Different Roles of $\alpha$- and $\beta$-Branch Xanthophylls in Photosystem Assembly and Photoprotection

Luca Dall’Osto$^{1, 2}$, Alessia Fiore$^{1, 2, 3}$, Stefano Cazzaniga$^{4}$, Giovanni Giuliano$^{2}$, and Roberto Bassi$^{3}$

From the $^1$Dipartimento Scientifico e Tecnologico, Università di Verona, Strada Le Grazie 15, 37134 Verona, Italy and the $^2$Ente per le Nuove Tecnologie, l’Energia e l’Ambiente, Dipartimento Biotecnologie, Centro Ricerche Casaccia, C.P. 2400, Rome 00100, Italy

Xanthophylls (oxygenated carotenoids) are essential components of the plant photosynthetic apparatus, where they act in photosystem assembly, light harvesting, and photoprotection. Nevertheless, the specific function of individual xanthophyll species awaits complete elucidation. In this work, we analyze the photosynthetic phenotypes of two newly isolated Arabidopsis mutants in carotenoid biosynthesis containing exclusively $\alpha$-branch (chy1chy2lut5) or $\beta$-branch (chy1chy2lut2) xanthophylls. Both mutants show complete lack of qE, the rapidly reversible component of nonphotochemical quenching, and high levels of photoinhibition and lipid peroxidation under photooxidative stress. Both mutants are much more photosensitive than npq1lut2, which contains high levels of viola- and neoxanthin and a higher stoichiometry of light-harvesting proteins with respect to photosystem II core complexes, suggesting that the content in light-harvesting complexes plays an important role in photoprotection. In addition, chy1chy2lut5, which has lutein as the only xanthophyll, shows unprecedented photosensitivity even in low light conditions, reduced electron transport rate, enhanced photobleaching of isolated LHCCI complexes, and a selective loss of CP26 with respect to chy1chy2lut2, highlighting a specific role of $\beta$-branch xanthophylls in photoprotection and in qE mechanism. The stronger photosystem II photoinhibition of both mutants correlates with the higher rate of singlet oxygen production from thylakoids and isolated light-harvesting complexes, whereas carotenoid composition of photosystem II core complex was not influential. In depth analysis of the mutant phenotypes suggests that $\alpha$-branch (lutein) and $\beta$-branch (zeaxanthin, violaxanthin, and neoxanthin) xanthophylls have distinct and complementary roles in antenna protein assembly and in the mechanisms of photoprotection.

Carotenoids are a group of C40 terpenoid compounds with a wide distribution in several biological taxa, ranging from archaea to bacteria, fungi, algae, and higher plants. Xanthophylls form a subgroup of oxygenated carotenoids, whose importance in the oxygenic photosynthesis is well known. Xanthophylls play essential roles in higher plant photosynthesis, as components of the photosynthetic apparatus of the chloroplast. In higher plants, $\beta$-carotene binds to reaction center subunits of both photosystem I (PSI) and II (PSII), whereas xanthophylls are both accessory pigments and structural elements of light-harvesting complexes (Lhc). Together with $\beta$-carotene, they act both as chromophores, absorbing light energy that is used in photosynthetic electron transport, and as photoprotectants of the photosynthetic apparatus from excess light and from the reactive oxygen species (ROS) that are generated during oxygenic photosynthesis. A remarkable characteristic of higher plant xanthophylls is that they show very similar spectral properties in the visible region. This evidence is apparently incoherent with the high conservation of their relative abundance across a range of plant taxa, which suggests that each xanthophyll species serves a specific role. Xanthophyll biosynthesis in plants is divided into two distinct branches; hydroxylation of $\alpha$-carotene gives rise to lutein (Lute), the most abundant xanthophyll in leaves (Fig. 1), whereas hydroxylation of $\beta$-carotene gives rise to zeaxanthin (Zea). In normal light conditions, zeaxanthin is epoxidized into antheraxanthin and violaxanthin (Viola) (Fig. 1), whereas in excess light, de-epoxidation prevails, leading to the accumulation of Zea (1, 2).

LHCCI, the major light-harvesting complex of photosystem II, binds Lute, Viola, and neoxanthin (Neo) (Fig. 1) (3). The minor complexes CP24, CP26, and CP29 bind the same pigments and, in excess light, Zea (4, 5). The specific function of each xanthophyll species in Lhc complexes is the subject of intense debate; lack of Lute and/or Zea decreases the capacity for photoprotection in high light, as suggested by the photosensitivity of Arabidopsis and Chlamydomonas mutants lacking both xanthophylls (6–9). Lute binds to site L1 of all Lhc pro-

---

$^*$This work was supported by the Italian Ministry of Research Special Fund for Basic Research Grant FIRB RBLA0345F_002 and Provincia Autonoma di Trento Grant SAMBAx2. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

$^1$The on-line version of this article (available at http://www.jbc.org) contains supplemental Table a1 and Figs. a1–a3.

