

# Arabidopsis Carotenoid Mutants Demonstrate That Lutein Is Not Essential for Photosynthesis in Higher Plants

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Lutein, a dihydroxy  $\beta,\epsilon$ -carotenoid, is the predominant carotenoid in photosynthetic plant tissue and plays a critical role in light-harvesting complex assembly and function. To further understand lutein synthesis and function, we isolated four lutein-deficient mutants of *Arabidopsis* that define two loci, *lut1* and *lut2* (for lutein deficient). These loci are required for lutein biosynthesis but not for the biosynthesis of  $\beta,\beta$ -carotenoids. The *lut1* mutations are recessive, accumulate high levels of zeinoxanthin, which is the immediate precursor of lutein, and define *lut1* as a disruption in  $\epsilon$  ring hydroxylation. The *lut2* mutations are semidominant, and their biochemical phenotype is consistent with a disruption of  $\epsilon$  ring cyclization. The *lut2* locus cosegregates with the recently isolated  $\epsilon$  cyclase gene, thus providing additional evidence that the *lut2* alleles are mutations in the  $\epsilon$  cyclase gene. It appears likely that the  $\epsilon$  cyclase is a key step in regulating lutein levels and the ratio of lutein to  $\beta,\beta$ -carotenoids. Surprisingly, despite the absence of lutein, neither the *lut1* nor *lut2* mutation causes a visible deleterious phenotype or altered chlorophyll content, but both mutants have significantly higher levels of  $\beta,\beta$ -carotenoids. In particular, there is a stable increase in the xanthophyll cycle pigments (violaxanthin, antheraxanthin, and zeaxanthin) in both *lut1* and *lut2* mutants as well as an increase in zeinoxanthin in *lut1* and  $\beta$ -carotene in *lut2*. The accumulation of specific carotenoids is discussed as it pertains to the regulation of carotenoid biosynthesis and incorporation into the photosynthetic apparatus. Presumably, particular  $\beta,\beta$ -carotenoids are able to compensate functionally and structurally for lutein in the photosystems of *Arabidopsis*.

## INTRODUCTION

The carotenoids are a diverse group of pigments that are widely distributed in nature and found in all photosynthetic organisms as well as in many nonphotosynthetic bacteria and fungi. Carotenoids are often responsible for the red, orange, and yellow colors of fruits, flowers, and tubers, and the epoxy xanthophylls are precursors of the phytohormone abscisic acid (Rock and Zeevart, 1991; Pfander and Packer, 1992). Nutritionally, carotenoids are important components of mammalian diets as a source of vitamin A and as possible protectants against serious disorders, such as cancer, heart disease, and degenerative eye disease (Krinsky, 1989). In plants, carotenoids are synthesized and accumulate in plastids, where they are essential for viability because they function in photoprotection by quenching triplet chlorophyll, singlet oxygen, and other reactive species (Siefermann-Harms, 1987). Carotenoids play additional roles as accessory pigments in light harvesting and in thermal dissipation of excess light energy (Demmig-Adams and Adams, 1992).

There are two main classes of carotenoids: the carotenes that are cyclized or uncyclized hydrocarbons (for example,

$\beta$ -carotene), and the xanthophylls that are oxygenated derivatives of carotenes (for example, lutein, violaxanthin, and neoxanthin). Higher plant chloroplasts typically accumulate lutein,  $\beta$ -carotene, violaxanthin, and neoxanthin (in order of abundance) in the thylakoid membrane-bound photosystems (Peter and Thornber, 1991; Ryberg et al., 1993). Both the location and the photochemical properties of the different carotenoids have been used to infer function in vivo.

Photosystems I and II are pigment-protein complexes consisting of a reaction center surrounded by antennae that harvest and transfer light energy to the reaction center. In general, the reaction center contains  $\beta$ -carotene and chlorophyll *a* (Chl *a*) as its only pigments, whereas the adjacent core complex proteins contain  $\beta$ -carotene and lutein as their only carotenoids (Peter and Thornber, 1991; Bassi et al., 1993).  $\beta$ -Carotene performs the critical role of photoprotection in the reaction center by quenching triplet chlorophyll and singlet oxygen, and it can undergo rapid degradation during photooxidation (Young, 1993a). In contrast, the surrounding antenna complexes are comprised of the xanthophylls (lutein, violaxanthin, and neoxanthin), light-harvesting complex (LHC) proteins, and both Chl *a* and chlorophyll *b* (Chl *b*) (Peter and Thornber, 1991; Bassi et al., 1993). Antenna complex xanthophylls are presumed to

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act as accessory light-harvesting pigments, which augment energy absorption and transfer singlet-excitation energy to chlorophyll (Frank and Cogdell, 1993). In addition, the xanthophyll zeaxanthin accumulates due to deepoxidation of part of the violaxanthin pool under high-light stress and is thought to provide photoprotection by increasing thermal energy dissipation within the antenna complexes (Demmig-Adams and Adams, 1993). This reversible interconversion of violaxanthin and zeaxanthin, via the monoepoxide antheraxanthin, is referred to as the xanthophyll or violaxanthin cycle (Pfundel and Bilger, 1994).

Lutein is the most abundant carotenoid in the chloroplast and often accounts for >50% of the total carotenoid pool. It has been localized in the crystallized structure of the LHC and is the only xanthophyll detected in the photosystem II core (Bassi et al., 1993; Kuhlbrandt et al., 1994). In addition, lutein is required for the *in vitro* reconstitution of LHCs, and consequently, a critical structural and functional role for lutein in photosynthesis has been assumed (Plumley and Schmidt, 1987; Cammarata et al., 1990; Paulsen et al., 1990).

The first committed step in lutein synthesis is the formation of  $\alpha$ -carotene from lycopene, as shown in Figure 1. In plants, cyclization of the end groups of lycopene produces  $\beta$ -carotene ( $\beta,\beta$ -carotene), which contains two  $\beta$  rings, and  $\alpha$ -carotene ( $\beta,\epsilon$ -carotene), which contains a  $\beta$  and an  $\epsilon$  ring (Figure 1; Britton, 1985, 1990). Isotopic labeling has shown  $\epsilon$  and  $\beta$  cyclization to be distinct processes (Britton, 1985, 1990), and the formation of  $\epsilon$  rings and production of  $\beta,\epsilon$ -carotenoids are two of the key differences distinguishing carotenoid biosynthesis in plants from that in cyanobacteria, fungi, and bacteria. Lutein is formed from  $\alpha$ -carotene by the stereochemically distinct hydroxylation of the 3 and 3' carbons of the  $\beta$  and  $\epsilon$  rings, respectively. Zeaxanthin is formed from  $\beta$ -carotene by two  $\beta$  ring hydroxylations and is in turn epoxidated to form violaxanthin, which is the precursor of neoxanthin (Figure 1; Britton, 1988). Although considerable progress has been made in recent years in defining the molecular genetics of carotene synthesis in plants (Cunningham et al., 1994; Sandmann, 1994; Albrecht et al., 1995; Huguency et al., 1995; Norris et al., 1995), relatively little is known about the enzymes and genes that regulate xanthophyll biosynthesis. None of the genes encoding the xanthophyll-forming enzymes proposed in Figure 1 has been isolated from plants, and the only genetically defined step is zeaxanthin epoxidase by the Arabidopsis abscisic acid-deficient (*aba*) mutant (Rock and Zeevart, 1991).

