The Arabidopsis szl1 Mutant Reveals a Critical Role of β-Carotene in Photosystem I Photoprotection

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Carotenoids are polyisoprenoid pigments that are ubiquitously distributed among oxygenic photosynthetic organisms, from cyanobacteria to land plants (Britton et al., 2004). A molecular feature of these $C_{40}$ molecules is a conjugated double-bond system, which is responsible for the strong absorption in the visible region of the spectrum and the antioxidant capacity of these pigments. In photosynthetic tissues of higher plants, carotenoids are mainly accumulated in the thylakoid membranes. Carotenoid composition is remarkably conserved among plant taxa, consisting of the hydrocarbons $\alpha$- and $\beta$-carotene (accounting for one-fourth of total carotenoids) and their oxygenated derivatives called xanthophylls. The latter group include the $\beta$-$\beta$-xanthophyll lutein, the most abundant plant carotenoid, and the $\beta$-$\beta$-xanthophylls violaxanthin, neoxanthin, antheraxanthin, and zeaxanthin (Demmig-Adams and Adams, 1992), whose biosynthesis is tightly controlled during plant acclimation to stressful conditions (Hirschberg, 2001; Alboresi et al., 2011). The carotenoid biosynthesis pathway from phytoene include a series of four desaturation reactions, leading to the formation of the $C_{40}$ compound lycopene, which is then cyclized at both ends by $\beta$-cyclase (LCYB) to produce $\beta$-carotene. Alternatively, $\beta$-cyclization occurs at a single end, the other being processed by $\epsilon$-cyclase (LUT2) to produce $\alpha$-carotene. Thus, there exist two distinct branches in plant carotenoid biosynthesis, one leading to synthesis of $\beta$-$\beta$-hydroxylated xanthophylls from $\beta$-carotene, and the other to lutein from $\alpha$-carotene. The hydroxylation of either $\alpha$- or $\beta$-carotene is catalyzed by multiple enzymes with overlapping substrate specificity belonging to two different classes: CHY1 and CHY2 (ferredoxin-dependent diiron oxygenases) catalyze $\beta$-ring hydroxylation, while LUT1 and LUT5 (cytochromes P450) are involved in the hydroxylation of both $\alpha$-ring and $\beta$-ring of $\alpha$-carotene (Tian et al., 2003, 2004; Fiore et al., 2006; Kim and DellaPenna, 2006; Kim et al., 2009). Complete lack of xanthophylls in the
The chy1chy2lut1lut5 mutant (Kim et al., 2009) confirms that these four genes encode the complete enzymatic complement catalyzing carotene hydroxylation in Arabidopsis (Arabidopsis thaliana).

The distribution of each carotenoid species between the different components of the photosynthetic machinery is the basis for their specific functions. Thus, a minor fraction is free in the lipid phase of thylakoids where it serves as antioxidant (Havaux et al., 2004) and modulates the fluidity of the lipid bilayer (Gruszeczki and Szczałka, 2005). However, carotenoids are located mostly within specific binding sites of pigment-protein complexes, contributing to both light harvesting and photoprotection of these PS subunits. β-Carotene is bound to reaction center subunits of both PSI and PSII, whereas xanthophylls are bound to peripheral light-harvesting complexes (Lhc) subunits that comprise the antenna system. Core complexes of PSI and PSII bind, respectively, 15 and 11 β-carotenes (Amunts et al., 2010; Umena et al., 2011). Lhc proteins, constituting the antenna system of PSII, bind lutein, violaxanthin, and neoxanthin at four distinct binding sites (Liu et al., 2004). Zeaxanthin, upon its synthesis from violaxanthin under excess light, can also bind to these antenna components in exchange for violaxanthin, in site V1 in the case of the major LHCCI trimeric complex (Caffarri et al., 2001) or in site L2 in the case of the monomeric subunits CP26 (Lhcb5), CP29 (Lhcb4), and CP24 (Lhcb6; Croce et al., 2003; Betterle et al., 2010; Pan et al., 2011). PSI antenna proteins (Lhca1–4) and CP24 lack neoxanthin (Jensen et al., 2007; Passarini et al., 2009).

Besides their role as structural determinants, carotenoids are involved in photoprotective mechanisms. Indeed, they have coevolved with oxygenic photosynthesis to avoid photooxidative damage derived from photosensitizing action of porphyrins and reduction of oxygen by univalent photosynthetic electron transporters. This is particularly important under rapid fluctuations in light intensity, when photochemical quenching activity is exceeded, leading to photoinhibition (Külheim et al., 2002). Carotenoids protect chloroplasts from excess light by (1) modulating the nonradiative dissipation of excess excitation energy (Niyogi et al., 1998; Dall’Osto et al., 2005), and (2) by mediating direct quenching of chlorophyll (Chl) triplets (3Chl*) or (3) by scavenging the reactive oxygen species (ROS) generated during photosynthesis (Niyogi, 2000; Dall’Osto et al., 2005, 2007b; Havaux et al., 2007). The essential role of carotenoids in photoprotection was evidenced by the phenotype of carotenoid-less plants, unable to perform photoautotrophic growth (Herrin et al., 1992; Trebst and Depka, 1997; Kim et al., 2009).

The roles of xanthophylls have been subjected to dissection through genetic analysis. Several mutants with altered xanthophyll composition are impaired in photoprotection, implying that these pigments have a key role in plant fitness. Lutein depletion in lut2 plants resulted in higher photosensitivity in high light (HL) with respect to the wild type (Pogson et al., 1996), due to impaired 3Chl* quenching within LHCII (Dall’Osto et al., 2006). Lack of both lutein and zeaxanthin further enhances the photodamage in both HL-treated plants and green algae (Niyogi et al., 1997, 2001; Gilmore, 2001; Baroli et al., 2003; Dall’Osto et al., 2006). Mutation of three β-carotene hydroxylases CHY1, CHY2, and LUT5 in Arabidopsis, yielded a plant with lutein as the only xanthophyll, revealing unprecedented photosensitivity (Fiore et al., 2006; Dall’Osto et al., 2007b; Kim et al., 2009) and implying that singlet oxygen (1O2) scavenging is a constitutive component of photoprotection in antenna proteins together with Chl triplet quenching. Finally, a neoxanthin-less mutant showed enhanced sensitivity to superoxide anion (Dall’Osto et al., 2007a).

While xanthophyll biosynthesis mutants of Arabidopsis and Chlamydomonas have revealed distinct photoprotective roles in vivo for xanthophyll species, until recently no photoautotrophic mutant showing a selective β-carotene loss has been described, to our knowledge. Therefore, the role of β-carotene has been more difficult to identify. Early reports showed that isolated PSI reaction centers form 1O2 with high yield, thus causing photooxidation of P680 and other Chls (Barber et al., 1987; Telfer et al., 1994b). Indeed, the primary event upon PSI photoinhibition is the damage to the D1 subunit (Aro et al., 1993), and restoration of photosynthetic electron transport requires degradation and de novo synthesis of this subunit. These observations are consistent with the idea that the PSI reaction centers are a major source of 1O2 within the chloroplasts (Krieger-Liszkay, 2005; Telfer, 2005), and led to the conclusion, although indirect, that β-carotene ligands in the core complex have a special role in scavenging 1O2 (Telfer et al., 1994a; Telfer, 2005).

