.3 ± 0.1	25.0 ± 7.8
Phytofluene[a]		3.3 ± 0.9		7.5 ± 2.8
ζ-Carotene[a]		4.6 ± 1.9		8.8 ± 1.3
Cis-neurosporene				2.6 ± 0.1
Neurosporene		1.6 ± 1.1		2.0 ± 0.1
Di-*cis*-lycopene				4.0 ± 3.8
Prolycopene		1.0 ± 0.9		30.2 ± 2.8
Lycopene		1.8 ± 1.8		
γ-Carotene		3.2 ± 1.6		
β-Carotene	1.1 ± 1.7	5.5 ± 1.4	0.8 ± 0.7	3.5 ± 1.6
Rubixanthin		11.6 ± 4.5		1.8 ± 2.6
β-Cryptoxanthin		2.5 ± 0.7	0.9 ± 1.1	
Violaxanthin	37.0 ± 7.1	11.0 ± 6.1	33.2 ± 6.4	
Neoxanthin	59.4 ± 7.0	36.5 ± 9.7	57.7 ± 6.3	9.7 ± 6.5
Lutein	2.5 ± 1.0	4.8 ± 0.6	7.1 ± 2.3	
Others/unidentified		1.5		4.9
Total carotenoids (μg g fresh weight)	770 ± 112	490 ± 327	1350 ± 521	998

Numbers correspond to percentage of total carotenoids. Total carotenoid concentration is per fresh weight.
[a] All isomers.

cyanobacterial PDS and ZDS from tomato. The data clearly indicate that poly-*cis*-carotenes occur as a default in the general pathway of phytoene desaturation in both chloroplasts and chromoplasts of plants and in cyanobacteria (our unpublished data), as was suggested by Bartley et al. (1999). CRTISO possibly is required as well for isomerization of *cis*-ζ-carotene and *cis*-neurosporene, because these species accumulate when the enzyme is deficient (Tables 1 and 3). Either CRTISO must rearrange the ζ-carotene molecule to enable ZDS activity, or ZDS catalysis, which is performed in conjunction with CRTISO, is required in the dark or in nonphotosynthetic tissues to enable the pathway to proceed to all-*trans*-lycopene and downstream to cyclic carotenoids. Support in favor of the latter hypothesis comes from in vitro experiments with the conversion of ζ-carotene to lycopene with purified ZDS from *Capsicum annuum* that was expressed in *E. coli* (Breitenbach et al., 1999). In this system, different lycopene isomers were formed according to the type of isomer that was used as a substrate. All-*trans*-ζ-carotene gave all-*trans*-lycopene, whereas *cis*-ζ-carotene yielded

Table 2. Carotenoid Composition in Leaves of Wild-Type (Micro-Tom) and *tangerine*^{mic} Seedlings Growing on Murashige and Skoog (1962) Medium 7 Days (Dark) or 10 Days (Light) after Germination

	Light		Dark	
Carotenoid	Micro-Tom	*tangerine*^{mic}	Micro-Tom	*tangerine*^{mic}
Phytoene[a]		2.1		17.8
Phytofluene[a]				7.7
ζ-Carotene[a]				24.2
Cis-neurosporene				15.1
Di-*cis*-lycopene				1.2
Prolycopene				33.6
β-Carotene	28.5	33.2	4.0	
Violaxanthin	3.3	22.5	19.8	
Neoxanthin	7.9	10.6		
Lutein	59.6	29.2	76.2	
Others	0.7	0.4		0.4

Numbers correspond to percentage of total carotenoids (average of two measurements).
[a] All isomers.

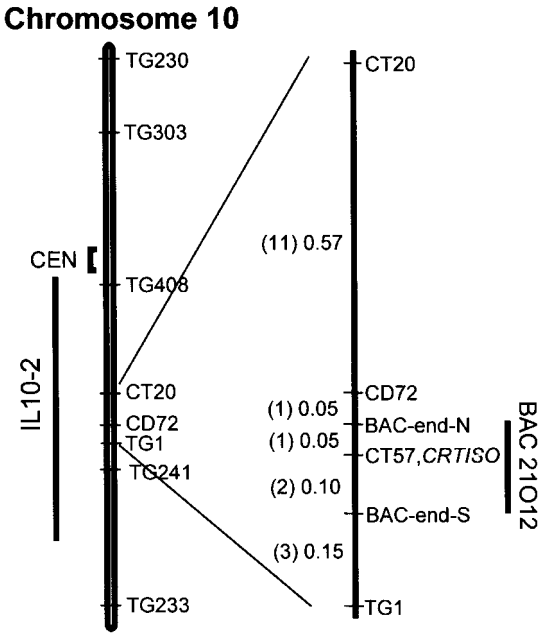

Figure 4. Map-Based Cloning of the *tangerine* Locus.