As a step toward advancing our understanding of xanthophyll biosynthesis, incorporation, and function, we have identified mutants that are deficient in the accumulation of lutein, the most predominant plant xanthophyll. We discuss two mutants, *lut2* and *lut1* (for lutein deficient), that disrupt lutein biosynthesis in different ways. Growth and development of *lut* mutants were not visibly affected by the absence of lutein, despite its presumed critical role in photosynthesis. The results are discussed in terms of the control of carotenoid accumulation by biosynthesis and/or incorporation and in relation to the proposed functions of lutein and other xanthophylls in photosynthesis.

## RESULTS

### Isolation of Four Arabidopsis Mutants Defective in Lutein Biosynthesis

The hallmark phenotypes that indicate disruption of a biosynthetic pathway are the absence of the subsequent product(s) of the pathway and often, but not always, the accumulation of compounds before the site of blockage. The xanthophylls are accessory light-harvesting pigments, so although alterations in xanthophyll composition may reduce light-harvesting efficiency, they would not necessarily be lethal. Based on this reasoning, disruptions specific to xanthophyll synthesis were targeted in our screen by analyzing only mutagenized plants that were phototrophic. This approach selected against mutations earlier in the pathway that would accumulate only noncyclized carotenoids, such as lycopene and phytoene, which cannot protect against photooxidation, thus making the mutations lethal (Young, 1993a). More than 4000 individual soil-grown  $M_2$  mutant lines were screened for abnormal pigment profiles by HPLC. Figure 2A shows a typical profile of wild-type leaf pigments absorbing at 440 nm. Lutein is the predominant carotenoid; the other carotenoids that accumulate, in order of abundance, are  $\beta$ -carotene, violaxanthin, and neoxanthin. This normal pigment profile was observed in the vast majority of lines examined. However, four lines were identified that are deficient in lutein content.

The reduction in lutein for the four mutant lines ranged from 80 to 100% (see Figures 2B and 2C and Table 1); however, all mutants were viable as homozygotes, and under the growth conditions used, there were no obvious differences in growth or morphology when compared with wild-type plants. The four mutants were placed into two classes based on their biochemical phenotype. The *lut2* class had no detectable lutein and had increased amounts of  $\beta$ -carotene and the xanthophyll cycle pigments (violaxanthin, antheraxanthin, and zeaxanthin); however, it did not accumulate any additional carotenoids (Figures 2B and 2E). The *lut1* class had severely reduced lutein and increased amounts of the xanthophyll cycle pigments; in addition, it accumulated a carotenoid with a retention time of 24 min that was not detected in the wild type or *lut1* (compare Figures 2A and 2D with 2C and 2F).

### *lut1* and *lut2* Define Two Loci Required for Lutein Biosynthesis: *lut1* Is Recessive and *lut2* Is Semidominant

Allelism tests were performed by analyzing  $F_1$  progeny of reciprocal crosses of the four mutants, and the results are presented in Table 2. All  $F_1$  progeny of six crosses between homozygous *lut1-1* and *lut1-2* were reduced in lutein, indicating that *lut1-1* and *lut1-2* are allelic. Similarly, *lut2-1* and *lut2-2* were shown to be allelic because all progeny of six crosses were also reduced in lutein. However, all crosses between the *lut1* and *lut2* mutants (four crosses of *lut1-1*  $\times$  *lut2-1* and two



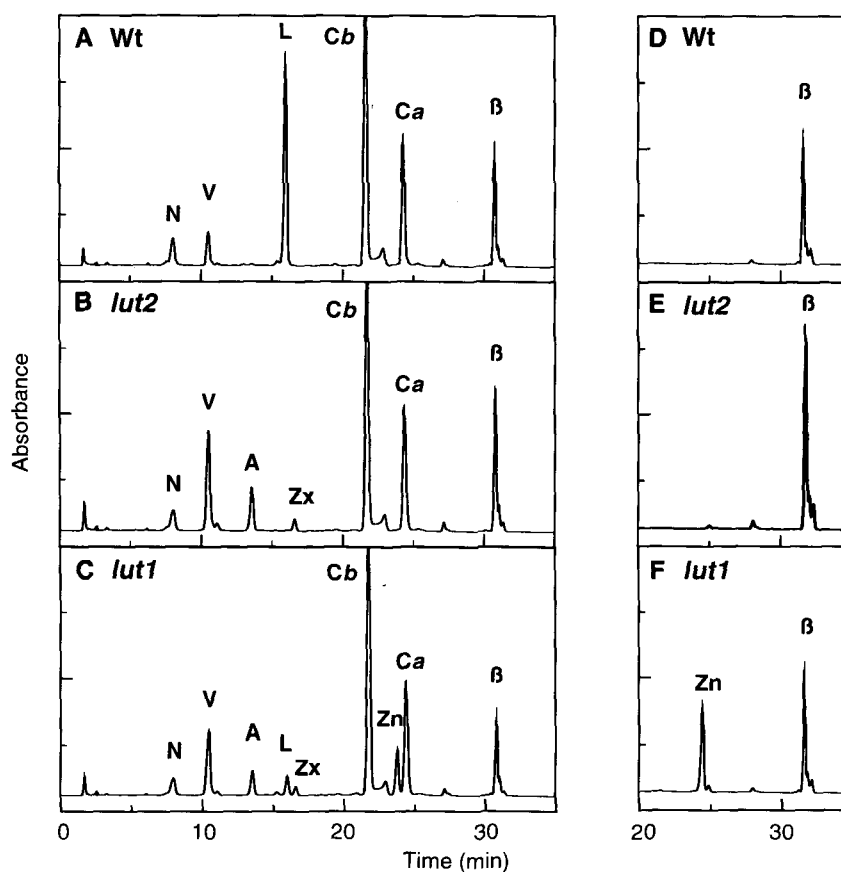
type lutein to lutein deficient, which is consistent with a 3:1 ratio by chi-square analysis ( $P > 0.2$ ; Table 2).

#### Identification of the Additional Carotenoid of *lut1* as Zeinoxanthin ( $\beta,\epsilon$ -Caroten-3-ol)

The additional compound that accumulated in the *lut1* mutants was identified by its HPLC retention time (24 min) and UV visible and mass spectra. The chromatographic behavior (when analyzed by thin-layer chromatography and HPLC) of the compound was characteristic of a monohydroxycarotenoid, for example, zeinoxanthin ( $\beta,\epsilon$ -caroten-3-ol),  $\alpha$ -cryptoxanthin ( $\beta,\epsilon$ -caroten-3'-ol), or  $\beta$ -cryptoxanthin ( $\beta,\beta$ -caroten-3-ol), and not of a dihydroxycarotenoid, such as lutein ( $\beta,\epsilon$ -caroten-3,3'-ol) or

zeaxanthin ( $\beta,\beta$ -caroten-3,3-ol) (Figure 2C). The additional compound was not affected by saponification and is therefore neither a xanthophyll (lutein) ester nor a chlorophyll (Figure 2F) (Eugster, 1995).