$^2$Both authors contributed equally to this work.

$^3$Supervised by Prof. Laura Spano (University of L’Aquila) for doctoral work.

$^4$To whom correspondence should be addressed: Dip. Scientifico e Tecnologico, Università di Verona, Strada Le Grazie 15, 37134 Verona, Italy. Tel.: 39-045-8027916; Fax: 39-045-8027929/8027035; E-mail: bassi@sci.univr.it.
Photosynthetic Functions of $\alpha$- and $\beta$-Xanthophylls

Unstacked thylakoids were isolated from leaves as previously described (33). Membranes corresponding to 500 $\mu$g of chlorophylls were washed with 5 mM EDTA and then solubilized in 1 ml of 0.6% $\alpha$-DM at 10 $\mu$M HEPES, pH 7.5. Solubilized samples were then fractionated by ultracentrifugation in a 0.1–1 M sucrose gradient containing 0.06% $\alpha$-DM, 10 mM HEPES, pH 7.5 (22 h at 280,000 $\times$ g, 4°C).

*Gel Electrophoresis and Immunoblotting—SDS-PAGE analysis was performed with the Tris-Tricine buffer system as previously described (34). For immunotitration, thylakoid samples corresponding to 0.5, 1, 2, and 4 $\mu$g of chlorophylls were loaded for each sample and electrophoresed on nitrocellulose membranes. Filters were incubated with antibodies raised against Lhcb1, Lhcb2, Lhcb3, CP29 (Lhcb4), CP26 (Lhcb5), CP24 (Lhcb6), or CP47 (PsbB) and were detected with alkaline phosphatase-conjugated antibody, according to Ref. 35. Signal amplitude was quantified using the GelPro 3.2 software (Bio-Rad).
Photosynthetic Functions of α- and β-Xanthophylls

Electron Microscopy—Intact leaf fragments from wild-type and mutant 3-week-old leaves were fixed, embedded, and observed in thin section as previously described (36).

Spectroscopy—Spectra were obtained using samples in 10 mM HEPES, pH 7.5, 0.06% α-DM, 0.2 M sucrose. Absorption measurements were performed using an Aminco DW-2000 spectrophotometer (SLM Instruments, Rochester, NY) at room temperature. Fluorescence emission spectra were measured at room temperature using a Jobin-Yvon Fluoromax-3 spectrofluorimeter at room temperature.

Measure of ΔpH—The kinetics of ΔpH formation across the thylakoid membrane were measured using the method of 9-aminocadine fluorescence quenching, as previously described (37). Reaction buffer contained 50 mM Tricine, pH 8.0, 50 mM NaCl, 2 μM 9-aminoacridine. The chlorophyll concentration in the reaction buffer was adjusted to 10 μg/ml.

Determination of the Sensitivity to Photooxidative Stress—Photooxidative stress was induced in detached leaves by a strong light treatment at low temperature. Detached leaves on wet filter paper were exposed to high light (1000 μmol m⁻² s⁻¹ for 5 h) in a growth chamber at low temperature (10 °C) and then immediately frozen in liquid nitrogen. Photooxidative stress was assessed by measuring malondialdehyde (MDA) formation, as indirect quantification of lipid peroxidation. MDA is a reactive, low molecular weight aldehyde derived from radical attack of polyunsaturated fatty acids; in our measurement, leaf MDA levels were stabilized through the formation of a colored, thiobarbituric acid adduct. The MDA-(thiobarbituric acid adduct)₂ complex was separated from other thiobarbituric acid adducts and quantified by HPLC as previously described (38).

Measurements of Singlet Oxygen Production—Measurements of singlet oxygen (¹O₂) production either from thylakoids and purified pigment-protein complexes were performed with singlet oxygen sensor green (SOSG; Molecular Probes, Eugene), SOSG is a fluorescent probe highly selective for ¹O₂ that increases its 530 nm emission band in the presence of this ROS (39). Thylakoids were resuspended in a reaction buffer (0.33 M sorbitol, 10 mM NaCl, 10 mM KCl, 5 mM MgCl₂, 10 mM Hepes, pH 7.5, 30 mM ascorbate, 100 μM methyl viologen, 2 μM SOSG) at a final Chl concentration of 20 μg/ml and kept under continuous stirring. Pigment-protein complexes were harvested from sucrose gradient and diluted in a reaction buffer (10 mM Hepes, pH 7.5, 0.06% α-DM, 2 μM SOSG) to the same absorption area in the wavelength range 600–750 nm (around 2.2 μg Chls/ml). Thylakoids and isolated complexes were illuminated with red light (λ > 600 nm) for 5 min; fluorescence yields of SOSG (λₑₓ 480 nm, λₑᴍ 530 nm) were determined before and after light treatment in order to quantify ¹O₂-dependent fluorescence increase.

