Defining the primary route for lutein synthesis in plants: The role of Arabidopsis carotenoid β-ring hydroxylase CYP97A3

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Lutein, a dihydroxy derivative of β-carotene, is the most abundant carotenoid in photosynthetic plant tissues where it plays important roles in light-harvesting complex-I structure and function. The synthesis of lutein from lycopene requires at least four distinct enzymatic reactions: β- and ε-ring cyclizations and hydroxylation of each ring at the C-3 position. Three carotenoid hydroxylases have already been identified in Arabidopsis, two nonheme diiron β-ring monoxygenases (the B1 and B2 loci) that primarily catalyze hydroxylation of the β-ring of β,β-carotenoids and one heme-containing monoxygenase (CYP97C1, the LUT1 locus) that catalyzes hydroxylation of the ε-ring of β,ε-carotenoids. In this study, we demonstrate that Arabidopsis CYP97A3 (the LUT5 locus) encodes a fourth carotenoid hydroxylase with major in vivo activity toward the β-ring of α-carotene (β,ε-carotene) and minor activity on the β-rings of β-carotene (β,β-carotene). A CYP97A3-null allele, lut5-1, causes an accumulation of α-carotene at a level equivalent to β-carotene in wild type, which is stably incorporated into photosystems, and a 35% reduction in lut5-1 activity on the lut5-1 allele.

In vivo data suggest that carotene synthesis is subject to substrate channeling. These data suggest that e-ring double-bond is not in conjugation, and e-rings have relatively free rotation around the C6–C7 carbon. Unlike the ubiquitous β-ring, which is found in organisms as diverse as archaeobacteria and plants, e-rings occur exclusively in green plants (5–7), red algae (8), and one extant prochlorophyte (9, 10). Therefore, ε-ring formation and modifications should postdate that of β-rings in evolutionary time and would be expected to have evolved only in this subgroup of ε-ring carotenoid-containing organisms.

Lutein (3R,3′R-β,ε-carotene-3,3′-diol) is a dihydroxy β,ε-carotene and the most abundant carotenoid in plant photosynthetic tissues (e.g., accounting for >50% of the carotenoids in an Arabidopsis leaf). Lutein synthesis requires hydroxylation of C-3 of both the β- and ε-rings by the action of β-ring and ε-ring hydroxylases. Numerous studies have shown that a class of nonheme diiron monoxygenases (nonheme) that are present in most carotenoid-containing organisms (11) can efficiently hydroxylate the β-rings of several carotenoid substrates (12, 13). Arabidopsis contains two genes encoding nonheme β-ring hydroxylases (the B1 and B2 loci). Their primary in planta substrates are β-rings of β,β-carotenoids (Fig. 1) because a b1b2 double-null mutant reduced ββ-carotene-derived xanthophyll (ββ-xanthophyll) levels 80%, but the level of lutein (βε-xanthophyll) was slightly increased relative to the WT (14). These data suggest that ββ- and βε-xanthophyll synthesis operate relatively independently of each other; which presumably reflects the independent evolution of the two pathway branches in plants.

Recently, two heme-containing cytochrome P450 monoxygenases (P450) have been identified that define two new classes of carotenoid hydroxylases. The archetypal members are the Arabidopsis ε-ring hydroxylase (CYP97C1) encoded by the LUT1 locus (15) and the β-carotene hydroxylase (CYP175A1) of the eubacteria, Thermus thermophilus (16). Expression of T. thermophilus CYP175A1 in Escherichia coli engineered to produce β-carotene resulted in the production of zeaxanthin, a dihydroxy ββ-carotene, and is a clear example of convergent evolution in β-ring hydroxylases (the nonheme and P450 classes). In Arabidopsis, a T-DNA insertion mutant in the LUT1 gene accumulates zeaxanthin (βε-carotene with a hydroxylated β-ring) in place of lutein, consistent with LUT1 being the primary enzyme responsible for ε-ring hydroxylation in Arabi...
dopsis. In the triple carotenoid hydroxylase mutant, \textit{b1b2lut1}, \textit{b1b2lut1}/H9252/H9252, /H9252/H9252-xanthophylls are reduced 80\% relative to WT, but zeinoxanthin is still accumulated to high levels suggesting at least one additional carotene hydroxylase must exist in \textit{Arabidopsis} with activity toward the /H9252/H9252-rings of /H9252/H9252, /H9255/H9255-, and /H9252/H9252-carotenoids (14). In this article, we report that a second member of the \textit{Arabidopsis} CYP97 P450 family, CYP97A3, encodes a /H9252/H9252-ring hydroxylase with major activity toward the /H9252/H9252-ring of /H9252/H9252-carotene (\textit{H}20862), and, to a lesser extent, the /H9252/H9252-rings of /H9252/H9252-carotene.