Fine mapping of chromosome 10 was performed with the restriction fragment length polymorphism markers indicated in the map. The chromosomal segment in the introgression line IL 10-2 that overlaps *tangerine* is depicted as a bar. Numbers correspond to centimorgans. Numbers in parentheses describe recombination events. Markers were TG408, CT20, CD72, CT57, TG1, and TG241. CEN, centromere.

prolycopene via the corresponding *cis*-neurosporene isomer. This result indicated that although ZDS could use *cis*-isomers as substrates, it was unable to change their *cis-trans* molecular configuration, suggesting that the isomer of lycopene formed during desaturation depends strictly on the isomeric state of the substrate. We propose that CRTISO might operate in vivo together with ZDS and hypothesize that the two enzymes form a complex.

A dinucleotide binding motif in the N terminus of the CRTISO polypeptide is characteristic of all carotenoid desaturases identified to date and is present as well in lycopene cyclases. Its existence suggests that the carotene isomerase, possibly a flavoprotein, is engaged in a redox-related reaction in which a temporary abstraction of electrons takes place to allow the rotation of the molecule around the specific C-C bond, which is followed by reconfiguration of the double bond.

Requirement for *cis-trans* Isomerization for Lycopene Cyclization

The reason for the accumulation of prolycopene in the absence of CRTISO is unclear at present. The most plausible explanation is that prolycopene cannot be cyclized by lycopene cyclases because of steric hindrance. This hypothesis is supported by the correlation observed in flowers of *tangerine* between the accumulation of di-*cis*-lycopene and the presence of γ-carotene and rubixanthin (Table 1). We have observed a similar correlation in the *Synechococcus* PCC6803 mutant, which lacks the isomerase ortholog (data not shown). Formation of the one-ringed carotenoids could have occurred by cyclization at the side of the molecule that is not bent by the *cis* configuration.

It was determined that the bacterial-type lycopene cyclase, CRTY, converts all-*trans*-lycopene to β-carotene and does not accept tetra-*cis*-prolycopene as a substrate (Schnurr et al., 1996). Likewise, it was shown that the plant-type lycopene cyclases efficiently convert all-*trans*-lycopene to β-carotene or δ-carotene (Cunningham et al., 1993, 1996; Pecker et al., 1996; Ronen et al., 1999). It is inexplicable, therefore, that only tetra-*cis*-prolycopene was accepted as a substrate for cyclization in isolated membranes of *Narcissus pseudonarcissus* (Beyer et al., 1994). Because the cyclized carotenes were formed in this system in the all-*trans* configuration, it was assumed that isomerization occurred during the cyclization of lycopene independent of the cyclization reaction (Beyer et al., 1991). Our data clearly indicate that isomerization takes place independent of cyclization in both plant tissues and *E. coli*.

Carotenoid Isomerases in Other Species

CRTISO is conserved among photosynthetic organisms in which phytoene conversion to lycopene is performed by two enzymes, PDS and ZDS. In Arabidopsis, a gene designated Pdh encodes a polypeptide that is 75% identical to CRTISO from tomato (86% identical in the predicted mature polypeptide region). Evidence that the Arabidopsis CRTISO ortholog is involved in carotenoid biosynthesis is described by Park et al. (2002). The similarity between the *CRTISO* genes in tomato and Arabidopsis extends to their genomic structure: both contain 12 introns in the same position of the coding sequence. In the cyanobacterium *Synechocystis*

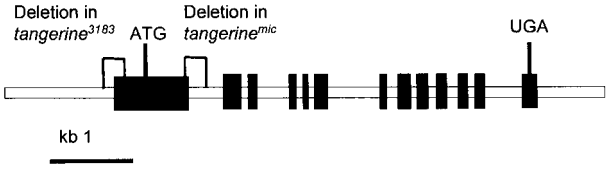

Figure 5. Organization of the Genomic Sequences of *CRTISO*.

Closed boxes represent exons. Deletions found in *CRTISO* of *tangerine* alleles are indicated. ATG, initiation codon; UGA, stop codon.