RESULTS

Pigment Composition—Wild type and single and triple mutants chy1chy2lut5 and chy1chy2lut2, described by Fiore et al. (26), were grown in low light conditions (45 μmol m⁻² s⁻¹) for 3 weeks. None of the single or double mutants displayed a visible phenotype, whereas the triple mutants chy1chy2lut2 and chy1chy2lut5 showed, respectively, paler leaves and a highly retarded growth. Since Viola and Neo are precursors of the plant growth regulator abscisic acid (ABA) (40), the lack of β-β-xanthophylls in chy1chy2lut5 and the concomitant reduction in ABA content could be in principle a cause for some of the chy1chy2lut5 phenotypes. However, chy1chy2lut5 plants sprayed daily with ABA did not show a visible phenotypic reversion (data not shown). This result is in agreement with a recent report showing that ABA deficiency is not responsible for increased photooxidation of thylakoid membranes (20).

The lut2 and lut5 mutants were included in this characterization, respectively, as lutein-less and increased α-carotene internal controls, whereas mutants npq1 (16) and npq1lut2 (7, 41) were used as a reference for increased photosensitivity (7, 18). The steps affected by the various mutations are described in Fig. 1A, whereas Fig. 1B and Table 1 show the leaf pigment composition of the mutants analyzed (see additional results in supplemental Table a1 for a complete description of leaf pigment composition of all genotypes used in this characterization).

At 45 μmol m⁻² s⁻¹, the triple mutants had Fᵥ/Fₘ ratios (the maximal photochemical yield of PSII) decreased with respect to WT (Table 1); chy1chy2lut5 scored a value of 0.68 versus 0.77 for chy1chy2lut2. In these conditions, lut2npq1, the most lightsensitive xanthophyll mutant so far described (7), had an Fᵥ/Fₘ ratio of 0.8, the same as WT (data not shown). These mutants showed a strong increase in chlorophyll a/b ratios as well as a reduced Chl content per leaf area with respect to WT and lut2 (chy1chy2lut2 45% reduction) and chy1chy2lut5 (35% reduction) (Table 1) and a severely reduced xanthophyll content with respect to WT and lut2 plants, the main leaf carotenoids being α- and/or β-carotene (Fig. 1B). β-Carotene normally binds photosynthetic reaction centers and Lhca, whereas α-carotene binds the same sites as β-carotene and is found in shade-adapted plants (42, 43). In this case, its accumulation is induced by a knock-out mutation in the LUT5 gene, which encodes a cytochrome P450 carotenoid hydroxylase (25, 26). Both lut2 and lut5 mutations induced a slight increase in the chlorophyll/xanthophyll ratio, which led to a slight decrease in functional antenna size (Table 1). Chlorophyll/xanthophyll ratio was essentially the same in both triple mutants. Still they show distinct compositions of the xanthophyll fraction; Lute and the only oxygenated carotenoid in chy1chy2lut5, whereas chy1chy2lut2 leaves contain Neo, antheraxanthin, Viola, and Zea in approximately equal amounts. lut2 and lut5 had similar functional antenna size, measured from kinetics of fluorescence rise in 3-(3,4-dichlorophenyl)-1,1-dimethyleurea, whereas chy1chy2lut5 antenna size was 57% with respect to WT, and chy1chy2lut2 antenna size was 45% with respect to lut2, consistent with their lower Chl b content (Table 1).

Chloroplast Ultrastructure and Pigment-Protein Complex Composition—Carotenoids are important ligand and structural elements of several photosynthetic subunits; thus, changes in thylakoid pigment composition can result in structural modification of photosynthetic membranes as well as in changes of the relative amount of pigment-protein complexes. Electron microscopy analysis was performed to verify if the thylakoid structure was changed as an effect of the different carotenoid composition (Fig. 2A). The mutants showed a membrane organization very similar to that of wild-type chloroplasts. All gen-
otypes formed well defined grana, containing $\sim 9.1 \pm 3.3$ (WT, lut5), $8.9 \pm 2.5$ (chy1chy2lut5), $10.1 \pm 3.6$ (lut2), $6.9 \pm 2.7$ (chy1chy2lut2) stacks. Statistical analysis revealed that only the chloroplasts from chy1chy2lut5 plants formed grana with a number of stacks significantly lower with respect to all other genotypes (Student's t test, $p < 0.05$, $n > 15$). One striking observation was the difference in the number of osmiophilic globules found; whereas WT and lut2 showed few of these globular structures (5 and 4, respectively), the two triple mutants showed a strongly increased incidence of these structures: 14 and 18, respectively, for chy1chy2lut5 and chy1chy2lut2. Moreover, these structures were also larger in the latter genotypes.