Recently, the Arabidopsis mutant sz11 was identified (Li et al., 2009) that carries a point mutation of LCYB gene, and thus exhibits a less-active lycopene β-cyclase with respect to the wild type. Due to the activity of the four carotene hydroxylase enzymes that catalyze the subsequent reactions leading to xanthophyll synthesis, a depletion in carotene with respect to wild-type plants is produced (Li et al., 2009), offering the opportunity of specifically probing carotene function in vivo in the presence of xanthophylls.

In this work, we have addressed the question of the function for the carotene ligands of both PS, and their importance in the photoprotection of the chloroplast. To this aim, we have compared a panel of Arabidopsis mutants affected in the biosynthesis of either xanthophylls or carotenoids, and analyzed the effect of their depletion on the photodamage of chloroplast in vivo. We show that, although the sz11 mutation does not affect photosynthetic electron transport rate (ETR), mutant plants show a higher sensitivity to photooxidative stress with respect to the wild type when exposed to HL at low temperature (8°C). Interestingly, sz11 plants revealed stronger photoinhibition of PSI compared with PSII. These findings imply that β-carotene ligands of PSI have a crucial role in
the photoprotection of the complex, especially in low-temperature conditions.

RESULTS

The szl1 Mutant of Arabidopsis Has Lower Carotene and Higher Xanthophyll Content Than Wild-Type Plants

The szl1 mutant (Li et al., 2009) showed, under our growth conditions (100 μmol photons m$^{-2}$ s$^{-1}$, 8 h light, 23°C/16 h dark, 20°C), similar leaf morphology and development rate with respect to wild-type plants. Pigment content of leaves from both genotypes was analyzed through diode array HPLC of leaf acetone extracts (Table I). We tested dark-adapted plants, a condition in which wild-type leaves accumulate violaxanthin, and plants transferred for 60 min under HL (550 μmol photons m$^{-2}$ s$^{-1}$), a condition in which violaxanthin is largely deepoxidized into zeaxanthin. Chl content, Chl a/b, and Chl/Car ratios were essentially the same in both genotypes (Table I), whereas β-carotene content was 60% lower in szl1 leaves (Table II), as reported previously (Li et al., 2009). The szl1 mutant showed a slight accumulation of α-carotene (a lutein precursor normally found in small amount in wild-type plants), a lower content in β,β-xanthophylls, and a higher content in lutein, thus a far lower β,β/ε,ε-xanthophylls ratio than in the wild type. When plants were exposed to HL, deepoxidation was 30% lower in szl1 with respect to the wild type (Table II).

The aim of this work was to address the function for carotene molecules bound to PS, and their relative importance in the photoprotection of the chloroplast. However, previous characterization of xanthophyll biosynthesis mutants (Dall’Osto et al., 2007b; Kim et al., 2009) clearly showed that xanthophyll composition of the LHCs also affects photoprotection. In particular, distinct roles were identified for β,β- and ε,ε-xanthophylls. Relevant to this study, depletion of β,β-xanthophylls increased photosensitivity (Dall’Osto et al., 2007b). Since szl1 plants, besides a lower carotene content, also have a lower β,β/ε,ε-xanthophylls ratio than the wild type, it is important to distinguish the effect of carotene depletion in core complexes from the increased lutein to β,β-xanthophyll ratio in the antenna moiety of the PS. Therefore, we included the chy1chy2 and lut5 genotypes in this characterization as controls (Tables I and II). The chy1chy2 double mutant has a reduced conversion of β-carotene into β,β-xanthophylls, yielding the same β,β/ε,ε-xanthophylls ratio as the szl1 plants. In addition, we analyzed the lut5 genotype as a control with respect to the α-carotene accumulation. In fact, α-carotene competes with β-carotene binding sites on pigment-protein complexes (Dall’Osto et al., 2007b), and thus it might be responsible, in part, for changes in photoprotection activity. chy1chy2 and lut5 plants showed leaf Chl content and Chl a/b ratio identical to wild-type plants. Xanthophyll content per Chl was significantly lower than in the wild type (Table I), as expected from previous reports (Fiore et al., 2006; Kim et al., 2009).

| Genotypes | Chls/cm$^2$ | Chl a/b | Chls/Cars | Xanthophyll/Cars | Carotene/Cars | F$\text{m}$ | F$\text{m}$ | F$\text{m}$/F$\text{m}$ | T$\text{2/3}$ | μg |
|-----------|------------|--------|----------|-----------------|---------------|--------|--------|-----------------|--------|
| WT        | 20.8 ± 1.1$^a$ | 3.0 ± 0.1$^a$ | 3.7 ± 0.2$^b$ | 0.7 ± 0.01$^a$ | 0.3 ± 0.01$^a$ | 388 ± 42$^a$ | 2136 ± 149$^a$ | 0.82 ± 0.01$^a$ | 4.3 ± 0.4$^a$ | 10$^3$, ms$^{-1}$ |
| szl1      | 17.5 ± 1.6$^b$ | 2.9 ± 0.1$^b$ | 3.5 ± 0.1$^b$ | 0.9 ± 0.01$^b$ | 0.1 ± 0.01$^b$ | 515 ± 39$^b$ | 1858 ± 165$^b$ | 0.72 ± 0.01$^b$ | 4.6 ± 0.4$^b$ |
| chy1chy2  | 20.2 ± 1.9$^c$ | 2.9 ± 0.1$^c$ | 4.0 ± 0.2$^c$ | 0.7 ± 0.01$^c$ | 0.3 ± 0.01$^c$ | 348 ± 35$^c$ | 1766 ± 173$^c$ | 0.80 ± 0.01$^c$ | 3.7 ± 0.4$^c$ |
| lut5      | 19.4 ± 3.5$^a$ | 3.0 ± 0.2$^a$ | 4.1 ± 0.2$^a$ | 0.6 ± 0.01$^a$ | 0.4 ± 0.02$^a$ | 388 ± 16$^a$ | 1883 ± 131$^a$ | 0.82 ± 0.01$^a$ | 3.1 ± 0.3$^a$ |

Table I. Pigment content and Chl fluorescence induction parameters

Measurements were done on dark-adapted leaves of Arabidopsis wild type and mutants szl1, chy1chy2, and lut5. Data are expressed as mean ± SD (n ≥ 4). Chls, Total chlorophylls; Cars, total carotenoids; $T_{2/3}$, time corresponding to two-thirds of the induction fluorescence rise in DCMU-treated leaves; a/u., arbitrary units. Values marked with the same letters are not significantly different from each other within a column (Student’s t test, P < 0.05).