The identification of the additional carotenoid as a  $\beta,\epsilon$ -carotenoid ( $\alpha$ -carotene derivative) followed from the UV visible absorption spectrum shown in Figure 4. Carotenoids typically show three characteristic absorption peaks, and the  $\lambda_{\max}$ , shape, and ratios of those peaks (in particular, the peak III-to-peak II ratio, which is called the spectral fine structure) are important parameters in identifying a carotenoid (Britton, 1995). The additional carotenoid had  $\lambda_{\max}$  values at 424, 446, and 476 nm and a peak III-to-peak II ratio of 60%, which is typical of a  $\beta,\epsilon$ -carotenoid in 50% acetonitrile and 50% ethyl acetate (Figure 4). A  $\beta,\beta$ -carotenoid, for example, zeaxanthin,



**Figure 2.** Pigment Analysis of Wild-Type, *lut2*, and *lut1* Leaves.

(A) to (C) HPLC separation of lipid-soluble pigments from leaves of 4-week-old plants.

(D) to (F) Saponified extract of carotenoids from leaves of 2-week-old plants. Chlorophylls were removed from the carotenoid-containing phase by saponification. The 20- to 35-min portion of the gradient is shown.

(A) and (D) show the wild-type profiles; (B) and (E) show the *lut2-1* profiles; (C) and (F) show the *lut1-1* profiles. Each profile represents absorbance at 440 nm of pigments extracted from 5 mg fresh weight of tissue.

A, antheraxanthin;  $\beta$ ,  $\beta$ -carotene; Ca, Chl a; Cb, Chl b; L, lutein; N, neoxanthin; V, violaxanthin; Wt, wild type; Zn, zeinoxanthin; Zx, zeaxanthin.

**Table 1.** Carotenoids in Wild-Type, *lut1*, and *lut2* Leaves<sup>a</sup>

Line	Neoxanthin	Violaxanthin	Antheraxanthin	Lutein	Zeaxanthin	Zeinoxanthin	$\beta$ -Carotene	Total
Wild type	35	30	0	133	0	0	57	255
<i>lut1-1</i>	26	63 <sup>b</sup>	22 <sup>b</sup>	20 <sup>b</sup>	6 <sup>b</sup>	39 <sup>b</sup>	50	226
<i>lut1-2</i>	27	65 <sup>b</sup>	23 <sup>b</sup>	24 <sup>b</sup>	6 <sup>b</sup>	32 <sup>b</sup>	52	230
<i>lut2-1</i>	31	103 <sup>b</sup>	44 <sup>b</sup>	0 <sup>b</sup>	9 <sup>b</sup>	0	75 <sup>b</sup>	261
<i>lut2-2</i>	27	90 <sup>b</sup>	36 <sup>b</sup>	0 <sup>b</sup>	7 <sup>b</sup>	0	71	232

<sup>a</sup> Carotenoid content, expressed as micrograms per gram fresh weight of tissue, was quantified from HPLC analyses as described in Methods.

<sup>b</sup> The mean amount of the indicated carotenoid in the mutant is significantly different from the mean for the wild type ( $P < 0.05$ ). The population standard error for a significant difference ( $P < 0.05$ ) between means for each carotenoid is nine for neoxanthin, 16 for violaxanthin, five for antheraxanthin, 19 for lutein, one for zeaxanthin, eight for zeinoxanthin, 17 for  $\beta$ -carotene, and 70 for total carotenoids.

had  $\lambda_{\max}$  values at 432, 454, and 480 nm and a peak III-to-peak II ratio of 25% in acetonitrile-ethyl acetate buffer. The spectrum of the additional carotenoid was unchanged following treatment with dilute acid, indicating a lack of any 5,6-epoxide groups, such as those in violaxanthin or antheraxanthin, which have similar absorption spectra (Eugster, 1995).

The identification of the unknown carotenoid in *lut1* as the  $\beta,\epsilon$ -caroten-3-ol zeinoxanthin was confirmed by chemical ionization mass spectrometry (MS), shown in Figure 5. This analysis was used to determine the mass of the protonated molecule (MH) of lutein (MH<sub>L</sub>), zeaxanthin (MH<sub>Zx</sub>), and the unknown compound (MH<sub>Zn</sub>) as well as the ionization profiles of each of these carotenoids. First, the quasimolecular ion (H+ adduct) of the novel carotenoid (MH<sub>Zn</sub>) was at an  $m/z$  of 553, corresponding to a carotenol C<sub>40</sub>H<sub>56</sub>O, which is the mass of a monohydroxycarotenoid (Figure 5C) and is consistent with the chromatographic retention time (Figures 2C and 2F). Second, the location of the hydroxy group on the  $\beta$  ring of the additional carotenoid was deduced from the ionization profile. Carotenoids that have a hydroxy group in an allylic position, for example, at C-3 of an  $\epsilon$  ring, readily eliminate water. This was observed in the MS of lutein in which the intensity of the MH<sub>L</sub> minus H<sub>2</sub>O peak was greater than that of the adduct molecular ion (MH<sub>L</sub>) (Figure 5A). In contrast to zeaxanthin, in which both hydroxy groups are nonallylic, the loss of water was weak (Figure 5B). In the MS of the unknown compound, the fragment ion at an  $m/z$  of 535 corresponding to the loss of a water molecule was of low relative intensity (MH<sub>Zn</sub> minus H<sub>2</sub>O), as in the MS of zeaxanthin, indicating that the hydroxy group is located in a nonallylic position, that is, at position 3 in the  $\beta$ -ring (Figures 5B and 5C).

Tandem MS-MS analysis of the molecular ion of this carotenoid revealed the presence of significant fragment ions at an  $m/z$  of 497 (MH-56) and 430 (MH-123), characteristic of an unsubstituted  $\epsilon$  ring (data not shown). The retention time, MS, and UV visible spectrum are entirely consistent with identification of the unknown carotenoid in *lut1* as zeinoxanthin ( $\beta,\epsilon$ -caroten-3-ol); the alternative possibilities,  $\alpha$ -cryptoxanthin

( $\beta,\epsilon$ -caroten-3'-ol) and  $\beta$ -cryptoxanthin ( $\beta,\beta$ -caroten-3-ol), are excluded. Although the UV visible spectrum is characteristic of the all-*E* (all-*trans*) isomer, the chirality at C-3 and C-6' (3*R* and 6'*R* in lutein) was not proved by the techniques used.

#### Carotenoid Composition but Not Chlorophyll Content Is Altered in *lut1* and *lut2*

The loss of lutein (80 to 100%) in *lut1* and *lut2* is compensated for by an equimolar change in the abundance of other carotenoids (Table 1). In the *lut1* mutants, there was a significant increase in the xanthophyll cycle pigments (violaxanthin, antheraxanthin, and zeaxanthin) and in zeinoxanthin. In the *lut2* mutants, there was also an increase in the xanthophyll cycle pigments plus an increase in  $\beta$ -carotene; however, no precursors of lutein (for example, zeinoxanthin,  $\alpha$ -carotene,  $\gamma$ -carotene,