Chlorophyll proteins from wild type, lut2, lut5, chy1chy2lut5, and chy1chy2lut2 were fractionated by sucrose gradient ultracentrifugation (Fig. 2B). The amount of LHCII trimers was found to be roughly proportional to the Lute/chlorophyll ratio, in agreement with previous results (44). The chy1chy2lut5 pattern was very similar to that of WT, with abundant LHCII trimers and a low monomer level; the major difference consisted in a higher PSII core/Lhc ratio. These results show that even severe changes in $\beta$-xanthophyll content, although causing a strong reduction of biochemical antenna size, still do not yield major qualitative changes in the organization of thylakoid membranes and of the photosynthetic apparatus: the PSI-LHCl complexes were unaffected, whereas in PSII-LHCII,
Photosynthetic Functions of α- and β-Xanthophylls

the trimeric organization of LHCCI is disrupted only in the genotypes lacking Lute (Fig. 2B). Green bands were harvested from the gradient and analyzed by optical spectroscopy, and their pigment composition was determined by HPLC (Table 2). The PSI-LHCCI complexes from the two triple mutants had very similar chlorophyll/carotenoid ratios and absorption spectra with changes in the 450–500 nm range, which reflect the carotenoid composition (data not shown). The trimeric LHCCI complex, found in band 3 of WT and lut5, had a xanthophyll content per polypeptide of 4.0 (3, 45, 46), whereas LHCCI from chy1chy2lut5 has only 2.9 bound Lute molecules per 14 Chl; this evidence suggests that in this mutant site, N1 is empty, a condition already described in the absence of violaxanthin (47), and that site V1 binds Lute in the absence of Violax (45). Monomeric and trimeric LHCbs from lut5 showed a slightly lower content in β-β-xanthophylls than the corresponding fractions from WT. lut2 and chy1chy2lut2 did not have trimers; thus, the monomeric Lhc band was analyzed. Again, the xanthophyll content per Chl was lower in chy1chy2lut2 with respect to lut2 although to a lesser extent with respect to WT versus chy1chy2lut5. The pigment composition indicates that in lut2, Lute is substituted predominantly by violaxanthin, whereas in chy1chy2lut2, a more balanced content of β-β-xanthophylls is observed, including significant levels of Zea and Antherax. Separation of thylakoid proteins on SDS-PAGE, followed by quantitative immunoblotting with specific antibodies (48) revealed comparable reductions of most antenna proteins in the two triple mutants (Fig. 2C). A clear difference was observed in CP26, which was completely absent in chy1chy2lut5.

PSII Function and Excess Energy Dissipation—The chlorophyll-protein analysis (Fig. 2B) clearly showed a strong impact of the xanthophyll composition on the PSI antenna system rather than on the PSI-LHCCI complex, which appeared essentially unaffected. We then proceeded to analyze PSII function and qE. Chlorophyll fluorometry at room temperature revealed a significant reduction in Fm/Fo ratio on both triple mutants (Table 1), a parameter that reflects changes in PSII photochemical efficiency (49). We thus measured both relative ETR and qP on intact leaves and trans-thylakoid ΔPH following 9-aminoacridine quenching in isolated chloroplasts. Results are shown in Fig. 3.

Wild-type and lut5 plants showed saturation of ETR at approximately 600 μmol m⁻² s⁻¹, whereas lut2 plants, which were reported to have a reduction in antenna size (13) and, to a larger extent, chy1chy2lut2, saturated at higher light intensities. The behavior of chy1chy2lut5 was different; ETR was lower than in WT even at very low light and reached lower values at saturation (Fig. 3A), implying that efficient light use is compromised by lack of β-β-xanthophylls. It is worth noting that the chy1chy2lut2 mutant, with even stronger reduction in outer antenna content (Fig. 2C), showed ETR similar to WT and lut2 at low light and a higher rate at saturation. Thus, reduction in electron transport rate in chy1chy2lut5 cannot be exclusively related to the smaller antenna size of PSII. 1-qP, a measure of the fraction of QA reduced, was significantly lower in the triple mutants with respect to WT, lut2, and lut5 at light intensity below 800 μmol m⁻² s⁻¹, consistent with the smaller antenna size (Fig. 3B). Analysis of 9-aminoacridine quenching, a meas-

FIGURE 2. Photosystem organization. A, transmission electron micrographs of plastid from mesophyll cells of wild type and mutants. Osmiophilic globules are labeled. B, sucrose gradient fractionation of thylakoid membranes. Solubilization was performed with 0.6% α-DM. For each gradient, fractions harvested are indicated. Fractionations were repeated two times. C, quantitative Western blotting of thylakoid proteins, separated on SDS-PAGE. CP47 (PsbB)-specific signal was used as an internal standard for normalization to photosystem II reaction centers. Values significantly different from the wild type (p < 0.05) (*) or the lut2 ($) are marked as indicated. Data are expressed as means ± S.D. (n = 3).
ure of lumen acidification on isolated chloroplasts, was in accordance with ETR results, showing 50% lower H⁺ accumulation in chy1chy2lut5 with respect to chy1chy2lut2. The dependence of H⁺ accumulation on light intensity also showed that the light intensity needed for 50% of maximal 9-aminoacridine quenching was 20% higher in chy1chy2lut5 with respect to chy1chy2lut2 (Fig. 3C).