Table 3. Functional Expression of *CRTISO* in *E. coli*

Genes	Phytoene	Phytofluene	ζ-Carotene	Neurosporene	Di-*cis*-lycopene	Prolycopene	Lycopene	Others
Light								
crtE, crtB	98.4							1.6
crtE, crtB, Zds, CRTISO	99.4							0.6
crtE, crtB, crtP	12.2 ± 1.8	6.8 ± 0.2	79.2 ± 1.5					1.8
crtE, crtB, crtP, CRTISO	15.2 ± 4.6	7.0 ± 0.6	75.3 ± 4.7					2.5
crtE, crtB, crtP, Zds	17.9 ± 0.1	10.4 ± 0.2	54.6 ± 0.6		3.6 ± 0.2	6.9 ± 0.7	6.2 ± 0.5	0.4
crtE, crtB, crtP, Zds, CRTISO	19.7 ± 3.1	11.4 ± 1.2	38.5 ± 5.6				30.1 ± 9.7	0.3
Dark								
crtE, crtB	99.0							1.0
crtE, crtB, Zds, CRTISO	100							
crtE, crtB, crtP	8.9 ± 1.5	6.0 ± 0.8	83.2 ± 2.2					1.9
crtE, crtB, crtP, CRTISO	11.7 ± 0.9	7.6 ± 0.1	78.5 ± 1.3					2.2
crtE, crtB, crtP, Zds	13.7 ± 1.2	11.1 ± 1.6	66.2 ± 1.9			8.3 ± 1.4		0.7
crtE, crtB, crtP, Zds, CRTISO	23.2 ± 1.1	16.5 ± 0.9	50.0 ± 1.9	0.6 ± 0.9			8.5 ± 2.4	1.2

Cells of *E. coli*, all carrying plasmid with the gene *Ipi*, were transfected with different combinations of carotenoid biosynthesis genes. *crtE*, geranylgeranyl diphosphate synthase; *crtB*, phytoene synthase; *crtP*, phytoene desaturase; *Zds*, ζ-carotene desaturase; *CrtiSO*, carotenoid isomerase. Numbers correspond to percentage of total carotenoids.

PCC6803, the polypeptide encoded by sll0033 (http://www.kazusa.or.jp/cyano/) is 60% identical to the presumed mature CRTISO polypeptide. We have generated in this cyanobacterium a null mutation in the gene sll0033 by insertion mutagenesis (Δsll0033; data not shown). Cells of this mutant contain a significant proportion of prolycopene and other *cis*-carotenoids, similar to the phenotype observed in young or dark-grown green leaves of *tangerine*^{mic} tomato (data not shown).

Interestingly, the *CRTISO* sequences are similar to those of *crtI*, the bacterial-type PDS. However, CRTISO itself does not have PDS activity (Table 3). A phylogenetic tree based on amino acid sequence similarity demonstrates this relationship (Figure 7). It is possible that *CRTISO* originated in evolution from *crtI*. It is important to note that the bacterial phytoene desaturase, CRTI, produces all-*trans*-lycopene from phytoene through four dehydrogenation reactions, which apparently are mechanistically different in the plant-type enzymes PDS and ZDS.

Photoisomerization in Green Tissues

We observed that light could substitute for the lack of isomerase activity in leaves of *tangerine*^{mic}, in the outer green tissues in immature fruit of *tangerine* that are exposed to light, and in the Δsll0033 mutant of *Synechocystis* PCC6803 (data not shown) but not in nonphotosynthetic tissues of flowers (petals), ripe fruit, and the innermost parts of the green fruit of *tangerine*. A similar light-induced isomerization of prolycopene to all-*trans*-lycopene was observed in cells of the mutant C-6D of the unicellular green alga *Scenedesmus obliquus* (Romer et al., 1991; Sandmann, 1991), suggesting that *CRTISO* is impaired in this mutant.

The chemical mechanism of photoisomerization is unclear. We exclude the possibility that light interacts directly with the *cis*-isomers because we see no effect of light on prolycopene in chromoplasts of fruit and flowers and in *E. coli* cells. The differences in carotenoid production between light- and dark-grown *E. coli* (Table 3) are attributed to photoisomerization of 15-*cis*-ζ-carotene produced by PDS,

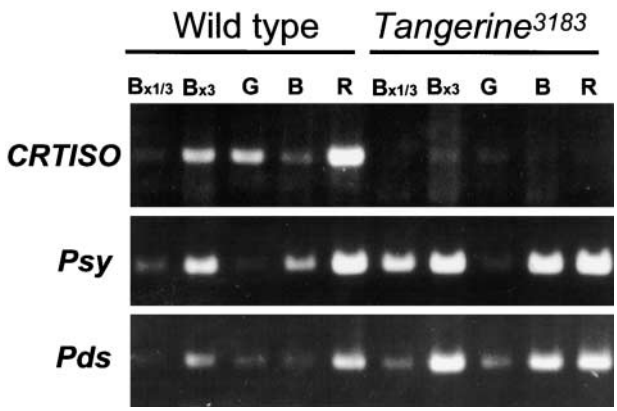

Figure 6. Expression of *CRTISO* during Tomato Fruit Development.