Organization and Stoichiometry of Chl-Binding Proteins

The organization of pigment-protein complexes in wild-type and mutant genotypes was analyzed by non-denaturing Deriphat-PAGE (Fig. 1). In agreement with a previous report (de Bianchi et al., 2008), seven major green bands were resolved upon solubilization of thylakoid membranes with 0.8% dodecyl-α-ω-maltoside (α-DM). The uppermost band contained the PSII-LHCII supercomplex whose dissociation into components yielded the PSII core and the antenna moieties, namely CP29-CP24-(LHCII)$_1$ supercomplex (Bassi and Dainese, 1992), trimeric LHCII, and monomeric Lhcb. The major green band just below the PSII supercomplex contained the PSI-LHCI supercomplex, which, different from PSI, is stable and does not yield dissociation products upon mild solubilization of wild-type thylakoids. Finally, the lowest band was composed of free pigments that dissociated during solubilization, mainly carotenoids. The distribution of Chl between PSI-LHCl, PSI core, and Lhcb components was determined from the densitometric analysis of the Deriphat-PAGE patterns. In szl1, the PSI-LHCI complex relative abundance was reduced versus wild-type thylakoids (−27%). Consistently, a higher PSI core/PSI-LHCI ratio was found in szl1 (0.61) with respect to the wild type (0.41). Minor differences were observed in the Lhcb/PSI core ratio, which was slightly lower (approximately 4.0) in chy1chy2 and lut5 with respect to the wild type (5.5), accompanied by a higher PSI core/PSI-LHCI ratio (approximately 0.5 versus 0.4).
Table II. Photosynthetic pigment content of the wild type and mutants

Pigment content was determined before and after leaves were illuminated for 60 min at 550 μmol photons m⁻² s⁻¹. Data are normalized to 100 Chl a + b molecules and are expressed as mean ± so (n = 3). Values marked with the same letters are not significantly different from each other within a column and a light regime (Student’s t test, P < 0.05).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Neoxanthin</th>
<th>Violaxanthin</th>
<th>Antheraxanthin</th>
<th>Lutein</th>
<th>Zeaxanthin</th>
<th>α-Carotene</th>
<th>β-Carotene</th>
<th>β,β,e,β Xanthophylls Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.4 ± 0.4ᵇ</td>
<td>2.8 ± 0.4ᵇ</td>
<td>13.0 ± 0.1ᵃ</td>
<td>0.2 ± 0.04ᵃ</td>
<td>6.9 ± 0.4ᵃ</td>
<td>0.6 ± 0.01ᵃ</td>
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</tr>
<tr>
<td>szl1</td>
<td>1.3 ± 0.1ᵇ</td>
<td>0.8 ± 0.2ᵇ</td>
<td>22.3 ± 0.5ᵇ</td>
<td>1.2 ± 0.2ᵇ</td>
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<td>0.1 ± 0.01ᵇ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chy1chy2</td>
<td>0.7 ± 0.2ᵇ</td>
<td>0.7 ± 0.1ᵇ</td>
<td>15.6 ± 0.8ᵇ</td>
<td>0.2 ± 0.1ᵇ</td>
<td>8.0 ± 0.2ᵇ</td>
<td>0.1 ± 0.03ᵇ</td>
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</tr>
<tr>
<td>lut5</td>
<td>2.9 ± 0.4ᶜ</td>
<td>2.0 ± 0.2ᶜ</td>
<td>10.3 ± 0.5ᵈ</td>
<td>6.3 ± 0.4ᶜ</td>
<td>2.9 ± 0.2ᵇ</td>
<td>0.5 ± 0.01ᶜ</td>
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<td>HL</td>
<td>4.2 ± 0.1ᵃ</td>
<td>1.4 ± 0.1ᵃ</td>
<td>0.3 ± 0.1ᵃ</td>
<td>12.6 ± 0.3ᵃ</td>
<td>1.1 ± 0.2ᵃ</td>
<td>0.2 ± 0.1ᵃ</td>
<td>6.1 ± 0.3ᵃ</td>
<td>0.6 ± 0.01ᵃ</td>
</tr>
<tr>
<td>szl1</td>
<td>1.3 ± 0.1ᵇ</td>
<td>0.6 ± 0.1ᵇ</td>
<td>0.1 ± 0.01ᵇ</td>
<td>20.8 ± 1.0ᵇ</td>
<td>0.8 ± 0.1ᵇ</td>
<td>1.1 ± 0.2ᵇ</td>
<td>2.4 ± 0.5ᵇ</td>
<td>0.1 ± 0.01ᵇ</td>
</tr>
<tr>
<td>chy1chy2</td>
<td>0.8 ± 0.2ᶜ</td>
<td>0.6 ± 0.1ᵇ</td>
<td>0.1 ± 0.01ᵇ</td>
<td>15.2 ± 0.4ᶜ</td>
<td>0.6 ± 0.1ᵇᶜ</td>
<td>0.2 ± 0.1ᶜ</td>
<td>7.6 ± 0.4ᶜ</td>
<td>0.1 ± 0.02ᵇ</td>
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<tr>
<td>lut5</td>
<td>3.1 ± 0.4ᵈ</td>
<td>1.4 ± 0.1ᵃ</td>
<td>0.1 ± 0.04ᵇ</td>
<td>11.0 ± 0.7ᵈ</td>
<td>0.6 ± 0.04ᶜᵈ</td>
<td>5.9 ± 0.4ᶜ</td>
<td>3.0 ± 0.1ᵇ</td>
<td>0.5 ± 0.04ᶜ</td>
</tr>
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</table>

We first proceeded to determine whether the light harvesting and energy transfer to reaction center activity was affected by the mutations. The functional antenna size of PSII was measured on leaves by estimating the rise time of Chl fluorescence in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). chy1chy2 leaves showed a significant reduction in the PSII antenna size with respect to the wild type (Table I), thus suggesting that carotenoid depletion did impair the overall light-harvesting capacity, as suggested by densitometric analysis of the Deriphat-PAGE. However, the PSII functional antenna size was not significantly affected by the szl1 mutation.

Green bands were eluted from the gel, and their pigment composition was determined by HPLC (Table III). Monomeric and trimeric Lhcb complexes from mutant genotypes showed a lower content in carotenoids per unit Chl versus the corresponding fractions from the wild type (around -20%). Furthermore, antenna proteins isolated from szl1 and chy1chy2 thylakoids had a lower content in β,β-xanthophylls (~90% in monomeric Lhcb, ~75% in trimeric LHCII) and a compensatory increase in lutein (+25% in monomeric Lhcb, +10% in trimeric LHCII), while the relative abundance of e,β- and β,β-xanthophylls were similar in antenna proteins from the wild type and lut5. The PSII core complexes purified from wild-type and mutant thylakoids only bound Chl α,β-carotene and α-carotene. However, while the Chl/Car ratios were essentially the same in the wild type, chy1chy2, and lut5, the PSII core complex purified from szl1 showed a far lower content in carotenes (~40%). Moreover, 33% of bound carotenoids in the PSII core complex from szl1, are made by α-carotene, versus 72% in lut5; PSII core complexes bound almost exclusively β-carotene in both the wild type and chy1chy2.

A similar effect was observed on the pigment composition of the PSI-LHCI complexes purified from wild-type and mutant thylakoids: Carotenoid content per unit Chl was essentially the same in the wild type, chy1chy2, and lut5, while it was reduced in szl1 due to a lower carotene content (~30%). The relative abundance of e,β- and β,β-xanthophylls was similar in PSI-LHCI from the wild type and lut5, while complexes isolated from szl1 and chy1chy2 had a far lower content in β,β-xanthophylls and a compensatory increase in lutein. While β-carotene is the main carotene found in PSI-LHCI from the wild type and chy1chy2, α-carotene accounts for 28% and 62% of total carotenoids of the complexes in szl1 and lut5, respectively.