NPQ was measured on leaves (Fig. 4, A and B). Wild-type plants, upon illumination for 9 min at saturating light intensity, showed a rapid rise of NPQ, reaching a maximum value of 2.7 and relaxing to 0.25 upon 9 min of dark recovery (Fig. 4, A and B). In this analysis, we included genotypes previously described in the literature as a reference, since it was reported that low light conditions, as used in this work, may well affect both amplitude and kinetics of NPQ (50); lut2, npq1, and npq1lut2 showed NPQ kinetics in agreement with published results (7, 11, 51) with amplitude of WT > lut2 > npq1 >> npq1lut2. The triple mutants showed a strong reduction in NPQ, scoring 1.0 in chy1chy2lut2 and 0.8 in chy1chy2lut5. These values are higher than those found in npq1lut2. However, upon correction for residual quenching after 9 min of dark relaxation (photoinhibitory quenching, qE), both triple mutants showed very little or no recovery (Fig. 4, A and B), suggesting that they were photoinhibited. Net qE values are plotted as a function of light intensity (Fig. 4A) and show that, indeed, residual qE activity (0.2–0.3) could be measured in both chy1chy2lut5 and chy1chy2lut2. The two genotypes differed with respect to the light dependence of their small qE; although in chy1chy2lut5 the activity increased steadily with light intensity, in chy1chy2lut2 it was saturated already at 200 μmol m⁻² s⁻¹. lut5, a genotype used in the construction of chy1chy2lut5 (25, 26), was also analyzed in order to exclude the possibility that α-carotenoid accumulation affects NPQ. lut5 showed a maximum value of NPQ lower than WT and similar to the lut2 mutant; however, the early kinetic phase was similar to WT and faster than lut2. Fig. 4E shows that PsbS is present in all genotypes in comparable amounts.

Besides NPQ, regulation of PSII excess energy is achieved by state 1-state 2 transitions. State transitions consist in the reversible movement of LHCII from PSII and its association to PSI in order to balance the excitation pressure on both photosystems (52). We thus investigated the relationship between plant carotenoid composition and extent of state transition mechanism, using the well established chlorophyll fluorescence methods (53) with either PSII- or PSI-exciting light and saturation pulses. This function was measured in the five genotypes (supplemental Fig. a1). In WT and lut5, the transition from PSI to PSII light yielded a 7% decrease in Fm′, whereas chy1chy2lut5 yielded a 3.5% decrease only. In lut2 and chy1chy2lut2, state transitions were much smaller, yielding a 0.8% quenching. During this measurement, it was noticed that WT and lut2 underwent a decrease of Fv at the onset of far red light, implying that a fraction of PSII centers was reduced in continuous low light conditions. In contrast, both chy1chy2lut5 and chy1chy2lut2 did not show such a Fv decrease (Fig. a1), implying that the plastoquinone pool was constitutively oxidized under low light intensities (40 μmol m⁻² s⁻¹).

**Photosensitivity under Short and Long Term Stress Conditions**—Treatment of leaves with strong light produces a phototoxic oxidative stress, which can be measured as a decrease in the PSII photochemical efficiency (Fv/Fm ratio) and increase in oxidation of membrane lipids (8, 18, 38). These measurements thus allow quantifying the effect of carotenoid composition on the capacity for photoprotection. Lipid peroxides can be quantified by measuring the level of MDA (malondialdehyde, a byproduct of lipid peroxidation) as thiobarbituric acid-reactive substances before and after stress (38). Detached leaves were floated on water-imbibed filter paper and treated with 1000 μmol m⁻² s⁻¹ white light, 10 °C for 5 h, during which a time course experiment of MDA accumulation was performed (Fig. 5A). Peroxidation levels were similar for all genotypes during the first 90 min of treatment, after which the two triple mutants underwent a dramatic increase after 3 and 5.5 h. A wider analysis of MDA production, including single and double mutants, is reported as supplemental results (supplemental Fig. a2). During the whole period of high light treatment, WT, lut2, and lut5 did not show an increase of lipid peroxidation, whereas the two triple mutants not only did undergo lipid peroxidation, but the effect was even higher than in npq1lut2, the xanthophyll mutant with the highest light sensitivity described so far (7). chy1chy2lut5 was significantly more sensitive than chy1chy2lut2 (Fig. 5A). The sensitivity to light stress conditions is reported as supplemental results (supplemental Fig. a1).
was also assessed on whole plants, by exposing wild-type and mutant genotypes to two different light intensities, 750 and 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), and measuring the time course of PSII photo-inhibition. At 750 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), the half-time for complete photo-inhibition of the triple mutants was less than 1 h versus 3 h for \( \text{lut}5 \), \( \text{lut}2 \), and WT. At 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), an intensity that was never described as photo-inhibitory for a xanthophyll biosynthesis mutant, \( \text{chy1chylut}5 \) plants still showed a decline of \( F_{v}/F_{m} \) (Fig. 5C), implying an unprecedented level of photosensitivity in this genotype. Measurements of \( F_{v}/F_{m} \) recovery after a photo-inhibitory treatment clearly showed that WT and \( \text{chy1chylut}5 \) leaves have the same kinetics of PSII quantum efficiency recovery (Fig. 5D), implying that the extreme photosensitivity of lutein-only plants is due to a less effective photoprotection rather than to impaired PSII repair mechanism (30).