Steady state levels of mRNA of *CRTISO*, *Psy*, and *Pds* were measured by reverse transcriptase–mediated PCR from total RNA isolated from different stages of fruit development in wild-type tomato (cv M82) and mutant *tangerine*^{3183}. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. G, mature green fruit; B, breaker stage; R, ripe stage 7 days after breaker; $B_{\times 1/3}$ and $B_{\times 3}$, samples that contained one-third and three times the total RNA, respectively, from breaker-stage fruit.

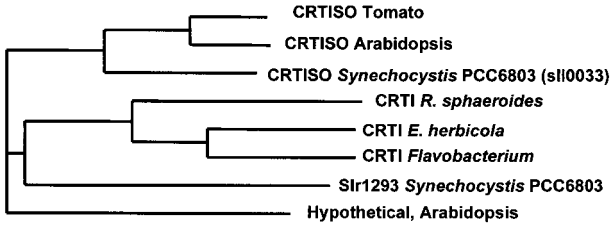

Figure 7. Phylogenetic Tree of *CRTISO* and Related Genes.

CRTI, phytoene desaturase from bacteria. Slr1293 is a hypothetical gene from *Synechocystis* PCC6803. CRTI *R. sphaeroides*, CRTI *Rhodobacter sphaeroides*.

which enables the subsequent desaturation by ZDS. This type of photoisomerization had been described previously in *Euglena gracilis* (Cunningham and Schiff, 1985).

Cis-to-*trans* isomerization was observed upon incubations of all-*trans*-isomers of β-carotene, lycopene, and canthaxanthin with HTMD (3-hydroxymethyl-3,4,4-trimethyl-1,2-dioxetane), a thermodissociable source of electronically excited ketones (Sundquist et al., 1993). The ability of chlorophyll compounds to sensitize the photoisomerization of all-*trans*-β-carotene has been demonstrated (Oneil and Schwartz, 1995). Light-driven isomerization of all-*trans* violaxanthin to the conformation of 9-*cis* and 13-*cis* was found in the light-harvesting pigment-protein complex of photosystem II (Phillip et al., 1999). Up to 9% 13-*cis* isomer was detected in leaves of barley after illumination, and up to 15% *trans*-to-*cis* conversion was achieved in the isolated light-harvesting pigment-protein complex of photosystem II of *Secale cereale* via a presumed triplet-sensitized photoisomerization reaction with blue light absorbed by chlorophylls and carotenoids as well as by red light absorbed exclusively by chlorophyll pigments (Grudzinski et al., 2001). In *Dunaliella salina*, the ratio of 9-*cis*- to all-*trans*-β-carotene is $>$2:1 under low light, and this is decreased to $<$0.45:1 upon exposure to high irradiance (Orset and Young, 2000). However, this isomerization probably occurs after cyclization of lycopene. We conclude that photoisomerization of ζ-carotene, neurosporene, and prolycopene, which occurs in chloroplasts, is associated with the existence of the photosynthetic apparatus. It is unclear at this time whether a component of this system can elicit isomerization using absorbed light energy or if this is an indirect effect of photosynthesis that relates to redox reactions necessary for isomerization.

In view of the possibility of substituting the activity of the carotenoid isomerase for light in green cells, it is hypothesized that the function of carotene isomerase in plants is to enable carotenoid biosynthesis in nonphotosynthetic tissues or in the dark. This is essential in germinating seedlings, as shown by Park et al. (2002) in roots and chromoplasts in the absence of chlorophyll sensitization.