Results

Mutation of \textit{Arabidopsis} CYP97A3 Causes Accumulation of High Levels of \textit{\alpha}-Carotene. Our initial search for a fourth \textit{Arabidopsis} carotenoid hydroxylase focused on members of the CYP97 clade because it already contains one carotenoid hydroxylase (CYP97C1, the \textit{LUT1} locus). Although all three \textit{Arabidopsis} CYP97 clade members are predicted to be targeted to the chloroplast by CHLOROP 1.1 (17), CYP97A3 (At1g31800) was selected because it has the highest (52\%) amino acid identity with CYP97C1. To determine whether loss of CYP97A3 activity affected the carotenoid composition in \textit{Arabidopsis} leaf tissue, 5-week-old leaves of two independent \textit{cyp97a3} mutant alleles were analyzed. \textit{lut5-1} contains a T-DNA insertion in the third exon of \textit{CYP97A3}, whereas \textit{lut5-2} contains a single amino acid change (E283K).

Fig. 2 shows the HPLC profiles of leaf extracts from WT, \textit{b1b2}, \textit{lut1-4}, and \textit{lut5-1}. All lines are in the Columbia-0 (Col-0)

<image>
Types (19, 20). The photosystems are the most likely place for complex comigrated and are well separated from light-and analyzed their pigment compositions (Table 2). Fig. 3 shows membranes from WT, is generally more severe than and elevated levels of anthin, violaxanthin, and lutein) and one carotene (β-carotene). Although the total carotenoid level is not significantly different (data not shown), and only Col-0 is shown for WT. WT accumulates four major carotenoids: three xanthophylls (neoxanthin, violaxanthin, and lutein) and one carotene (β-carotene). Table 1. Leaf tissue carotenoid composition of the indicated genotypes. Each value is the mean result of four experiments ± SD. Student’s t test for two samples. VAZ, the sum of violaxanthin, antheraxanthin, and zeaxanthin; tot.car, total carotenoids; Hβ, the moles of hydroxylated β-rings; βε/ββ, the molar ratio of total βε-carotenoids to total ββ-carotenoids. ND, not detectable. *P < 0.05.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>βε-carotene</th>
<th>ββ-carotene</th>
<th>VAZ</th>
<th>Neoxanthin</th>
<th>tot.car</th>
<th>Hβ</th>
<th>βε/ββ</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>127.0* (51)</td>
<td>0.7* (0.3)</td>
<td>55.4* (4.0)</td>
<td>22.1*</td>
<td>5.1 (13)</td>
<td>32.5* (3.8)</td>
<td>13</td>
</tr>
<tr>
<td>lut5-1</td>
<td>4.6* (14)</td>
<td>2.7* (8)</td>
<td>4.8* (15)</td>
<td>2.4* (9)</td>
<td>2.7* (11)</td>
<td>4.8* (14)</td>
<td>1.1</td>
</tr>
<tr>
<td>lut5-2</td>
<td>110.7* (18)</td>
<td>3.1* (13)</td>
<td>13.6* (24)</td>
<td>2.3* (9)</td>
<td>1.1* (11)</td>
<td>13.6* (24)</td>
<td>1.2</td>
</tr>
<tr>
<td>lut5-1l1l4</td>
<td>0.4* (0.2)</td>
<td>ND</td>
<td>52.8* (5.2)</td>
<td>53.9* (23)</td>
<td>37.9* (16)</td>
<td>69.6* (30)</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Carotenoids are expressed as millimoles of pigment per moles of chlorophyll a + b, with the relative molar percentage of each carotenoid given in parentheses. Each value is the mean result of four experiments ± SD. Student’s t test for two samples. VAZ, the sum of violaxanthin, antheraxanthin, and zeaxanthin; tot.car, total carotenoids; Hβ, the moles of hydroxylated β-rings; βε/ββ, the molar ratio of total βε-carotenoids to total ββ-carotenoids. ND, not detectable. *P < 0.05.