Figure 1. Analysis of pigment-protein complexes of the wild type and mutants. Thylakoid pigment-protein complexes were separated by nondenaturing Deriphat-PAGE upon solubilization with 0.8% α-DM. Thylakoids corresponding to 25 μg of Chls were loaded in each lane. Composition of each band is indicated. [See online article for color version of this figure.]
Table III. Pigment composition of Chl proteins purified from thylakoids of the wild type, sz11, chy1chy2, and lut5

Data are normalized to 100 Chl a + b molecules and are expressed as mean ± SD (n = 3). See “Materials and Methods” for details of purification. Abbreviations: Chls, Total Chl; Xanths, total xanthophylls; Cars, total carotenoids; ε,β,ε,β-xanthophylls, β,β,β,xanthophylls. Values marked with the same letters are not significantly different from each other within a column and a complex (Student’s t test, P < 0.05).

<table>
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<tr>
<th>Complexes</th>
<th>Monomeric Lhcb</th>
<th>PSII core complex</th>
<th>Trimeric LHCB</th>
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<tbody>
<tr>
<td></td>
<td>Chl a</td>
<td>Chl b</td>
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</tr>
<tr>
<td>WT</td>
<td>64.6 ± 0.34a</td>
<td>35.5 ± 0.24</td>
<td>62.4 ± 0.15a</td>
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<tr>
<td>sz11</td>
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<td>62.4 ± 0.14d</td>
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<tr>
<td>chy1chy2</td>
<td>60.3 ± 0.5b</td>
<td>39.7 ± 0.5b</td>
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<tr>
<td>lut5</td>
<td>87.0 ± 0.2c</td>
<td>13.0 ± 0.2c</td>
<td>62.4 ± 0.14d</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pigment/100 Mol Chls</th>
<th>α-Carotene</th>
<th>ε-Carotene</th>
<th>Bacteriopheophytin</th>
<th>Neoxanthin</th>
<th>Violaxanthin</th>
<th>Lutein</th>
<th>α-Carotene</th>
<th>ε-Carotene</th>
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<th>Violaxanthin</th>
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<tbody>
<tr>
<td>WT</td>
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<tr>
<td>lut5</td>
<td>87.0 ± 0.2c</td>
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Photosynthesis-Related Functions: ETR and Excess Energy Dissipation

Analysis of the fluorescence yield in dark-adapted leaves (Butler, 1978) revealed a significant decrease of the PSII maximum quantum efficiency (Fm/Fo) in sz11 with respect to the other genotypes (Table I). In all mutants, the absolute values of Fm are slightly reduced with respect to the wild type, while only in sz11 the F0 value is 35% higher than the corresponding wild type. Thus, the decline in Fm/Fo in sz11 is mainly due to F0 rise (Table I), meaning that a larger fraction of absorbed energy is lost as fluorescence in this mutant; it suggests either that the connection between the major LHC and PSII reaction center is less efficient in carotenedepleted plants, or that PSII reaction center trapping efficiency is reduced.

To test the hypothesis that caroten content might affect photosynthetic electron transport, PSII function during photosynthesis was analyzed by Chl fluorometry. sz11, chy1chy2, and lut5 showed no major differences with respect to wild-type plants either in the linear ETR or in the Qo redox state (qL), as measured on leaves at different light intensities in the presence of saturating CO2 (Fig. 2, A and B).

Capacity for Chl fluorescence quenching (qE), the rapidly reversible component of nonphotochemical quenching (NPQ), was plotted as a function of light intensity (Fig. 2C), and a reduction in qE activity was measured in chy1chy2 and lut5 plants. These results are consistent with previous reports (Niyogi et al., 1998; Dall’Osto et al., 2007b; Kim et al., 2009) showing a correlation between xanthophyll content, accumulation of zeaxanthin, and amplitude of qE. sz11 leaves were also analyzed for their fluorescence quenching capacity, to investigate if carotene depletion affected qE amplitude. sz11 showed a maximum value of qE lower than the wild type but similar to that of the other mutants. Since PsbS content was the same in wild-type and sz11 plants (Li et al., 2009), we determined the capacity of intact chloroplasts to produce changes in luminal pH by following the light-induced quenching of 9-aminoacridine (AA; Johnson et al., 1994). All mutants performed similar to the wild type at all light intensities (Fig. 2D). This suggests that the reduction of qE in sz11 can be attributed to its lower xanthophyll cycle pool size, similar to the wild type at all light intensities (Fig. 2D). This suggests that the reduction of qE in sz11 can be attributed to its lower xanthophyll cycle pool size, similar to the wild type at all light intensities (Fig. 2D). This suggests that the reduction of qE in sz11 can be attributed to its lower xanthophyll cycle pool size, similar to the wild type at all light intensities (Fig. 2D). This suggests that the reduction of qE in sz11 can be attributed to its lower xanthophyll cycle pool size, similar to the wild type at all light intensities (Fig. 2D).

Photosensitivity under HL at Chilling Temperature

Treatment of plants with strong light produced photodinitive stress, whose severity was enhanced by low temperature. Under these conditions, enhanced release of ·O2 produced bleaching of pigments, lipid peroxidation, and PSII photoinhibition (Zhang and Scheller, 2004). To analyze the effect of missing carotenoids on the sensitivity to photodinitive stress, leaf discs from the wild type and mutants were subjected to HL + cold stress (2,400 μmol photons m−2 s−1, 8°C), then the time course of pigment photobleaching was measured (Fig. 3A). Results indicate that the Chl bleaching rate was higher in sz11 leaves, lower in the wild type and chy1chy2, while lut5 leaves showed an intermediate behavior.

The level of stress caused by HL + cold treatment in the wild type and mutants was further investigated by measuring the extent of lipid peroxidation as detected by thermoluminescence (TL; Ducruet and Vavilin, 2012).
Data are expressed as means ± SD (n = 4). Figure 3B shows plots of TL amplitudes at different time points during exposure of leaf discs to HL + cold stress (800 μmol photons m⁻² s⁻¹, 8°C). The highest levels of lipid peroxidation upon HL treatment was observed in szl1, followed by lut5 while chy1chy2 was only slightly more photosensitive than the wild type. Measurement of the \( ^1 \)O₂ production in leaves (Fig. 3C) was consistent with pigment bleaching and lipid peroxidation measurements: At the end of the treatment, the wild type showed the lowest level of singlet oxygen sensor green (SOSG) fluorescence, while szl1 the highest; chy1chy2 and lut5 had intermediate behavior. Instead, after illumination with HL, szl1 leaves showed significantly lower yield in reduced forms of ROS (namely, hydrogen peroxide, O₂⁻, and OH⁻) with respect to all the other genotypes at each time point (Supplemental Fig. S1).

The enhanced photosensitivity of szl1 plants could be caused by impaired photoprotection at either PSII, PSI, or both. To determine the primary target of photodamage in szl1 leaves upon exposure at HL and low temperature, kinetics of PSII and PSI photoinhibition were determined. The sensitivity to photodamage stress in wild-type and mutant plants was assessed upon their transfer from control conditions to HL + cold stress (550 μmol photons m⁻² s⁻¹, 8°C), upon which the levels of \( F_v/F_m \), the maximal photochemical yield of PSII, were monitored for 9 h; results are reported on Figure 4A. In wild-type plants \( F_v/F_m \) gradually decreased from 0.8 to 0.6 during the treatment (halftime of PSII photoinhibition of approximately 20 h), similar to the behavior of chy1chy2. In lut5, however, \( F_v/F_m \) was more affected by the treatment, down to a value of 0.4 at the end of the treatment. The szl1 plants were as photosensitive as lut5, since their \( F_v/F_m \) decreased to 0.35 at the end of the treatment, corresponding to a halftime for PSII photoinhibition of approximately 5.5 h. Measurements of \( F_v/F_m \) recovery after photoinhibitory treatment clearly showed the same rate of PSII quantum efficiency recovery in all genotypes (Supplemental Fig. S2), implying that the higher photosensitivity is due to a less-effective photoprotection rather than to impaired PSII repair mechanism (Aro et al., 1994).