METHODS

Plant Material and Growth Conditions

Tomato (*Lycopersicon esculentum* cv M82) and the introgression line IL 10-2 (Eshed and Zamir, 1995) served as the wild-type lines. The *tangerine* mutant LA3183 (*tangerine*^{3183}), which was kindly provided by Roger Chetelat (Tomato Genetics Resource Center, University of California, Davis), was used to map the *tangerine* locus and to characterize the phenotype. The mutant *tangerine*^{mic} was identified among M2 plants of a fast neutron–mutagenized population (David-Schwartz et al., 2001) in the background of the miniature cultivar Micro-Tom (Meissner et al., 1997) and was kindly donated by Dr. Avi Levy (Weizmann Institute, Rehovot, Israel). The two mutations were found to be allelic by genetic crossing (data not shown).

Recombinant plants in the F2 generation of a cross between *tangerine*^{3183} and IL10-2 were selfed, and the F3 progeny were screened for homozygous recombination products. Fixed recombinant plants were used to fine map the *tangerine* locus and served as isogenic lines for carotenoid analysis and measurement of gene expression. Lines 98-802 and 98-818 served as the wild type, and lines 98-823 and 104 served as *tangerine*^{3183} (see Results for details).

Seed of the different lines were sterilized by soaking in 70% ethanol for 2 min, in 3.3% NaOCl and 0.1% Tween 20 for 10 min, followed by three washes with sterile water. Seed were sowed on Murashige and Skoog (1962) basal salt mixture with 3% sucrose. The seedlings were grown at 23°C in dark or light for 2 weeks before leaves were analyzed. Plants were grown in the field for crossing and in the greenhouse for fruit analysis.

Carotenoid Analysis

Extraction of carotenoids from tomato fruit followed previously described protocols (Ronen et al., 1999, 2000). Leaf pigments were extracted from ~70 mg of fresh cotyledons of dark- or light-grown seedlings. Fresh tissue was minced in acetone and filtered. The solvent was dried under a stream of nitrogen and dissolved in acetone. Flower pigments were extracted from petals of fresh single flowers (for cv Micro-Tom, two flowers were extracted for each sample). The tissues were ground in 2 mL of acetone, 2 mL of dichloromethane was added, and the samples were agitated until all pigments were extracted. Saponification of flower carotenoids was performed in 60% (w/v) ethanol/KOH (9:1) for 16 hr at 4°C. The carotenoids were extracted with ether after the addition of NaCl to a final concentration of 1.2%. The samples were dried and dissolved in acetone. Analysis by HPLC using a photodiode array detector has been described previously (Ronen et al., 1999, 2000). Carotenoids were identified by their characteristic absorption spectra, distinctive retention times, and, in some cases, comparison with standards. Quantification was performed by integrating the peak areas of the HPLC results using Millennium chromatography software (Waters, Milford, MA).

Map-Based Cloning

Genomic DNA was prepared from 5 g of leaf tissue as described (Eshed and Zamir, 1995). Restriction fragment length polymorphism in genomic DNA from tomato was performed with markers TG408, CT20, CD72, CT57, TG1, and TG241 (Tanksley et al., 1992). A ge-

nomic library in bacterial artificial chromosomes (BACs) of tomato (var Heinz1706; http://www.clemson.edu) was screened with the marker DNA CT57. Sequences at the ends of the insert in BAC 21O12 were amplified by polymerase chain reaction (PCR) using the primers BAC2FA (5′-TGTCATCACCCAATTTTCCA-3′; forward end of BAC2), BAC2FB (5′-TTCCAGGAACTTGGTTTCCTT-3′; forward end of BAC2), BAC2RA, (5′-TGAAAGGGCATACCAAAAGG-3′; reverse end of BAC2), and BAC2RB (5′-GGCTACGCCAAGAACTCTGA-3′; reverse end of BAC2). The amplified sequences were used as probes in hybridization with DNA from recombinant plants. DNA fragments of the BAC insert were subcloned in the plasmid vector pBS (Promega) and sequenced using the T3 and T7 primers. Assembly of sequences was accomplished with the Vector NTI Suit software package (InfoMax Inc., Bethesda, MD). cDNA clones were obtained by reverse transcription followed by PCR using total RNA isolated from flowers.