Effect of Mutating P450-Type Carotenoid Hydroxylase on the Expression of Other Carotenoid Biosynthetic Enzymes. As shown in Table 1, the ratio of total βε-carotenoids to ββ-carotenoids in lut5-1 was over twice that of WT, suggesting that β- and/or ε-cyclase activities might be affected in the mutant. Because it is not possible to directly assay carotenoid cyclase or hydroxylase activities in Arabidopsis leaf extracts, we determined the steady-state transcript levels of these genes to indirectly assess the effect of the lut5 and lut1 mutations on the pathway. As shown in Fig. 4, both cyclase mRNAs are modestly increased in lut1-4 and lut5-1 relative to WT, the β-cyclase more so than the ε-cyclase, but this increase appears unrelated to changes in the βε- to ββ-carotenoid ratios in the mutants. We also quantified mRNAs...
for the four known carotenoid hydroxylase genes (B1, B2, LUT1, and LUT5) to determine whether altered gene expression plays a role in compensating for the absence of P450-type carotenoid hydroxylases in the mutants. The detection of each P450 gene transcript in the corresponding gene-knockout mutant was possible because the real-time probe is positioned upstream of each T-DNA insertion site. With the exception of a slight increase in LUT5 mRNA levels, the expression of other carotene hydroxylases is not affected in the lut1-4 mutant. In contrast, B1, B2, and LUT1 mRNAs were all up-regulated in lut5-1 and lut5-1lut1-4, most notably LUT1 mRNA, which was >4-fold higher than WT.

**Discussion**

The current study and prior work (12, 13, 18) have defined a minimum of four carotenoid hydroxylase genes involved in xanthophyll biosynthesis in Arabidopsis: two nonheme β-ring hydroxylases (encoded by the B1 and B2 loci), a P450-type e-ring hydroxylase (CYP97C1, encoded by the LUT1 locus), and now a P450-type β-ring hydroxylase (CYP97A3, encoded by the LUT5 locus). Analyses of mutant defective in one or more carotenoid hydroxylase activities (Table 1) (14) provide insight into the specific and overlapping activities of each enzyme in vivo. All mutant genotypes exhibit specific alterations in xanthophyll and carotene compositions (Table 1) (14), indicating the loss of any single activity cannot be fully compensated by the remaining activities. The degree of compensation observed in a given mutant genotype reflects the preferential activity of the missing enzyme(s) and the regulation and substrate specificities of the remaining active enzymes in the mutant. For example, LUT1 is the primary e-ring hydroxylase activity in Arabidopsis because lutein synthesis is nearly completely blocked in the null lut1-3 (15) and lut1-4 (Fig. 2) alleles and the presumed LUT1 monohydroxy substrate, zeaxanthin, accumulates. Similarly, disruption of both nonheme β-ring hydroxylases in the b1b2 double-null mutant reduces β,β-xanthophylls 76% without affecting β-ring hydroxylation in lutein synthesis, suggesting that the β-rings of β,β-carotenoids are the preferred in planta substrates for B1 and B2. LUT5 is a P450 β-ring hydroxylase with activity toward the β-rings of both α-carotene and β-carotene in vivo. The major effect of the null lut5-1 mutation is an accumulation of α-carotene at a level equivalent to β-carotene in WT, consistent with the β-ring of α-carotene being a preferred LUT5 substrate in planta. Because lutein is only reduced 18% in lut5-1 relative to WT, at least one of the other carotene hydroxylases must also be able to catalyze hydroxylation of the β-ring of α-carotene, although less efficiently than LUT5. B1 and B2 are clearly able to hydroxylate the β-ring of α-carotene as in the lut5-1lut1-4 double mutant zeaxanthin is still present at levels similar to lut1-4. LUT1 may also have some level of β-ring hydroxylase activity in vivo because LUT1 expression is strongly up-regulated in the lut5-1 background, as one might expect for a compensating enzyme (Fig. 4), and the level of total hydroxylated β-rings is further reduced in the b1b2lut1-3 mutant relative to the b1b2 mutant (Table 1) (15).

LUT5 also appears to have a minor in vivo activity toward the β-rings of β-carotene as β,β-xanthophylls (primarily violaxanthin and neoxanthin) are reduced 35% in lut5-1 versus a 76% reduction in the b1b2 genotype (Table 1). Although LUT5 is likely to be responsible for synthesis of at least a portion of the β,β-xanthophylls present in b1b2, contributions from LUT1, an additional uncharacterized hydroxylase activity, or indirect effects resulting from the elevated levels of α-carotene produced in lut5-1 can also not be excluded. Unfortunately, attempts to assay LUT1 and LUT5 in vitro by heterologous expression in Saccharomyces cerevisiae and E. coli and in vivo in E. coli engineered to accumulate carotenoid substrates have not been successful (data not shown), and therefore, we cannot directly assay potential substrates for the two enzymes. The generation of triple mutant genotypes in which only one of the four carotenoid hydroxylase activities remains would be informative in this regard.