**Singlet Oxygen Production from Thylakoids and Isolated Proteins**—When photosynthetic organisms are exposed to light in excess, photo-oxidative stress occurs with production of singlet oxygen (54) deriving from the reaction of excited chlorophyll with molecular oxygen \( (\text{O}_2) \) to yield singlet oxygen \( (\text{O}_2) \), particularly in PSII (55). In order to compare the capacity for photoprotection of WT and mutants, we evaluated their ability in preventing \( \text{O}_2 \) production of either thylakoids and isolated pigment-protein complexes using SOSG, a highly selective probe for \( \text{O}_2 \) (see “Experimental Procedures” for details). After illumination of isolated thylakoids with increasing light intensity, both triple mutants showed, at each light intensity, higher \( \text{O}_2 \) production with respect to wild type, \( \text{lut}5 \), or \( \text{lut}2 \). The two triple mutants showed a different dependence of \( \text{O}_2 \) production on light intensity; at low light, \( \text{O}_2 \) release was significantly higher in \( \text{chy1chylut}5 \) (Fig. 6A). When \( \text{O}_2 \) production was measured on isolated pigment-protein complexes, PSII core complexes from the different genotypes did not show significant differences (Fig. 6C). Instead, the purified Lhcb fractions showed clear differences, with complexes from \( \text{chy1chylut}5 \) and \( \text{chy1chylut}2 \) yielding twice as much singlet oxygen as those from WT (Fig. 6B). Lhcb2 complexes showed an intermediate behavior between wild-type and triple mutants, consistent with previous results (13). Light-harvesting complexes form \( \text{lut}5 \) exhibited a significantly higher \( \text{O}_2 \) production with respect to WT Lhcb, consistent with their lower \( \beta-\beta \)-xanthophyll content (Table 2). It should be noted that at low light intensity, the difference was even higher, with \( \text{lut}2 \) and \( \text{lut}5 \) performing similar to WT.

**Photoprotection in Isolated Lhc Proteins**—In order to verify if the increased light sensitivity of the triple mutants was due to the disruption of mechanisms localized within antenna proteins, we measured the kinetics of photobleaching of isolated Lhc proteins under high light and low temperature (24, 46). This experiment has proven efficient in measuring ROS-mediated bleaching of antenna chromophores, by exploiting ROS produced by chlorophyll, a strong sensitizer. Photobleaching rate of isolated Lhc proteins is strongly related to photosensitivity in vivo (13, 20). Monomeric and trimeric LHCCI from \( \text{chy1chylut}5 \) are clearly more sensitive to photobleaching than the corresponding complexes isolated from...
WT and lut5 (Fig. 7). In the case of lut2 and chy1chy2lut2, this measurement was performed on monomers only; photobleaching kinetics were faster than in monomeric Lhcs from WT. When comparing Lhc monomers from lut2 and chy1chy2lut2, no significant differences in the sensitivity of the two mutants were detected. This is a clear discrepancy with respect to the data obtained from in vivo stress analysis and $^{1}$O$_{2}$ production in thylakoids and isolated Lhcb, where chy1chy2lut2 genotype proved more sensitive to photooxidation than lut2 (Fig. 7).

**DISCUSSION**

Reduced Xanthophyll Content Leads to Enhanced Light Sensitivity—The photoprotective action of carotenoids in the photosynthetic apparatus is well established (7, 18, 56) and is performed both by carotenes, bound to PSII core complexes, and by xanthophylls, mainly bound to Lhc proteins. In this work, we have analyzed two novel mutants that are both different in xanthophyll composition and reduced in the xanthophyll/carotene ratio. Irrespective of the xanthophyll composition, it can be observed that both chy1chy2lut5 and...
*chy1chy2lut2* are more sensitive to light stress with respect to WT and *lut2*. A striking difference between the WT and *lut2* on one hand and the two triple mutants on the other is the very low xanthophyll/carotene ratio in the latter. This results in a strong light sensitivity with respect to both WT and *lut2*, which appear to be independent from the xanthophyll composition, since it is found in both *chy1chy2lut5* and *chy1chy2lut2*, which exhibit mutually exclusive xanthophyll compositions. This sensitivity is associated with a high level of lipid peroxidation (Fig. 5A).