**Table 2.** Genetic Analysis of *lut1* and *lut2* Mutants<sup>a</sup>

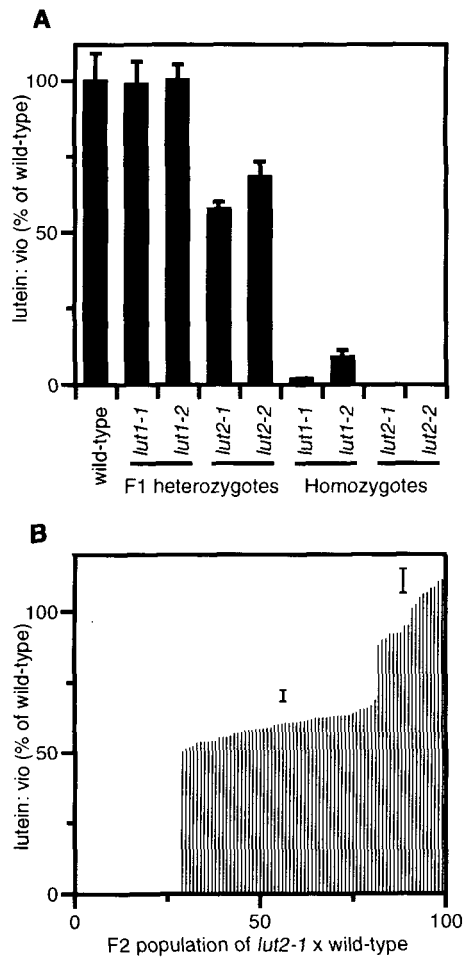
Cross	Wild Type <sup>b</sup>	Lutein Reduced <sup>b</sup>	Lutein Deficient <sup>b</sup>
<i>lut1-1</i> × <i>lut1-2</i> F <sub>1</sub>	0	0	30
<i>lut2-1</i> × <i>lut2-2</i> F <sub>1</sub>	0	0	38
<i>lut1</i> × <i>lut2</i> F <sub>1</sub>	26	0	0
<i>lut1-1</i> × <i>LUT1</i> F <sub>1</sub>	15	0	0
	F <sub>2</sub>	80	0
<i>lut2-1</i> × <i>LUT2</i> F <sub>1</sub>	0	24	0
	F <sub>2</sub>	19	53
			28 <sup>d</sup>

<sup>a</sup> The F<sub>1</sub> and F<sub>2</sub> progeny were analyzed for lutein content and the lutein/violaxanthin ratio by peak area ratios at 440 nm.

<sup>b</sup> Progeny were classed as wild type, lutein reduced (70 to 90% of wild-type lutein content; heterozygous), or lutein deficient (<20% of wild-type lutein content; homozygous).

<sup>c</sup>  $\chi^2 = 1.3$ ; calculated for a 3:1 ratio with  $P > 0.2$ .

<sup>d</sup>  $\chi^2 = 2.0$ ; calculated for a 1:2:1 ratio with  $P > 0.3$ .



**Figure 3.** *lut1* Alleles Are Recessive, and *lut2* Alleles Are Semi-dominant.

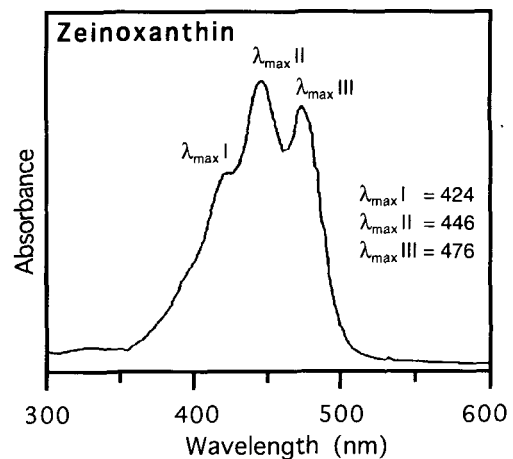
The peak area ratio of lutein to violaxanthin (vio; at 440 nm) as a proportion of the wild type was determined.

(A) Wild-type, heterozygous, and homozygous *lut1* and *lut2* alleles. (B) A segregating population of F<sub>2</sub> plants of *lut2-1* × *LUT2*. Standard deviations are shown.

or lycopene) were detected. Neoxanthin levels were not significantly altered in *lut1* or *lut2* mutants. The total amount of carotenoids does not vary significantly for the wild-type, *lut1*, and *lut2* plants when it is expressed either as micrograms (Table 1) or as nanomoles per gram fresh weight of tissue. The net increase in other carotenoids plus residual lutein in the *lut* mutants equal the micrograms (or nanomoles) of lutein in wild-type leaves. The net increase (micrograms per gram fresh weight of tissue) in other carotenoids plus residual lutein is 120 for *lut1-1*, 120 for *lut1-2*, 143 for *lut2-1*, and 117 for *lut2-2* compared with 133  $\mu$ g of lutein in the wild type; these means are not significantly different at  $P < 0.05$ .

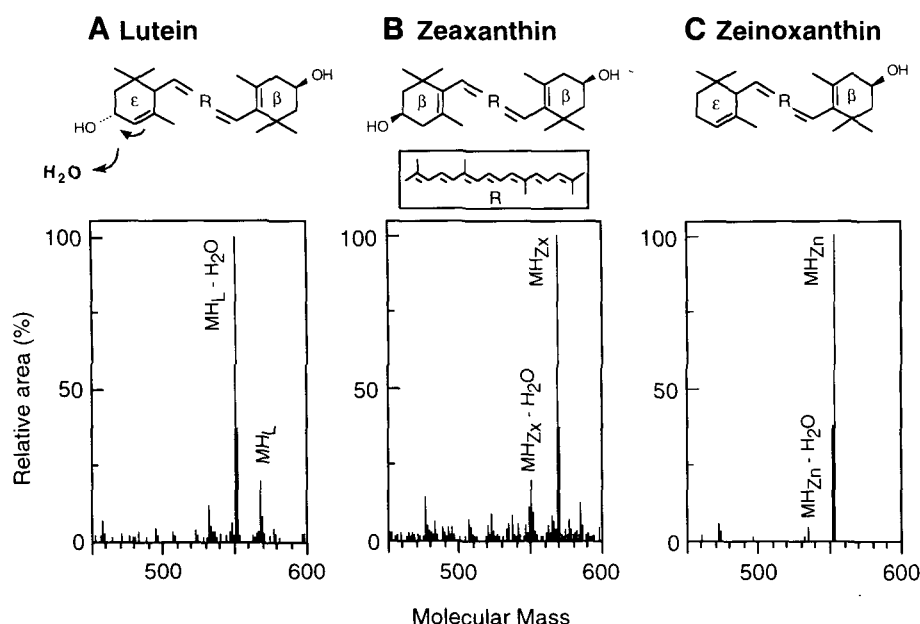
The interconversion of violaxanthin and zeaxanthin via antheraxanthin is referred to as the xanthophyll cycle and is a mechanism for adaptation to high-light stress (Demmig-Adams and Adams, 1992; Horton et al., 1994). One measure of the interconversion is the change in the deepoxidation state (the ratio of the deepoxy to the epoxy xanthophylls) under different light regimes (Ruban et al., 1994). Light stress results in the deepoxidation of a portion of the violaxanthin pool to form zeaxanthin and antheraxanthin. When light-stressed plants are placed in the dark, the transient pool of zeaxanthin and antheraxanthin that had been produced undergoes epoxidation to form violaxanthin, and the deepoxidation state should revert to zero (Demmig-Adams and Adams, 1993). Although all plants were grown under moderate light intensity such that there was no zeaxanthin accumulation in the wild type (the deepoxidation state was zero), both *lut1* and *lut2* plants had high deepoxidation state ratios (see Table 3). After plants were dark treated for 24 hr, the deepoxidation state did not revert to zero for *lut1* and *lut2*, although it declined significantly by 12 to 25%. Apparently, the vast majority of the xanthophyll cycle pigments in *lut1* and *lut2* are not involved in light-induced cyclic interconversions.