**A Model for Lutein Biosynthesis and Regulation in Plants.** As shown in Fig. 1, there are several potential biosynthetic routes leading from lycopene to lutein that have only partially been delineated by prior genetic studies in Arabidopsis (18, 24, 25). The reactions leading to lutein must be highly efficient or tightly associated because many of the potential pathway intermediates in WT Arabidopsis leaf tissue are near or below the 1-ng carotenoid HPLC detection limit (Table 1). The activities of Arabidopsis β- and e-cyclases expressed in lycopene producing E. coli suggested that the preferential substrate for the e-cyclase is lycopene rather than γ-carotene (24). Consistent with this, mutation of the Arabidopsis LUT2 locus (encoding the e-cyclase) eliminates

**Table 2. Carotenoid composition in photosystems (PS I holocomplex and PS II core complex) of the indicated genotypes**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lutein</th>
<th>α-Cryptoxanthin</th>
<th>Zeaxanthin</th>
<th>α-Carotene</th>
<th>β-Carotene</th>
<th>VAZ</th>
<th>Neoxanthin</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>54.0 ± 8.1 (28)</td>
<td>ND</td>
<td>ND</td>
<td>1.9 ± 0.3 (1)</td>
<td>112.6 ± 7.7 (59)</td>
<td>26.1 ± 3.1 (12)</td>
<td>0.8 ± 0.4 (0.6)</td>
</tr>
<tr>
<td>lut1-4</td>
<td>1.3 ± 1.6* (1.7)</td>
<td>ND</td>
<td>14.6 ± 0.9* (12)</td>
<td>1.3 ± 0.5 (1)</td>
<td>86.1 ± 2.9* (68)</td>
<td>21.6 ± 1.2 (17)</td>
<td>0.5 ± 1.1* (0.5)</td>
</tr>
<tr>
<td>lut5-1</td>
<td>51.4 ± 3.3 (26)</td>
<td>1.0 ± 1.1 (0.5)</td>
<td>ND</td>
<td>77.3 ± 5.8* (39)</td>
<td>47.7 ± 1.7* (24)</td>
<td>17.9 ± 1.2 (9.1)</td>
<td>0.6 ± 1.1* (0.5)</td>
</tr>
</tbody>
</table>

The amount of carotenoid is expressed as millimoles of pigment per moles of chlorophyll a. Each value is the mean result from three experiments ± SD, with the relative molar percentage of each carotenoid given in parentheses. Student’s t test for two samples; ND, not detectable; VAZ, the sum of violaxanthin, antheraxanthin, and zeaxanthin. *, P < 0.05.
lutein synthesis without causing accumulation of the pathway intermediates rubixanthin or \( \gamma \)-carotene (18). These data indicate that \( \varepsilon \)-ring cyclization of lycopene to produce \( \delta \)-carotene is the first step in lutein synthesis. \( \delta \)-Carotene is undetectable in WT \textit{Arabidopsis} leaf tissue, but \( \alpha \)-carotene is detectable suggesting that the \( \delta \)-carotene produced by LUT2 is efficiently utilized by the \( \beta \)-cyclase. The isolation and characterization of mutations in the \textit{LUT5} and \textit{LUT1} loci now allow us to infer the primary reaction sequence for lutein synthesis in plant tissues from among the remaining possible steps in Fig. 1.