Upon exposure to photooxidative stress, \( F_v \) and \( F_m \) changes showed different kinetics in wild-type and mutant leaves (Supplemental Fig. S3). Stress treatment resulted in a decrease of \( F_m \) in all genotypes, likely due to photoactivation of PSIII reaction centers, which then dissipate excitation energy as heat rather than as photochemistry (Baker, 2008). Instead, while HL + cold stress was associated with an increase in \( F_0 \) in chy1chy2, lut5, and wild-type plants, as expected upon oxidative damage of PSI RC, szl1 plants showed a slight reduction of \( F_0 \) value with time of treatment (Supplemental Fig. S3). The szl1 \( F_0 \) changes could be traced back to the massive Chl bleaching of this genotype upon photodamage stress (Fig. 3A) that are likely to affect the fluorescence emission per leaf surface area.

The kinetic of PSI photoinhibition was assessed by measuring the maximum content of photooxidizable P700 upon transfer of plants from control conditions to HL + cold stress. These stress conditions had a much more dramatic effect on photoinhibition of PSI with respect to that of PSII in Arabidopsis, in agreement with previous results (Zhang and Scheller, 2004).
Indeed, the photooxidizable P700 gradually decreased to 50% of its initial value in 4.5 h and to 30% at the end of the 9-h treatment in wild-type plants (Fig. 4B). The halftime of photoinhibition was shorter for chy1chy2 and lut5 genotypes (being 50% inhibited in 2.5 and 4 h, respectively). Surprisingly, the halftime of PSI photoinhibition was far shorter for szl1 plants (approximately 0.6 h) than in the other genotypes.

To further quantify the PSI damage, the maximum level of P700+ was measured upon a saturating flash under a far-red-light background, before and after HL treatment (Munekage et al., 2002). The decrease in the P700+ level might be caused not only by PSI photoinhibition, but also by an overreduced state of acceptors and activation of cyclic electron flow (Sonoike, 2011; Rutherford et al., 2012). To evaluate the extent of PSI photoinhibition, leaves were vacuum infiltrated with methyl viologen upon the HL treatment; electron acceptance from PSI by methyl viologen restored the maximum oxidation of P700. Results (Supplemental Table S1) show that in the wild type less than 25% of PSI was damaged, whereas in szl1 up to 80% of PSI was inactivated.

1O2 Production from Purified Pigment-Protein Complexes

The above results (Figs. 3 and 4) suggest a role for carotenes in photoprotection of both PSII and PSI reaction centers, possibly in limiting the 1O2 release into the lipid phase. Although this result is consistent with carotene location in PSI-LHCI and PSII core complexes, it is relevant to experimentally assess whether photodamage is due to the properties of the pigment-binding proteins or is caused by pleiotropic factors. To this aim, we purified PSII core, Lhcb antenna proteins, and PSI-LHCI supercomplex and determined their 1O2 production when illuminated with strong light (see "Materials and Methods" for details). When isolated Chl-binding complexes are exposed to strong light, 1O2 is produced as the main ROS involved in the photoinhibition of both PS (Triantaphylidès et al., 2008) deriving from the reaction of excited Chl (3Chl*) with molecular oxygen. The level of 1O2 production by isolated complexes strongly depends on carotenoid composition and is inversely correlated with the capacity of 3Chl* quenching and ROS scavenging by bound xanthophylls (Mozzo et al., 2008; Dall’Osto et al., 2010; de Bianchi et al., 2011).

Results of 1O2 production at 20°C by the different complexes isolated from the wild type and szl1 are reported in Figure 5. PSII core complexes from the wild type, chy1chy2, and lut5 showed a similar yield of 1O2 at each light intensity tested, up to 800 μmol photons m⁻² s⁻¹. The complexes from szl1 showed an

Figure 3. Photooxidation of Arabidopsis wild-type and mutant genotypes under photoxidative stress. A, Detached leaves floating on water were treated at 2,400 μmol photons m⁻² s⁻¹ at 8°C, and kinetics of Chl bleaching were recorded. Data are expressed as means ± sd (n = 6). B, Wild-type and mutant leaves floating on water were exposed to 800 μmol photons m⁻² s⁻¹ at 8°C, and photooxidation was estimated from the extent of lipid peroxidation measured by high-temperature TL. Each experimental point corresponds to a different sample. a.u., Arbitrary units. See "Materials and Methods" for details. C, Wild-type and mutant detached leaves were vacuum infiltrated with 5 μM SOSG, a 1O2-specific fluorogenic probe. SOSG increases its fluorescence emission upon reaction with 1O2. The increase in the probe emission was followed during illumination with red actinic light (550 μmol photons m⁻² s⁻¹) at 8°C. a.u., Arbitrary units. Values that are significantly different (P < 0.05) from the wild type are marked with an asterisk (*).
on PSI-LHCI supercomplexes. Clearly, the $^{1}$O$_{2}$ yield was 2-fold higher in the preparation from szl1 with respect to that of the wild type (Fig. 5D). In contrast, $^{1}$O$_{2}$ yield from the PSI-LHCI complex of chylchy2 and lut5 was only slightly higher than the wild type. Pigment-protein photoprotection was further evaluated from the ability to prevent Chl photobleaching via thermal deactivation of $^{3}$Chl$^{*}$ by carotenoids under strong white light illumination in atmosphere. The PSI core complex from szl1 did not show any reduction of its rate of bleaching ($t_{50\%}$ bleaching) with respect to the wild type. On the contrary, PSI-LHCI from szl1 plants was less photoprotected, as shown by the 28% reduction in $t_{50\%}$ bleaching (Supplemental Fig. S4).

**DISCUSSION**

In this work, we have investigated the role of carotene versus xanthophyll ligands in the photosynthetic apparatus, focusing on their photoprotective capacity for both PS. A panel of Arabidopsis mutants affected in the carotenoid biosynthesis pathways was compared: In fact, due to the intermediate position of carotenes in the carotenoids biosynthesis pathway, it is impossible to affect their abundance without inducing changes in the xanthophylls, which are downstream in the metabolic pathway. The major target of our analysis was the mutant szl1, depleted in carotenes due to a lower β-cyclase activity (Li et al., 2009). When exposed to HL at low temperature, szl1 plants showed the highest levels of pigment bleaching and lipid peroxidation among the genotypes considered in this study. Interestingly, carotene depletion in szl1 plants preferentially affects PSI activity, since mutant plants revealed far stronger photoinhibition of PSI with respect to PSII. Thus, it appears that photoprotection efficiency strongly depends on carotene content of PS. It should be noted, however, that the szl1 mutation, besides decreasing carotene content, also favors ε-branch versus β-branch xanthophylls, thus modifying the chromophore composition of the xanthophyll-binding Lhc subunits of the antenna system and increasing the α-carotene, usually a minor component, versus the normally found β-carotene. Comparison with the photoprotection phenotype of the chylchy2 and lut5 is thus important to assess if the phenotype can be attributed in part to changes in xanthophyll composition, which is very similar in szl1 and chylchy2, or to the enrichment in α-carotene, a feature observed in both szl1 and lut5.