The major common characteristic between the two mutants is the strong depletion in Lhc proteins with respect to core complexes (Fig. 2C). Xanthophylls are needed for folding of Lhc proteins *in vitro* (57); thus, it is not surprising that a strong decrease in their availability leads to a decreased content in Lhc proteins. It should, however, be noted that only Lhcb proteins, particularly LHCII, are affected, whereas the Lhca protein level, as well as their assembly into PSI-LHCI supercomplexes, is not disturbed, as shown by the identical migration rate of PSI-LHCI supercomplexes in sucrose gradients (Fig. 2B). As a result of the higher stability of Lhca versus Lhcb proteins, the PSI antenna function is maintained, whereas PSII antenna function is impaired, as shown by the more oxidized redox state of plastoquinone under low continuous light, in *chy1chy2lut2* and *chy1chy2lut5* versus WT, *lut5*, and *lut2* (Fig. 3B). These results strongly suggest that photoinhibition of *chy1chy2lut2* and *chy1chy2lut5* does not derive from overreduction of the plastoquinone pool, the most usual stress condition in WT plants (58). We conclude that carotenes cannot replace xanthophylls in stabilizing Lhc proteins, leading to their strong reduction. This implies that functional Lhcb proteins are essential for photoprotection. The molecular basis for these observations can be found in the measurements in Fig. 6; thylakoids of WT and *lut2* produce far less singlet oxygen at all light intensities with respect to both *chy1chy2lut2* and *chy1chy2lut5*, yet *O₂* production from purified PSII core complexes is independent from the genotype, suggesting that Lhc proteins, in PSI-LHCII supercomplexes, might act in preventing *O₂* produc-

---

**FIGURE 5.** Photoinhibition, lipid peroxidation, and PSII repair efficiency under photoxidative stress. A, detached leaves were treated at 1000 μmol m⁻² s⁻¹ for 5 h, and kinetics of MDA formation were recorded. B and C, *F₅/F₆* ratio was measured on whole plants at two different light intensities (B, 750 μmol m⁻² s⁻¹; C, 150 μmol m⁻² s⁻¹) for a different time interval (B, 5 h; C, 7 days). D, PSII repair efficiency was quantified by measuring *F₅/F₆* recovery on whole plants in low light (30 μmol m⁻² s⁻¹) after photoinhibitory treatment (1600 μmol m⁻² s⁻¹ for WT plants and 700 μmol m⁻² s⁻¹ for *chy1chy2lut5* plants for 1.5 h). Data are expressed as means ± S.D. (n > 4). Values significantly different (p < 0.05) from the wild type (*) or the *lut2* (§) are marked as indicated.
tion and/or scavenging reactive oxygen species, as recently shown for neoxanthin bound to Lhc proteins (20).

Extreme reduction in Lhc proteins is obtained with the ch1 mutation, impairing Chl b synthesis (supplemental Table a1) and thus preventing assembly of functional light-harvesting complexes (59). Although this mutant undergoes severe photoinhibition in high light conditions, it is not affected by growth in moderate light (Fig. a3). Thus, ch1 and chy1chy2lut2 plants (with a biochemical antenna size even smaller than chy1chy2lut5) can survive without any photoinhibition to the same light intensity (150 μmol m⁻² s⁻¹) that has a deleterious effect on chy1chy2lut5. We conclude that reduced Lhc content cannot be the only reason for the extreme light sensitivity of chy1chy2lut5.

**What Is the Origin of the Extreme Photosensitivity in Lutein-only Plants?**—Although the reduction in Lhcb proteins is obtained with the chl mutation, impairing Chl b synthesis (supplemental Table a1) and thus preventing assembly of functional light-harvesting complexes (59). Although this mutant undergoes severe photoinhibition in high light conditions, it is not affected by growth in moderate light (Fig. a3). Thus, ch1 and chy1chy2lut2 plants (with a biochemical antenna size even smaller than chy1chy2lut5) can survive without any photoinhibition to the same light intensity (150 μmol m⁻² s⁻¹) that has a deleterious effect on chy1chy2lut5. We conclude that reduced Lhc content cannot be the only reason for the extreme light sensitivity of chy1chy2lut5.

**FIGURE 6. Singlet oxygen production from thylakoid and pigment-protein complexes.** SOSG fluorescence increases as effect of the light-dependent singlet oxygen (¹O₂) production from thylakoids (A), isolated Lhcb (B), and isolated monomeric PSII core complex (C). The symbols and error bars show means ± S.D. (n = 3). In A and B, statistical analysis revealed that triple mutants showed significantly higher ¹O₂ production (p < 0.05) than wild-type and lut2 at each light intensity used, whereas significantly different values (p < 0.05) between triple mutants are marked by an asterisk. See “Experimental Procedures” for details.