The pathway shown in Fig. 1 has two possible routes leading to lutein from \( \alpha \)-carotene: \( \beta \)-ring hydroxylation to zeinoxanthin followed by \( \varepsilon \)-ring hydroxylation to lutein or \( \varepsilon \)-ring hydroxylation to \( \alpha \)-cryptoxanthin followed by \( \beta \)-ring hydroxylation to lutein. Whether one pathway is favored or both occur depends on the substrate specificities and regulation of the hydroxylases involved, LUT5 (CYP97A3) and LUT1 (CYP97C1). Accumulation of zeinoxanthin in \textit{lut1} while the \( \alpha \)-carotene level remains identical to that in WT is consistent with zeinoxanthin, rather than \( \alpha \)-carotene, being a preferred substrate for \( \varepsilon \)-ring hydroxylation by LUT1. If \( \alpha \)-carotene were a preferred LUT1 substrate, the \textit{lut5} mutant (which still has LUT1 activity) would be expected to accumulate significant amounts of \( \alpha \)-cryptoxanthin, which it does not. \textit{lut5} mut1 accumulates an \( \alpha \)-carotene level 10 times that of \( \alpha \)-cryptoxanthin, which is consistent with \( \alpha \)-carotene being a preferred substrate for \( \beta \)-ring hydroxylation by LUT5 and LUT1 having, at best, a minor activity toward the \( \varepsilon \)-ring of \( \alpha \)-carotene. Biochemical regulation (e.g., feedback inhibition by \( \alpha \)-cryptoxanthin) may also contribute to the \textit{lut5-1} phenotype, but this hypothesis seems less likely because the \textit{lut5-lut1-4} double mutant, which cannot synthesize \( \alpha \)-cryptoxanthin, has a phenotype that is essentially the combination of the \textit{lut1} and \textit{lut5} single mutants. If \( \alpha \)-cryptoxanthin played a regulatory role, one would expect a different phenotype when the compound was removed in the double mutant. Together, the data presented are consistent with the preferred pathway for lutein synthesis being two \( \varepsilon \)-ring cyclizations to yield \( \alpha \)-carotene and then proceeding to zeinoxanthin and lutein by the sequential action of LUT5 and LUT1.

The general reaction sequence for lutein synthesis, ring cyclizations followed by hydroxylations, is the same as that of its closest structural isomer, zeaxanthin, a dihydroxy \( \beta \)-carotene derivative. However, zeaxanthin is produced primarily by the action of the nonheme B1 and B2 \( \beta \)-ring hydroxylases, whereas lutein is produced primarily by the action of P450-mediated \( \alpha \)-carotene ring hydroxylases, and the regulatory mechanisms of these two classes of enzymes appear to differ significantly. In lutein synthesis the bacterial intermediate \( \alpha \)-carotene is barely detectable in WT, whereas the corresponding intermediate in zeaxanthin synthesis, \( \beta \)-carotene, is 22% of total WT leaf carotenoids. Accumulation of high levels of \( \alpha \)-carotene and zeinoxanthin in the \textit{lut5} and \textit{lut1} mutants excludes the possibility that these intermediates do not accumulate in WT simply because they are unstable \textit{in vivo}. The high level of \( \alpha \)-carotene in \textit{lut5} is especially intriguing because it suggests that the presence of LUT5 is specifically required for efficient lutein synthesis and that the two nonheme \( \beta \)-ring hydroxylases, B1 and B2, cannot substitute for LUT5 in this regard.

Why Would Two Members of the P450 Class of Carotenoid Hydroxylases Be Specifically Required for Efficient Lutein Biosynthesis \textit{in Vivo}?

We propose that in WT the reaction sequence from lycopene to lutein is catalyzed by a protein complex composed of two lycopene cyclases (the \( \beta \) and \( \varepsilon \)-cyclases) and two P450 class hydroxylases (CYP97A3 and CYP97C1) that allows channeling of substrates between reactions. There is precedent for this finding because cytochrome P450 enzymes are known to form dimers (26, 27), functionally interact with other cytochrome P450 enzymes (28–30), or act as anchors for soluble and membrane biosynthetic complexes (31) in other systems. Given the clear importance of lutein in LHC structure and photosystem function (20, 32, 33), it is relatively easy to rationalize a strong selective pressure for the evolution of \( \varepsilon \)-ring cyclization and hydroxylation activities for lutein synthesis. However, the forces driving the evolution of different biochemical machinery (P450 and nonheme carotenoid hydroxylases) for hydroxylation of \( \alpha \)-carotene and \( \beta \)-carotene are less obvious. Perhaps the answer lies in the need to efficiently synthesize lutein for LHC structure and function while simultaneously tightly controlling \( \alpha \)-carotene production, due to the potential negative consequences of producing \( \alpha \)-carotene-containing photosystems.