**Altered Xanthophyll Composition Affects Photoprotection Capacity**

Photoprotection by carotenoids is performed by multiple mechanisms including quenching of Chl triplet states (Peterman et al., 1995; Mozzo et al., 2008), scavenging of both superoxide and hydroxyl radicals (Trevithick-Sutton et al., 2006), and quenching of $^{3}$O$_{2}$, thus preventing lipid peroxidation (Havaux and Niyogi, 1999). In addition, ROS production can be approximately 30% increase in $^{1}$O$_{2}$ production at low and moderate light intensities while the differences were less evident at higher light when the signal from all complexes tended to saturation (Fig. 5A). Measurements of LhcB fractions (Fig. 5B) from the different genotypes did not show the same pattern. In fact, the complexes from chylchy2 and szl1 showed an approximately 70% increase in $^{1}$O$_{2}$ production with respect to the corresponding fraction from the wild type and lut5, consistent with the lower β,β-xanthophyll content (Dall’Osto et al., 2007b). Similar results were obtained by measuring PSI-LHCII: Supercomplexes from szl1 and chylchy2 showed an approximately 25% increase in $^{1}$O$_{2}$ production at moderate light intensities, with respect to both the wild type and lut5. In the case of PSI, the core complex and the antenna moieties form a stable complex that cannot be dissociated without some level of damage. We therefore measured $^{1}$O$_{2}$ production approximately 30% increase in $^{1}$O$_{2}$ production at low and moderate light intensities while the differences were less evident at higher light when the signal from all complexes tended to saturation (Fig. 5A). Measurements of LhcB fractions (Fig. 5B) from the different genotypes did not show the same pattern. In fact, the complexes from chylchy2 and szl1 showed an approximately 70% increase in $^{1}$O$_{2}$ production with respect to the corresponding fraction from the wild type and lut5, consistent with the lower β,β-xanthophyll content (Dall’Osto et al., 2007b). Similar results were obtained by measuring PSI-LHCII: Supercomplexes from szl1 and chylchy2 showed an approximately 25% increase in $^{1}$O$_{2}$ production at moderate light intensities, with respect to both the wild type and lut5. In the case of PSI, the core complex and the antenna moieties form a stable complex that cannot be dissociated without some level of damage. We therefore measured $^{1}$O$_{2}$ production
prevented by quenching of singlet Chl excited states, a function that is enhanced by lutein and zeaxanthin (Niyogi et al., 2001; Dall’Osto et al., 2005). The strong phenotypes of Arabidopsis mutants with altered xanthophyll composition (Niyogi et al., 2001; Dall’Osto et al., 2007b; Kim et al., 2009) showed that the presence and relative amounts of these pigments is relevant for plant fitness. We observed that chy1chy2 plants are more sensitive to photoxidative stress than the wild type (Fig. 3). The total xanthophyll content is only slightly reduced in this mutant (Table II), and yet the β,β/ε,β-xanthophyll ratio is 8-fold decreased in the mutant, and in its LHC proteins (Table III). Previous work showed that optimal photoprotection of Lhc proteins depends on the balanced activity in ROS scavenging by β,β-xanthophylls and Chl triplet quenching by lutein (Dall’Osto et al., 2007b). Indeed, we observed an increased rate of 1O2 release from antenna proteins purified from chy1chy2, with β,β-xanthophylls partially replaced by lutein, with respect to the corresponding preparation from the wild type (Fig. 5B). In particular, it appears that depletion in neoxanthin is a major factor, consistent with recent reports (Mozzo et al., 2008). Besides neoxanthin, violaxanthin and zeaxanthin are also effective in preventing photodamage since the effect of their depletion is observed in PSI-LHCI complex, which does not bind neoxanthin, isolated from chy1chy2 plants (Table III). It should be noted that the assembly of PSI-LHCI supercomplexes is not impaired in chy1chy2, as shown by the migration rate of the supercomplex in the native PAGE identical to the wild type (Fig. 1).

Since Lhca and Lhcb proteins from chy1chy2 and szl1 have essentially the same β,β/ε,β-xanthophyll ratio and the same total xanthophyll content, the extreme sensitivity of szl1 to excess light cannot be explained based on differences in the xanthophyll composition and/or organization of the antenna proteins, implying that additional sensitivity factors are present in szl1 plants.

Altered Xanthophyll/Carotene Ratio Leads to Enhanced Photoxidation

Kinetics of pigment bleaching and lipid peroxidation clearly showed that lut5 and szl1 are much more sensitive to photoxidative stress with respect to both the wild type and chy1chy2 (Fig. 3) while the discussion above led to the conclusion that this is not due to their xanthophyll composition. A striking difference between lut5 and szl1 plants is their carotenoid composition: a low xanthophyll content per Chl in the former (<25% with respect to the wild type, see Table II), and a high xanthophyll/carotene ratio in the latter (+50% compared with the wild type), and yet both conditions lead to a strong light sensitivity with respect to wild-type plants. We have shown above that the β,β/ε,β-xanthophyll ratio cannot be the cause for differential photosensitivity, since this is the same in chy1chy2 and szl1, which exhibit very different levels of lipid peroxidation (Fig. 3). Over-reduction of the plastoquinone pool is commonly associated with photoinhibition in wild-type plants, and yet the plastoquinone redox state was not significantly different in lut5 or szl1 with respect to chy1chy2 (Fig. 2). Zeaxanthin accumulation in HL, which provides photoprotection by both modulating qE and scavenging 1O2 in the lipid phase (Niyogi et al., 1998; Havaux and Niyogi, 1999), cannot account for the higher sensitivity of szl1 plants.
which evolves more 1O2, particularly at low-light intensity, a feature that correlates with the depletion in PSI-LHCI supercomplexes from this genotype did not show major differences in 1O2 production with respect to the complexes from the wild type (Fig. 5A), implying that α-carotene is not the major cause of photosensitivity.

What Is the Origin of the Extreme PSI Photosensitivity in szl1 Plants?

In the PSII core complex, most of the β-carotene molecules are in close contact with Chl s, as required for effective quenching of 3Chl*. (Ferreira et al., 2004). The only exception is represented by the two β-carotene ligands in the PSI reaction center, whose distance from the special pair P680 is higher, implying that they cannot quench 3P680* by triplet-triplet transfer and rather, they likely act as scavengers for 1O2 produced during charge recombination (Telfer et al., 1991, 1994b). PSI has been indicated as the primary target of photoinhibition (Andersson et al., 1992; Aro et al., 1993), since the D1 subunit is easily damaged in HL and rapidly turned over. Instead, P700+ was reported to be protective for PSI, since it can quench excitation energy and oxidize the reduced electron acceptor of PSI and remove excess reducing power (Sonoike, 2011). However, 3P680 and 3P700 lie close in energy level, thus both are prone to react with oxygen and yield 1O2. 3P700 results from charge recombination, therefore its yield is increased by acceptor side limiting conditions (Rutherford et al., 2012). Furthermore, exposure of PSI-LHCI to HL generates "Car" mainly associated with LHCI (Santabarbara and Carbonera, 2005), while a selective bleaching of lutein molecules located in the outer antenna was observed (Andreeva et al., 2007), thus raising the question of what the role for carotene ligands is.