In Table 4, the chlorophyll content of the *lut* mutants is summarized. Analyses of variance indicated no significant difference between any of the *lut* mutants and the wild-type plants for Chl a, Chl b, or the Chl a-to-Chl b ratio. Therefore, an 80 to 100% reduction in the most abundant carotenoid, lutein, and its replacement with equimolar amounts of other carotenoids did not markedly affect the amount and ratio of Chl a/b synthesized and incorporated into the photosynthetic apparatus.



**Figure 4.** UV Visible Absorption Spectrum of the Additional Carotenoid from *lut1* in Acetonitrile–Ethyl Acetate (1:1 [v/v]).

The three absorbance peaks,  $\lambda_{max I}$ ,  $\lambda_{max II}$ ,  $\lambda_{max III}$ , and their respective maximum wavelengths for the additional carotenoid that accumulated in *lut1* are shown.



**Figure 5.** MS Identifies the Additional Carotenoid from *lut1* as Zeinoxanthin ( $\beta,\epsilon$ -Caroten-3-ol).

The chemical structures of each compound and the loss of water from the hydroxylated  $\epsilon$  ring of lutein are shown.

(A) Lutein.

(B) Zeaxanthin.

(C) Zeinoxanthin, which is the additional carotenoid that accumulated in *lut1*.

The compounds were purified and analyzed by chemical ionization MS. The quasimolecular ion ( $H^+$  adduct) for lutein ( $MH_L$ ) at an  $m/z$  of 569, zeaxanthin ( $MH_{Zx}$ ) at an  $m/z$  of 569, and zeinoxanthin ( $MH_{Zn}$ ) at an  $m/z$  of 553 are shown. The quasimolecular ion for zeinoxanthin corresponds to a carotenol  $C_{40}H_{56}O$ . The fragment ions that correspond to the loss of a water molecule ( $MH - H_2O$ ) were of high relative intensity for lutein and of low relative intensity for zeaxanthin and zeinoxanthin. The data are presented as relative area (percentage) of the highest peak in each analysis.

### Mapping and Linkage Analysis of *lut* Mutants

Because the *lut2* mutants are inhibited in the production of lutein and do not accumulate any lutein precursors, it is likely that they are mutations that disrupt the  $\epsilon$  cyclase. In Figure 6, the first step toward confirming this and distinguishing between direct and indirect disruption is presented, that is, determining whether the *lut2* mutation cosegregates with the  $\epsilon$  cyclase gene. A cDNA for the  $\epsilon$  cyclase has recently been isolated from Arabidopsis and is characterized in detail in Cunningham et al. (1996). Homozygous *lut2-1* mutants in an Arabidopsis ecotype Columbia background were outcrossed with wild-type Landsberg erecta, and the resultant  $F_2$  mapping population was phenotyped by pigment analysis for lutein content and scored as wild type (lutein-to-violaxanthin ratio [L/V] of  $3.9 \pm 0.4$ ), lutein reduced (L/V of  $2.3 \pm 0.4$ ), and lutein deficient (L/V of  $0 \pm 0$ ). The same plants were then scored as Landsberg erecta (4.8 kb), heterozygous (4.8 and 4.0 kb), or Columbia (4.0 kb) for an  $\epsilon$  cyclase locus restriction fragment length polymorphism (RFLP). All homozygous *lut2* lines were homozygous for the Columbia restriction fragment, all heterozygous *lut2* lines were heterozygous for the RFLP, and all

wild-type lines were homozygous for the Landsberg erecta restriction fragment (for an example, see Figure 6). This places the two loci within 2 centimorgans (cM) of each other ( $P < 0.05$ ). There were no recombination events detected between *lut2*

**Table 3.** Deepoxidation State of Wild-Type, *lut1*, and *lut2* Leaves<sup>a</sup>

Plant Line	Light <sup>b</sup>	Dark <sup>c</sup>
Wild type	0.0	0.0
<i>lut1-1</i>	18.7	16.2 <sup>d</sup>
<i>lut1-2</i>	19.2	16.9 <sup>d</sup>
<i>lut2-1</i>	19.9	15.0 <sup>d</sup>
<i>lut2-2</i>	18.8	14.1 <sup>d</sup>

<sup>a</sup> The ratio of deepoxidated to epoxidated xanthophylls ( $Zx + 0.5A$ )/( $Zx + A + V$ ) (%) after light or dark treatment, where  $Zx$  is zeaxanthin,  $A$  is antheraxanthin, and  $V$  is violaxanthin.

<sup>b</sup> Leaves were harvested from plants grown in light and analyzed. See Methods for details.

<sup>c</sup> The same plants used for the light treatment were then stored in darkness for 24 hr before harvesting and analysis of tissue.

<sup>d</sup> Significantly less than corresponding light value ( $P < 0.05$ ).

**Table 4.** Chlorophyll Content in Wild-Type, *lut1*, and *lut2* Leaves<sup>a</sup>

Plant Line	Chl a	Chl b	Chl a/b
Wild type	1210	412	2.94
<i>lut1-1</i>	1010	354	2.86
<i>lut1-2</i>	1190	370	3.30
<i>lut2-1</i>	1120	385	2.91
<i>lut2-2</i>	970	283	3.41

<sup>a</sup>Chlorophyll content, expressed as micrograms per gram fresh weight of tissue, was measured from the same plants used for Table 1. There was no significant difference between the wild type and mutants for Chl a, Chl b, or the Chl a/b ratio. The population standard error for a significant difference ( $P > 0.05$ ) between means is 410 for Chl a, 143 for Chl b, and 0.87 for the Chl a/b ratio.

and the  $\epsilon$  cyclase gene, demonstrating tight linkage. Therefore, the *lut2* locus cosegregates with the  $\epsilon$  cyclase locus.

The  $\epsilon$  cyclase gene was mapped using the recombinant inbred lines (Lister and Dean, 1993) to the bottom of chromosome 5,  $\sim 6 \pm 4.7$  cM from markers m435 and g2368. Because the *lut2* and  $\epsilon$  cyclase loci are within 2 cM of each other, the *lut2* locus is also located between markers m435 and g2368. The *lut2* locus was also mapped by phenotypic markers to the bottom of chromosome 5 by showing linkage to *aba* ( $\chi^2$  of 4.3,  $P < 0.05$ ); in contrast, *lut1* segregated independently of *aba* ( $\chi^2$  of 0.02,  $P > 0.7$ ). These results confirm that *lut1* and *lut2* define two distinct loci.

## DISCUSSION

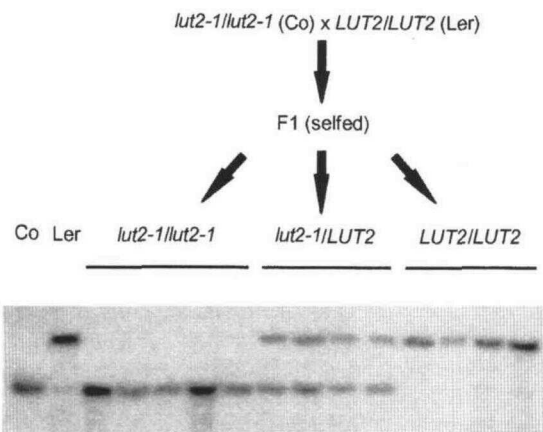
### Two Loci Required for Lutein Biosynthesis in Plants Are Defined by *lut1* and *lut2*

To further our understanding of xanthophyll biosynthesis and function in higher plants, we have isolated and characterized four *Arabidopsis* mutants impaired in their ability to accumulate lutein, the most abundant xanthophyll in plants. These mutants could be classified into two groups based on their biochemical phenotype: one group that accumulated a biosynthetic intermediate, zeinoxanthin, and a second group that did not accumulate intermediates (Figure 1 and Table 1). Allelism tests and linkage analysis confirmed the biochemical data defining two loci, *lut1* and *lut2*, that are required for lutein biosynthesis.