Plants produce \( \beta \)-carotene-containing photosystems almost exclusively under most environmental conditions (e.g., high light), \( \alpha \)-Carotene-containing photosystems are also produced in many plant genera but generally only in shade-grown or low-light-adapted plants, where \( \alpha \)-carotene can be present in excess of \( \beta \)-carotene (34–36). Presumably \( \alpha \)-carotene-containing photosystems provide a competitive advantage under low-light conditions, but at higher light levels, such photosystems show increased photooxidation, \( \alpha \)-carotene levels decrease, and \( \beta \)-carotene-containing photosystems predominate (37). These data suggest tight control of the \( \alpha / \beta \)-carotene ratio is an important adaptive response to low and high light (38, 39). Consistent with this hypothesis, the constitutive production of \( \alpha \)-carotene in \textit{lut5} renders the mutant much more sensitive than WT to high-light exposure (see Fig. 6, which is published as supporting information on the PNAS web site). LUT5 clearly plays a key role in carotenoid synthesis by allowing efficient lutein production while limiting \( \alpha \)-carotene accumulation and provides a straightforward biochemical mechanism for producing \( \alpha \)-carotene and lutein when both are needed under low-light conditions; this mechanism is by regulation of CYP97A3.

When taken together, the current genetic data in \textit{Arabidopsis} is consistent with \textit{in vivo} synthesis of the two major groups of xanthophylls in plants being preferentially catalyzed by two different classes of carotene hydroxylases: P450-type enzymes for synthesis of lutein and nonheme enzymes for synthesis of \( \beta \),\( \beta \)-xanthophylls. The evolution of what at first appears to be an unnecessarily complex system can be understood when considering the contrasting needs of plants in response to changing light conditions. In high (normal) light, it is competitively advantageous to produce lutein without \( \alpha \)-carotene, whereas under low-light conditions, it is advantageous to produce both lutein and \( \alpha \)-carotene. \( \beta \)-Carotene and \( \beta / \beta \)-xanthophylls are needed at different levels and ratios under these conditions. Thus, the P450 carotenoid hydroxylases may be best suited to fulfilling these contrasting demands by allowing formation of a separate biosynthetic complex for efficient metabolic channeling to lutein while providing a regulatory mechanism for \( \alpha \)-carotene production that is independent of the synthesis and regulation of \( \beta \)-carotene and \( \beta / \beta \)-xanthophylls. Such independent regulation of the two branches of the carotenoid pathway allows plants to respond efficiently, effectively, and adaptively to ever-changing light conditions.

Materials and Methods

Plant Material.

Plants were grown under a 12-h photoperiod (100–120 \( \mu \)mol m\(^{-2}\) \( \text{s}^{-1} \); 22°C) and 18°C at night. \textit{lut5-1} and \textit{lut5-1 lut1-4} are T-DNA-knockout mutant alleles of \textit{CYP97C1} (At3g53130) and \textit{CYP97A3} (At1g31800), respectively. \textit{lut5-2} was identified by HPLC screening of nine missense mutant alleles isolated by targeting induced local lesions in genomes (TILLING) screening (40). The \textit{lut5-1 lut1-4} double mutant was selected by PCR screening of \( F_{2} \) progeny from a cross of \textit{lut5-1} and \textit{lut1-4}. HPLC separation, identification, and quantification by spectra and retention time were performed as described in ref. 12, except that quantification of monohydroxy \( \alpha \)-carotenones was performed at 475 nm.
**TaqMan Real-Time PCR Assay.** The transcript levels of six different carotenoid biosynthetic genes (β-cyclase, s-cyclase, B1, B2, LUT1, and LUT5) were quantified by TaqMan real-time PCR by using elongation factor-1α mRNA levels for normalization. The LUT3 primers and TaqMan probe are as follows: 5'-GTTT-GATGGACGTGTTCTGACC-3' (forward primer), 5'-TTC-CGGACGCCGCTGAAT-3' (reverse primer), and 5'-ACCCC-AAGGTTCTCTAGGCTAAAGGT-3' (TaqMan probe). Primers and probes for other genes are described in ref. 14. The relative quantity of the transcripts was calculated by using the comparative threshold cycle (Ct) method (41).

**Isolation of Thylakoid Membranes and Nondenaturing PAGE.** Thylakoid membranes from Col-0, lut1-4, and lut5-1 were isolated, and the photosystems and peripheral LHCs were separated by non-denaturing PAGE as described in ref. 12. Individual pigment-containing bands were excised and homogenized in gel running buffer (19), and the pigments were extracted and analyzed as described in ref. 12.


A α-cryptoxanthin (13.75 min)

B zeinoxanthin (13.78 min)

C α-cryptoxanthin

D zeinoxanthin

E lutein (10.81 min)

F zeaxanthin (11.12 min)