When comparing photoinhibition of szl1 and lut5 plants at HL + cold stress, the halftime of Fv/Fm decay (PSII photoinhibition, see Fig. 4A), as well as PSI repair efficiency (Supplemental Fig. S2), were very similar for both genotypes, while the szl1 genotype was far more sensitive to PSI photoinhibition with respect to the other genotypes, as shown by a 6-fold-faster PSI photoinhibition rate (Fig. 4B). This is consistent with the quantification of 1O2 released in HL by purified pigment-protein complexes: The 1O2 yield was 2-fold higher in the PSI-LHCI from szl1 with respect to that from the wild type (Fig. 5C), while PSIII core complexes from all genotypes showed a similar yield of 1O2 at each light intensity tested (Fig. 5A). What is the origin of the extreme PSI photosensitivity in szl1 plants?

Besides carotene depletion, a further feature of szl1 mutants is the presence of α-carotene in both PSI-LHCI and PSII core complexes, partially replacing β-carotene and potentially being a cause for photosensitivity (Table III). However, the lut5 genotype has a far higher α/β-carotene ratio than szl1 in both thylakoid (Table II) and isolated PSI core complexes (Table III), and yet isolated PSI core complexes and PSI-LHCI from this genotype did not show major differences in 1O2 production with respect to the complexes from the wild type (Fig. 5A), implying that α-carotene is not the major cause of photosensitivity.
After light-induced damage, recovery of PSI from photoinhibition takes several days (Sonoike and Terashima, 1994; Sonoike, 2011); indeed, the damage to PSI is considered to be essentially irreversible and involves degradation and resynthesis of the whole complex.

Because of its irreversibility, PSI photoinhibition must be specially avoided. This is accomplished by a number of protective mechanisms, namely cyclic electron transport (Munekage et al., 2002) and the stromal scavenging enzyme systems superoxide dismutase and ascorbate peroxidase, which scavenge reduced ROS released by PSI (Asada, 1999). In several plant species including Arabidopsis, PSI becomes more sensitive to photoinhibition under specific environmental conditions such as chilling temperature, likely because the protective mechanisms are less efficient at low temperature (Sonoike, 2011); furthermore, the sink of reductants is decreased at low temperature, thus overreduction of the electron chain occurs and the yield of \( ^3 \text{P}0 \) and \( ^3 \text{O}_2 \) increases. Indeed, the primary target for PSI photoinhibition upon illumination was located in the iron-sulfur centers \( F_r \), \( F_y \), and \( F_A \), and was caused by ROS (Inoue et al., 1986; Tjus and Andersson, 1993). Here, we show that HL + cold stress is effective in damaging PSI even in wild-type plants (Fig. 4B), and that this effect is greatly enhanced in carotene-depleted \( szl1 \) plants, implying that carotene ligands in PSI are crucial in ensuring the maintenance of PSI activity under this condition.

When searching for the molecular mechanism(s) behind the preferential damage of PSI in \( szl1 \), it can be hypothesized that carotene composition might affect the susceptibility to photoinhibition of the mutant. Indeed, a clear difference between the wild type and \( szl1 \) is the presence of \( \alpha \)-carotene in PSI-LHCI of the mutant, partially replacing \( \beta \)-carotene. It is worth noting that PSI-LHCI from \( lut5 \) plants has a higher \( \alpha/\beta \)-carotene ratio than \( szl1 \), and roughly the same xanthophyll content (Table III). However, a faster PSI photoinhibition (Fig. 4B) and a higher release of \( ^3 \text{O}_2 \) from PSI-LHCI (Fig. 5C) were observed with respect to the wild type. On this basis, we cannot completely exclude the possibility that part of the PSI photosensitivity of \( szl1 \) plants was related to the \( \alpha \)-carotene content of either core complex or LHCI.

Moreover, PSI stability might be limited by the amount of carotene molecules available. Indeed, a recent improved model of plant PSI (Amunts et al., 2010) suggested that most of \( \beta \)-carotene molecules are coordinated by either different subunits or distant region of the same subunit; therefore, these pigments might have a key role for PSI structural integrity, and it is consistent with the high degree of conservation of their positions and coordination between plants and cyanobacteria (Amunts et al., 2010). In \( szl1 \) plants, a general weakening of the PSI-LHCI structure would make the complex more susceptible to ROS attack thus causing degradation, as shown by native-PAGE analysis (Fig. 1). However, the photobleaching kinetics of PSI-LHCI complexes, challenged with strong light (Supplemental Fig. S4), show that this pigment-protein complex from \( szl1 \) is rather stable.

Alternatively, rather than the PSI core complex, the peripheral light-harvesting system might be more affected by carotene depletion. Indeed, it should be stressed that \( \beta \)-carotene is a ligand not only of the wild-type PSI core complex, but also of LHCI antenna moiety (Wehner et al., 2004). A recent report (Alboresi et al., 2009) showed that preferential degradation of LHCI upon illumination of isolated PSI-LHCI is effective in protecting the catalytic activity of the complex. Recovery from PSI photoinhibition is an energetically demanding process, since it necessarily requires degradation and resynthesis of the whole complex; thus, sacrificing the antennae would be a photoprotective strategy evolved to limit photoxidative damage into LHCI moiety and preserve the integrity of iron-sulfur clusters. The role of LHCI proteins as safety valves for PSI is related to the red absorption forms (Carbonera et al., 2005; Alboresi et al., 2009), Chls of the outer antenna with low energy level that concentrate the excitation energy before transfer to the reaction center (Croce et al., 1996); \( ^3 \text{Chl}^* \) eventually formed by the red Chls are quenched by nearby carotenoids (Carbonera et al., 2005). This model implies that, as shown for Lhcb, Lhca proteins constitutively undergo formation of \( ^3 \text{Chl}^* \) and production of \( ^3 \text{O}_2 \). Carotenoid species bound to the LHCI, namely lutein, violaxanthin, and \( \beta \)-carotene, could be directly involved in triplet quenching and/or ROS scavenging, and likely occupy selective binding sites and serve distinct roles. Impairing one of these functions by changing the occupancy of specific carotenoid binding sites through mutations in the biosynthetic pathway leads to photosensitivity, similar to what observed previously within PSII antenna system (Dall’Osto et al., 2007).