The *lut1* mutants have an 80 to 85% reduction in lutein and accumulate a carotenoid that is not normally present in wild type. This carotenoid was identified as zeinoxanthin ( $\beta, \epsilon$ -caroten-3-ol) by its chromatographic properties and UV visible and mass spectra (Figures 2, 4, and 5). This result is critical for understanding the nature of the *lut1* mutation. The only

difference between lutein and zeinoxanthin is the presence of a hydroxy group on carbon 3 of the  $\epsilon$  ring in lutein. The decrease in lutein and its partial replacement by its immediate precursor, zeinoxanthin, define *lut1* as a mutation disrupting  $\epsilon$  ring hydroxylation (Figure 1). The *lut1* mutations are specific for  $\epsilon$  ring hydroxylation and do not impede other carotenoid biosynthetic enzymes, including, most significantly,  $\beta$  ring hydroxylation. Thus, the *lut1* mutations genetically define a minimum of two hydroxylation enzymes in carotenoid biosynthesis: one is specific for hydroxylation of the  $\epsilon$  ring, and a second, unaffected by *lut1*, is specific for hydroxylation of the  $\beta$  ring.

In contrast to *lut1*, the *lut2* mutants do not accumulate any lutein precursors, such as zeinoxanthin,  $\alpha$ -carotene ( $\beta, \epsilon$ -carotene),  $\gamma$ -carotene ( $\beta, \psi$ -carotene), or lycopene (Figure 2 and Table 1). The synthesis of  $\beta, \beta$ -carotenoids is unimpeded by *lut2* mutations, so *lut2* is not a  $\beta$  cyclase or  $\beta$  ring hydroxylase mutation. Also, because it is not allelic with *lut1* and did not accumulate zeinoxanthin, the mutation does not affect  $\epsilon$  ring hydroxylation. Thus, *lut2* most likely affects  $\epsilon$  cyclization, the first committed step in the biosynthesis of lutein (Figure 1). To identify and characterize the  $\epsilon$  cyclase, a cDNA encoding this enzyme has been isolated from *Arabidopsis* (Cunningham et al., 1996). No recombinations between the  $\epsilon$  cyclase gene and the *lut2* locus were observed, thus demonstrating tight link-

**Figure 6.** The *lut2-1* Locus Cosegregates with the  $\epsilon$  Cyclase Locus.

*lut2-1/lut2-1* (ecotype Columbia [Co]) was crossed with *LUT2/LUT2* (ecotype Landsberg *erecta* [Ler]). Individual plants from the  $F_2$  population were characterized by lutein and violaxanthin content as *lut2-1/lut2-1*, *lut2-1/LUT2*, or *LUT2/LUT2*. DNA gel blots of ecotypes Columbia, Landsberg *erecta*, and the  $F_2$  population were probed with the  $\epsilon$  cyclase cDNA. An RFLP using *Hha*I resulted in 4- and 4.8-kb fragments for Columbia and Landsberg *erecta*, respectively. Fifty-two homozygous *lut2* plants were homozygous for the Columbia restriction fragment, 23 *lut2* heterozygotes were heterozygous for the  $\epsilon$  cyclase RFLP, and six wild type were homozygous for the Landsberg *erecta* restriction fragment, demonstrating tight linkage of the  $\epsilon$  cyclase and *lut2* loci. A sample of the population is shown.



age. Both this tight linkage and the biochemical phenotype of *lut2* strongly indicate that *lut2* disrupts the  $\epsilon$  cyclase gene.

### Regulation of Lutein Biosynthesis

The results of this study provide some insight into the organization and regulation of carotenoid biosynthesis in Arabidopsis. The generally held theory is that carotenoid biosynthetic enzymes are aggregated into multienzyme complexes and that there may be a complex specific for each of the accumulating carotenoids (Britton, 1993). The biosynthesis of all carotenoids consists of a series of common steps leading from geranylgeranyl pyrophosphate to lycopene. After lycopene, the pathway can be considered to be two series of reactions operating independently: one series for the  $\epsilon$  ring end group and the other for the  $\beta$  ring end group. In the biosynthesis of an asymmetrical carotenoid such as lutein, if an enzyme that specifically operates on one end group is blocked, the activities associated with the production of the second end group should not be affected. This is seen with the *lut1* mutants, in which the  $\epsilon$  ring hydroxylase is blocked so that a monohydroxycarotenoid, zeinoxanthin ( $\beta,\epsilon$ -caroten-3-ol), accumulates, as well as  $\beta,\beta$ -carotenoids. In the same way, blockage of the  $\epsilon$  cyclase might be expected to cause the accumulation of  $\gamma$ -carotene ( $\beta,\psi$ -carotene) or its hydroxylated derivative, rubixanthin, as has been observed when carotenogenic systems are treated with known cyclization inhibitors, such as nicotine or 2-(4-chlorophenylthio)triethylamine (Bramley, 1993). However, this was not observed with *lut2*: lutein was replaced not by monocyclic precursors but by increased amounts of  $\beta,\beta$ -carotenoids. Possible explanations for this are that (1) the  $\epsilon$  cyclase in *lut2* was replaced in the putative lutein-biosynthetic complex by a second  $\beta$  cyclase, so additional quantities of  $\beta,\beta$ -carotenoids are made, or (2) in the absence of the  $\epsilon$  cyclase, the lutein-biosynthetic complex is not active. In this case, the additional  $\beta,\beta$ -carotenoids produced in *lut2* would result from either increased flow through existing  $\beta,\beta$ -carotenoid-biosynthetic complexes or from additional  $\beta,\beta$ -carotenoid-biosynthetic complexes.

### Incorporation into Pigment-Protein Complexes

Despite the dramatic changes in carotenoid composition in *lut1* and *lut2*, the total quantity of carotenoids remains the same as in the wild type (Table 1). A similar observation was reported for the *aba* mutation, which disrupts the epoxidase enzyme of the pathway (Rock and Zeevart, 1991). These combined observations are significant in that they suggest there is no net alteration in carbon flow to carotenoids in mutants disrupted in either branch of xanthophyll synthesis. Also, the observations suggest that incorporation of specific carotenoids into pigment-protein complexes is an important factor regulating carotenoid composition in these mutants. The fact that the majority of the zeaxanthin and antheraxanthin present in *lut1* and *lut2* does not undergo epoxidation under optimal condi-

tions for epoxidase activity suggests that unlike wild type, most of these pigments in *lut1* and *lut2* are no longer in a location accessible to the epoxidase (Table 3). The stable accumulation of zeaxanthin and antheraxanthin in the *lut* mutants is not consistent with their normal transient accumulation during high-light stress in wild-type plants (Demmig-Adams and Adams, 1993). The capacity to epoxidize zeaxanthin and antheraxanthin to violaxanthin in *lut1* certainly has not been exceeded because the violaxanthin content in *lut1* is 30% less than that in *lut2* (Table 1). Thus, accumulation of certain carotenoids in *lut* mutants, such as the majority of the zeaxanthin and antheraxanthin pool, is most likely the result of their preferential incorporation into sites normally occupied by lutein, thereby making them relatively inaccessible to further processing.