Functional Expression of *CRTISO* in *Escherichia coli*

Plasmid pAC-Zeta, which carries the genes *crtB* and *crtE* from *Erwinia* and *crtP* from *Synechococcus* PCC7942, has been described (Cunningham et al., 1994). Plasmid pGB-Ipi was constructed by inserting the cDNA of *Ipi* from *Haematococcus pluvialis* (Cunningham and Gantt, 2001) (kindly provided by F.X. Cunningham, University of Maryland, College Park) into the HindIII site of plasmid vector pGB2 (Churchward et al., 1984). Plasmid pCRTISO was constructed by subcloning a 1631-bp PCR-amplified fragment from the cDNA of the tomato (cv M82) *CRTISO*. The primers used for amplification were 5′-GTTCTAGATGTAGACAAAAGAGTGGA-3′ (forward) and 5′-ACATCTAGATATCATGCTAGTGTCCTT-3′ (reverse). Both primers contain a single mismatch to create an XbaI restriction site. The PCR fragment was cut with XbaI and subcloned into the XbaI site of vector pBluescript SK− (Stratagene). Plasmid pT-Zds was constructed by subcloning a 1643-bp PCR-amplified sequence from the tomato cDNA of *Zds*. This DNA fragment was obtained using the primers TZds248 (5′-GCTGATTTGGATATCTATGGTTTC-3′; forward) and TZds1901 (5′-AACTCGAGTTGTATTTGGATGATTTGCA-3′; reverse). Each primer contains a single mismatch to create an EcoRV (TZds248) and a XhoI (TZds1901) restriction site. The PCR fragment was cut with EcoRV and XhoI and subcloned into vector pBluescript SK−, which was cut with SmaI and XhoI. Plasmid pCRTISO-TZds was constructed by subcloning the *CRTISO* cDNA fragment, which was excised from pCRTISO with the restriction endonucleases Cfr42I and BcuI, into pT-Zds, which was cut with the same enzymes.

E. coli cells of the strain XL1-Blue carrying plasmid pGB-Ipi were cotransformed with plasmids pAC-Zeta, pT-Zds, pCRTISO, and pCRTISO-TZds in various combinations and selected on Luria-Bertani medium containing the appropriate antibiotics: spectinomycin (50 mg/L), ampicillin (100 mg/L), and chloramphenicol (50 mg/L). Cells were incubated either in the dark or under dim light of 10 to 30 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ photon flux at room temperature for 48 hr.

Measurement of mRNA by Reverse Transcriptase–Mediated PCR

Protocols for RNA extraction and reverse transcription have been described previously (Ronen et al., 1999, 2000). Total RNA was isolated from 1 g of fruit tissue using the TRI reagent protocol (Molecular Research Center, Cincinnati, OH). After reverse transcription of total mRNA, the cDNAs of *Psy*, *Pds*, and *CRTISO* were amplified by PCR for 24, 26, and 28 cycles, respectively, of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C. Various initial concentrations of mRNA, ranging over a ninefold difference, were used to demonstrate the linear ratio between the concentration of template mRNA and the final PCR products. The following primers were used for PCR amplification: for *Pds*, 5′-TTGTGTTTGCCGCTCCAGTGGATAT-3′ (forward) and 5′-GCGCCTTCCATTGAAGCCAAGTAT-3′ (reverse); for *Psy*, 5′-GGGGAATTTGGGCTTGTTGAGT-3′ (forward) and 5′-CCTTTGATTCAGGGGCGATACC-3′ (reverse); for *CRTISO*, 5′-GATCGCCAAATCCTTAGCAA-3′ (forward) and 5′-GCCCTGGGAAGAGTGTTTTT-3′ (reverse). The products of PCR amplification were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

DNA and Protein Sequence Analysis

The DNA sequence was determined with the ABI Prism 377 DNA Sequencer (Perkin Elmer) and processed with ABI sequence analysis software. Vector NTI Suit software was used for sequence analysis.

Accession Numbers

The GenBank accession numbers for the sequences mentioned in this article are AF416727 (*CRTISO*), AC011001 (Arabidopsis gene Pdh), and AF195507 (tomato gene *Zds*).

ACKNOWLEDGMENTS

We thank Dr. Avi Levy from the Weizmann Institute for providing the mutant *tangerine*^{mic}, David Kachanovsky for sharing unpublished data on the inactivation of sll0033 in *Synechocystis* PCC6803 and for technical assistance, and Dr. V. Mann for valuable comments on the manuscript. This work was supported in part by the European Commission, contract ProVitA (QLK3-CT2000-0809), and by the Israel Ministry of Science. Work in our laboratory is performed under the auspices of the Avron Even-Ari Minerva Center.

Received July 24, 2001; accepted October 30, 2001.

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Cloning of *tangerine* from Tomato Reveals a Carotenoid Isomerase Essential for the Production of β-Carotene and Xanthophylls in Plants

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Plant Cell 2002;14;333-342
DOI 10.1105/tpc.010303