CONCLUSION

Here we show that the \( szl1 \) plants, which carry a point mutation in the LCYB gene and thus a less-active \( \beta \)-cyclase than the wild type (Li et al., 2009), have a lower carotene content in both PS with respect to wild-type plants and altered xanthophyll composition of the light-harvesting component of the antenna systems. Physiological characterization of the \( szl1 \) mutant offered the possibility of probing carotene function in vivo differentially from the effect on xanthophyll complement. When challenged with HL + cold stress, \( szl1 \) mutant plants undergo more photodamage than the wild type, particularly within PSI-LHCI, despite the fact that PSI-LHCI was less depleted in carotenes than PSII. Comparison with the \( chy1chy2 \) and \( lut5 \) mutants, which respectively share with \( szl1 \) alterations in xanthophyll composition and \( \alpha \)-carotene accumulation, showed that these features were not the major factors causing enhanced susceptibility to photoinhibition and pointed to carotene depletion in photosynthetic core complexes as the major source of photodamage. It is
evident that regulation of PSI Chl excited states under HL + cold stress is crucial for protection of the photosynthetic apparatus.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type plants of Arabidopsis thaliana (ecotype Columbia) and mutants chy1chy2, lut5, and s2l were obtained as previously reported (Fiore et al., 2006; Li et al., 2009). T-DNA knockout lines used are: chy1 (SAIL line 49A07), chy2 (SAIL line 1242B12), and lut5 (SALK line 116660). Plants were grown for 4 weeks on Sundermisch potting mix (Granflor) in controlled conditions of 8 h light, 23°C/16 h dark, 20°C, with a light intensity of 100 μmol photons m−2 s−1.

Stress Conditions

For HL treatments, light was provided by 150-W halogen lamps (Focus 3, Prisma). Short-term HL treatment was performed for 1 h at 550 μmol photons m−2 s−1 at 8°C to measure maximum zeaxanthin accumulation on detached leaves floating on water. Samples for HPLC analysis were freshly frozen in liquid nitrogen prior to pigment extraction. Photooxidative stress was induced in either whole plants or detached leaves by a strong light treatment. Whole Arabidopsis plants were exposed to HL (550 μmol photons m−2 s−1) with a photoperiod day/night of 16/8 h) at 8°C for 14 h. Chloroplasts and Thylakoids Isolation

Chloroplasts and stacked thylakoid membranes were isolated from wild-type and mutant leaves as previously described (Casazza et al., 2001). Pigment Analyses

Pigments were extracted from leaves with 80% acetone, then separated and quantified by HPLC (Gilmore and Yamamoto, 1991). Chl content was determined by fitting the spectrum of the sample’s acetone extract with the spectra of individual pigments, as described previously (Croce et al., 2000).

Gel Electrophoresis

Nondenaturing Deriphat-PAGE was performed following the method described previously (Peter et al., 1991) but using 3.5% (w/v) acrylamide (38:1 acrylamide/bisacrylamide) in the stacking gel and in the resolving gel an acrylamide concentration gradient from 4.5% to 11.5% (w/v) stabilized by a glycerol gradient from 8% to 16%. Thylakoids concentrated at 1 mg/mL Chl were solubilized with either 0.8% DM or 1% DM, and 20 μg of Chls were loaded in each lane. Signal amplitude was quantified using the GelPro 3.2 software (BIORAD). Purified pigment-protein complexes were excised from gel and eluted with a pestle in a buffer containing 10 mM HEPES pH 7.5, 0.05% a-DM.

Analysis of Chl Fluorescence and P700 Redox Kinetics

PSII function during photosynthesis was measured through Chl fluorescence on whole leaves at room temperature with a PAM 101 fluorometer (Walz, Effeltrich, Germany), using a saturating light pulse of 4,500 μmol photons m−2 s−1, 0.6 s, and white actinic light ranging from 50 to 1,100 μmol photons m−2 s−1, supplied by a KL1500 halogen lamp (Schott). NPQ, φPSII, and photochemical quenching (qP) were calculated according to the following equations (Van Kooten and Snel, 1990; Baker, 2008): NPQ = (Fm − F0)/Fm, φPSII = (Fm − F0)/Fm, qP = (Fm − F0)/(Fm − F1), qL = qP Fm/F1, ETR = φPSII × ETR, and PAR = Amax/100 × qP. PAR was the maximal fluorescence from dark/light-adapted leaves measured after the application of a saturating flash, Fm, the stationary fluorescence during illumination, and PAR the photosynthetic active radiation; Amax (leaf absorptivity) was 0.67 ± 0.04 for the wild type, 0.59 ± 0.04 for chy1chy2, 0.61 ± 0.03 for lut5, 0.58 ± 0.05 for s2l.

Determination of the Sensitivity to Photooxidative Stress

HL treatment was performed for 2 d at 550 μmol photons m−2 s−1, 8°C on whole plants. Decay kinetics of maximal quantum yield of PSII photochemistry (Fv/Fm, Havaux et al., 2004) and maximum content of photooxidizable P700 (ΔAmax, at 705 nm; Yang et al., 2010) were recorded on detached leaves during illumination to assess inhibition of PSII and PSI, respectively. Content of oxidizable P700 (ΔAmax) was recorded during far-red-light illumination (2,500 μmol photons m−2 s−1, Amax = 720 nm); to have a precise estimation of the reaction center photoinhibition, ΔAmax has been determined in detached leaves, vacuolated with 50 μM dithrombomycin and 1 mM methyl viologen (Sonoke, 2011). The maximum contents of P700 were also determined on methylethiol-treat leaves using a saturating flash (3,000 μmol photons m−2 s−1) under a 520 μM far-red-light background (Munekage et al., 2002).

Photooxidative stress was measured on whole plants by thermoluminometry, with a custom-made apparatus that has been described (Ducruet, 2003). The amplitude of the TL peak at 135°C was used as an index of lipid peroxidation (Havaux, 2003). Chl bleaching was followed on 1-cm diameter leaf discs, harvested from mature leaves. Leaf discs were kept floating on water and then exposed to white light (2,400 μmol m−2 s−1, 8°C). Discs were rapidly frozen in liquid nitrogen prior to pigment extraction and quantification.

Measurements of ROS Production

Measurements of O2 production were performed with a spectrophotometer equipped with a fiber optic to measure emission of fluorescent probes on vacuum-infiltrated leaves. Measure of ΔPm—the kinetics of ΔPm formation across the thylakoid membrane was measured using the method of 9-AN fluorescence quenching (Johnson et al., 1994) with modifications described in de Bianchi et al. (2008).

Spectroscopy

Steady-state spectra were obtained using samples in 10 mM HEPES pH 7.5, 0.05% a-DM, 0.2 mM Suc. Absorption measurements were performed using a SLM-Amino DW-2000 spectrophotometer at room temperature. Fluorescence emission spectra were measured at room temperature using a Jobin-Yvon Fluoromax-3 spectrofluorometer, equipped with a fiber optic to measure emission of fluorescent probes on vacuum-infiltrated leaves. Measure of ΔPm—the kinetics of ΔPm formation across the thylakoid membrane was measured using the method of 9-AN fluorescence quenching (Johnson et al., 1994) with modifications described in de Bianchi et al. (2008).
Statistical Analysis

Significance analyses were performed using an ANOVA with a pairwise multiple comparison procedure in Origin. Error bars represent the s.e.

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers At1g25700 (chlA), At5g2570 (chlB), At1g31800 (lsl3), and At2g32640 (chlF).

Additional Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Production of reduced ROS in wild-type and mutant leaves.

Supplemental Figure S2. PSII repair efficiency under photodynamic stress.

Supplemental Figure S3. \( F_{v} \) and \( F_{m} \) changes upon PSI photoinhibition.

Supplemental Figure S4. Photobleaching of pigment-protein complexes purified from wild-type and szl1 plants.

Supplemental Table S1. Photosensitivities of PSI.

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