The *aba* mutation does not affect lutein levels and only results in accumulation of the precursor of the epoxidase, zeaxanthin, at equimolar amounts in place of violaxanthin and neoxanthin (Rock and Zeevart, 1991). This contrasts with *lut1* mutants that do not replace lutein with equimolar quantities of its precursor zeinoxanthin but instead replace the majority of the lutein with  $\beta,\beta$ -carotenoids (Table 1). One explanation for this difference is that  $\beta,\beta$ -carotenoid synthesis exceeds incorporation in wild-type plants. In this scenario, the accumulation of  $\beta,\beta$ -carotenoids may be limited in wild-type plants by the number of sites available for incorporation, with lutein being preferentially incorporated and the unincorporated  $\beta,\beta$ -carotenoids being turned over. Thus, in the absence of lutein in *lut* mutants, there are more sites available for  $\beta,\beta$ -carotenoids, so more accumulate. Conversely, lutein biosynthetic capacity may be at a maximum in wild type and *aba*; thus, there is no additional lutein available to compete with zeaxanthin for incorporation into the sites normally occupied by violaxanthin and neoxanthin in the *aba* mutants.

The semidominant nature of the *lut2* mutations lends support to the argument that lutein biosynthesis is limited by  $\epsilon$  cyclase activity. Semidominance is consistent with an enzyme, such as the  $\epsilon$  cyclase, being rate limiting so that the loss of one copy of the gene critically reduces total enzyme activity. If  $\epsilon$  cyclase activity is critically reduced in the *lut2* heterozygotes, then less lutein would be produced and thus less would accumulate. In vitro,  $\epsilon$  cyclase is only able to cyclize one end of lycopene to form  $\delta$ -carotene ( $\epsilon,\psi$ -carotene), whereas the  $\beta$  cyclase can react in vitro with  $\delta$ -carotene and lycopene to produce  $\alpha$ -carotene and  $\beta$ -carotene, respectively (Cunningham et al., 1996). Thus, provided that the  $\beta$  cyclase is in excess, the relative proportions of lutein to  $\beta,\beta$ -carotenoids can be regulated solely by the  $\epsilon$  cyclase at the biosynthetic level and subsequently by differential incorporation into pigment-protein complexes.

### Function of Lutein and the Carotenoids That Accumulate in Its Absence

Perhaps the most striking and unexpected finding in this study is that the complete elimination of lutein has no obvious deleterious effect on growth and development of the plant or on

chlorophyll content or Chl *a*-to-Chl *b* ratio under conditions of moderate light. Lutein normally plays a critical role in photosystem assembly and photosynthesis. It accounts for up to 50% of the total carotenoids in most plants, is the only carotenoid localized in the crystal structure of plant LHCs, is required for LHC reconstitution *in vitro*, and is the only xanthophyll detected in the photosystem II core (Plumley and Schmidt, 1987; Cammarata et al., 1990; Paulsen et al., 1990; Bassi et al., 1993; Kuhlbrandt et al., 1994). Despite these facts, in *lut1* and *lut2*, an 80 to 100% depletion of this most predominant carotenoid had no pronounced effect on chlorophylls that would be expected of destabilized or altered photosystems. However, more detailed structural studies under different light regimes may reveal an *in vivo* requirement for lutein for optimal photosystem assembly, function, or stability.

The most reasonable explanation for the viability of the *lut1* and *lut2* plants in the absence of lutein is that some or all of the other carotenoids that accumulate in its absence can functionally complement lutein. At this stage, we do not know which carotenoids are being incorporated into sites normally occupied by lutein, but inferences about likely substitutions can be made. *In vitro* studies of LHC assembly by Plumley and Schmidt (1987) demonstrated that although less than optimal, violaxanthin and neoxanthin together could enable LHC assembly in the absence of lutein. As discussed above, it appears that violaxanthin and antheraxanthin may be preferentially incorporated over zeinoxanthin in *lut1*. There are no reports of zeinoxanthin accumulating in any plant or algal species, although another monohydroxycarotenoid,  $\beta$ -cryptoxanthin, is a minor pigment in some species (Young, 1993b).

Why should it be violaxanthin that preferentially substitutes for lutein in *lut* mutants and not zeaxanthin, the closest structural homolog to lutein? After lutein, violaxanthin is the next most abundant xanthophyll in plant light-harvesting antennae and, along with lutein, is presumed to augment light harvesting. Conversely and by inference, the accumulation of zeaxanthin during high-light stress, and the resulting reduction in chlorophyll fluorescence, implies a role in thermal energy dissipation (Demmig-Adams and Adams, 1993). These observations, together with the results for the *lut* mutants, suggest that violaxanthin (rather than zeaxanthin) may more readily functionally replace and sterically substitute for lutein in the LHC. Interestingly, in a small number of species, notably lettuce, ~30% of the lutein in the LHC is replaced by lactucaxanthin ( $\epsilon,\epsilon$ -carotene-3,3'-diol).

Because the amount of lutein is generally directly proportional to Chl *b* content and  $\beta$ -carotene is proportional to Chl *a*, one would expect that a decrease in lutein and an increase in  $\beta$ -carotene (as observed in *lut2*) would be reflected by alterations in Chl *a*-to-Chl *b* and reaction center-to-LHC ratios (Peter and Thornber, 1991; Bassi et al., 1993; Juhler et al., 1993). In fact, such a correlation was observed in a mutant of the alga *Scenedesmus obliquus* in which a decrease in lutein was accompanied by a reduction in loroxanthin and Chl *b* and the near absence of LHC (Bishop et al., 1995). Contrary to the

algal mutant, the change in lutein and  $\beta$ -carotene content in *lut2* did not result in a change in the Chl *a*-to-Chl *b* ratio or in neoxanthin levels, which in turn implies no change in reaction center-to-LHC ratio in the *lut* mutants. We cannot determine whether the *S. obliquus* mutant is a homolog of *lut2*, because the pleiotropic effects and technical difficulties inherent to *S. obliquus* make definition of the mutation difficult. The increase in  $\beta$ -carotene in *lut2* may reflect increased flux down that side of the pathway due to the block in  $\epsilon$  cyclization and a rate-limiting catalysis of  $\beta$ -carotene by the  $\beta$  ring hydroxylase. Alternatively, it may represent stable incorporation of  $\beta$ -carotene and other carotenoids in sites in the LHC normally occupied by lutein. At this stage, we have no reason to conclude that the apparent carotenoid substitutions in *lut* mutants have substantially impeded their ability to photosynthesize.

## Conclusions

We identified two novel carotenoid biosynthetic loci, *lut1* and *lut2*, in Arabidopsis. They define two essential and sequential steps required for the biosynthesis of  $\beta,\epsilon$ -carotenoids such as lutein but not required for the biosynthesis of  $\beta,\beta$ -carotenoids. The *lut* mutations demonstrate genetically a requirement for at least two hydroxylases and two cyclases in plants: one set (a cyclase and a hydroxylase) specific for the  $\epsilon$  ring and the other for the  $\beta$  ring. The *lut* mutants also provide some insight into the regulation of carotenoid biosynthesis and incorporation into the photosynthetic pigment-protein complexes, because the total quantity of carotenoids did not change; instead, lutein was replaced by compensating molar increases in specific carotenoids. The  $\epsilon$  cyclase appears to be the key enzyme in the assembly of a functional multienzyme complex for lutein biosynthesis. These mutants should be useful for detailed studies of the regulation of carotenoid biosynthesis, especially with regard to the relationship between the  $\beta,\beta$ -carotenoids and the  $\beta,\epsilon$ -carotenoids.

Finally, and quite unexpectedly, the absence of lutein in photosynthetic tissue had no readily detectable effect on growth, development, and chlorophyll content in the *lut* mutants, suggesting that other carotenoids can functionally compensate for lutein. Further investigations into the nature of the *lut* mutations and structural and photochemical analyses of the *lut* mutants and double mutants (such as a *lut2*  $\times$  *aba* double mutant) may provide important insight into the functions of specific carotenoids in photosynthesis and the regulation of the carotenoid composition of pigment-protein complexes.

## METHODS

### Screening of Mutants and Plant Tissue

Two ethyl methanesulfonate-mutagenized populations of *Arabidopsis thaliana* ecotype Columbia (Lehle Seeds, Round Rock, TX; R.L.

Last, Boyce Thompson Institute, Ithaca, NY) were screened for abnormal carotenoid accumulation by HPLC pigment analysis. More than 4000 individual lines were analyzed by extracting the pigments from leaf tissue of each plant. The plants were grown in soil under 16 hr per day of light of  $\sim 150 \mu\text{E m}^{-2} \text{sec}^{-1}$ . The tissue was harvested and extracted immediately or stored at  $-80^\circ\text{C}$  until extraction.

### Carotenoid and Chlorophyll Analysis and Quantification

Pigments were extracted in a microcentrifuge tube by grinding with a micropestle in 250  $\mu\text{L}$  of acetone-ethyl acetate (3:2 [v/v]). Water (200  $\mu\text{L}$ ) was added, the mixture was centrifuged, and the ethyl acetate upper phase was recovered. The extract was either stored at  $-20^\circ\text{C}$  or analyzed immediately by HPLC, as described by Norris et al. (1995). Alternatively, extracts were saponified in 100  $\mu\text{L}$  of 80% methanol containing 6% KOH and centrifuged; the pellet was then reground in 100% methanol containing 6% KOH. The supernatants were pooled and incubated for 1 hr at  $37^\circ\text{C}$ . Petroleum ether-diethyl ether (1:1 [v/v]; 200  $\mu\text{L}$ ) was added, followed by 400  $\mu\text{L}$  of  $\text{H}_2\text{O}$ . The solution was centrifuged, and the upper organic layer was recovered. The residual solution was reextracted with the petroleum-diethyl ether mixture. The recovered organic phases were pooled and washed three times with 400  $\mu\text{L}$  of  $\text{H}_2\text{O}$  followed by centrifugation and isolation of the organic phase, which was dried under nitrogen and resuspended in ethyl acetate for HPLC (Davies, 1976). Carotenoids were identified by comparing retention times and spectra with those of standards. Mass spectrometry of zeinoxanthin, lutein, and zeaxanthin was performed as described by Norris et al. (1995).

For quantification, 10 mg of tissue from 31-day-old plants was extracted, and half of this was loaded onto the HPLC. There was a minimum of three replicate plants per line. The means are presented in Table 1, and the deepoxidation state was determined from this data set. The plants were then stored for 24 hr in darkness, and the analysis was repeated using more tissue from the same plants for the determination of the dark treatment deepoxidation state. HPLC peak areas at 440 nm were integrated and calibrated to micrograms of carotenoid per unit area by loading known quantities of purified carotenoids (calculated by spectroscopy and published absorption coefficients) and preparing a standard curve for each carotenoid (Davies, 1976; Britton, 1995; Schiedt and Liaaen-Jensen, 1995). The correlation coefficient ( $R^2$ ) for the standard curve for each of the carotenoids ranged from 0.972 to 0.998, and the curve was linear from <20 to >500 ng per injection.

Chlorophyll content of leaves was quantitatively determined spectrophotometrically, with equations adjusted for a DU-40 spectrophotometer (Beckman Instruments, Fullerton, CA), as described by Lichtenthaler (1987). The tissue analyzed came from the same plants at the same age (31 days) as that used for determining carotenoid content.

Analyses of variance were undertaken with Excel 5.0 (Microsoft Corp., Redmond, WA). The determination of significant difference between means was calculated by using the Bayes  $k$  ratio least square significant difference (K-LSD) rule, which is a population standard error (Waller and Duncan, 1969).

### Genetic Analysis

To determine allelism, reciprocal crosses between homozygous mutants were performed and the  $F_1$  progeny were analyzed for lutein

content. To determine dominance, homozygous mutants were outcrossed with both ecotypes Columbia and Landsberg *erecta*. HPLC peak areas at 440 nm of carotenoid extracts from plants of  $F_1$  and  $F_2$  progeny aged 2 to 3 weeks were integrated. For the experiment shown in Figure 3, there were nine replicates per sample in each  $F_1$  population, and 100  $F_2$  individuals were analyzed for each of *lut1-1*  $\times$  Landsberg *erecta* and *lut2-1*  $\times$  Landsberg *erecta*. There was no difference between Columbia and Landsberg *erecta* ecotypes for the lutein-to-violaxanthin ratio, and there was no significant effect on the progeny if the wild-type parent was Columbia or Landsberg *erecta*. Only the progeny of mutants crossed with Landsberg *erecta* are shown. The ratios of carotenoids vary under different growth conditions and during development, so wild-type plants were always grown at the same time as a control.

Plants homozygous for the *lut2* mutation were crossed with Landsberg *erecta* plants, and cosegregation was determined by restriction fragment length polymorphism (RFLP) linkage analysis in  $F_2$  progeny with the  $\epsilon$  cyclase cDNA (Cunningham et al., 1996). DNA from Landsberg *erecta* and Columbia was digested with 60 different endonucleases, and an RFLP for the  $\epsilon$  cyclase cDNA probe was obtained by digestion with HhaI. Genomic DNA was purified by the modified miniprep method (DellaPorta et al., 1983) or, for larger scale preparations, by the macroprep method (Tai and Dahlbeck, 1993). DNA was fractionated on a 0.8% agarose gel and transferred to a nylon membrane (Micron Separations, Westborough, MA). Hybridizations were performed as described by Pogson et al. (1995), except that the probe,  $\epsilon$  cyclase cDNA, was prepared by using the random priming protocol of Gibco BRL, and the highest stringency wash was at  $65^\circ\text{C}$  in  $0.2 \times \text{SSC}$  ( $1 \times \text{SSC}$  is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS.

Mapping was undertaken for the  $\epsilon$  cyclase locus by using 18 recombinant inbred lines (Lister and Dean, 1993), and data were analyzed by using Map Manager version 2.6 (Kenneth F. Manly, Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY). For linkage analysis of *lut1*  $\times$  *aba* and *lut2*  $\times$  *aba*, 75 and 64  $F_2$  progeny were used, respectively.

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