**FIGURE 7. Photobleaching of isolated Lhcb.** Monomeric Lhcb isolated from solubilized thylakoids of WT, lut2, lut5, and triple mutants (A) and trimeric LHCII from WT, lut5, and chy1chy2lut5 (B) were analyzed by following the Qy transition absorbance decay during strong illumination, as described under “Experimental Procedures.” Chlorophyll concentrations of Lhcb were set to 8 μg/ml. Samples were cooled to 10 °C during measurements. Data are expressed as means ± S.D. (n = 3).
explained on this basis. In fact, this genotype shows higher levels of photoinhibition and lipid peroxidation under stress conditions than any other xanthophyll mutant described to date, including npq1lut2 (7). Moreover, transfer from 45 μmol m⁻² s⁻¹ to moderate light conditions (150 μmol m⁻² s⁻¹) caused photoinhibition and visible bleaching of this mutant but not of any of the other genotypes tested. This suggests that additional sensitivity factors are present in chy1chy2lut5.

A clear difference between the two triple mutants is the presence of α-carotene in both PSI and PSII core complexes of chy1chy2lut5, partially replacing β-carotene (25). This condition mimics acclimation to deep shade (42, 43), a condition increasing sensitivity to moderate light stresses (60). However, the lut5 genotype has the same α/β-carotene ratio as chy1chy2lut5, yet no increase in lipid peroxidation under stress was observed (supplemental Fig. a2); nor did isolated PSI or PSII core complexes show differences in O₂ production (Fig. 6C). Measurements in vivo (Fig. a2) and with isolated pigment proteins (Fig. 6C) consistently show that accumulation of α-carotene in the lut5 mutant does not affect photoprotection. Additional measurements, performed upon high light stress at both 10 and 25 °C (not shown) confirmed equal sensitivity of WT and lut5, in contrast with a previous report (25).

Viola and Neo are biosynthetic precursors of ABA, suggesting that reduction in ABA synthesis in chy1chy2lut5 might affect stress resistance. Nevertheless, aba mutants show much lower levels of photosensitivity than the chy1chy2lut5 mutant, and no rescue of the chy1chy2lut5-photosensitive phenotype was observed by spraying with exogenous ABA (data not shown). A further peculiarity of chy1chy2lut5 is the complete lack of CP26 (Lhcb5), which is involved in qI (61). A decrease in CP26 was reported in the Zea-accumulating genotypes npq2 and npq2lut2 (19) and a neoxanthinless mutant (20), thus suggesting that this subunit was destabilized in vivo by lack of Viola and/or Neo. The data shown here imply that accumulation of CP26 requires at least one β-β-xanthophyll species. It is, however, unlikely that lack of CP26 can explain the extreme photosensitivity of chy1chy2lut5, since antisense inhibition of lhcb5 does not significantly affect photosensitivity (48). The efficiency of PSI repair process was comparable in WT versus lutein-only plants (Fig. 5D), thus suggesting that β-β-xanthophylls do not affect the recovery of PSI quantum efficiency after photoinhibition. Once the above options are excluded, we observe that a putative cause for chy1chy2lut5 sensitivity can be found in the properties of isolated Lhc proteins.

In fact, Lhc is the thylakoid fraction showing the highest contribution to singlet oxygen production (Fig. 6B), whereas chy1chy2lut5 exhibits the highest levels of lipid peroxidation upon stress (Fig. 5A) and the highest singlet oxygen production from whole thylakoid membranes in low light conditions (up to 400 μmol m⁻² s⁻¹) (Fig. 6A), suggesting that an impaired function within Lhcb proteins is responsible for increased oxidative stress. The experiments in Figs. 6 and 7 clarify this point; although purified Lhcb from chy1chy2lut5 and from chy1chy2lut2 have similar levels of singlet oxygen production (Fig. 6B), the photobleaching measurements (Fig. 7A) show that bleaching is much faster with Lhcb from chy1chy2lut5. In photobleaching experiments, singlet oxygen is produced by sensitization of chlorophyll and is scavenged to a different extent by carotenoids bound to the protein, the final rate of bleaching being determined by the balance between the two processes (46). The comparison between singlet oxygen production (Fig. 6B) and photobleaching rates (Fig. 7A) clearly shows that Lhc proteins from chy1chy2lut5 are less efficient than those from chy1chy2lut2 in scavenging ROS, thus preventing photobleaching. We conclude that the extreme photosensitivity of lutein-only plants (Fig. 5C) derives from a deficit of O₂, scavenging in their Lhc proteins with respect to those of chy1chy2lut2, binding only β-β-xanthophylls. We notice that reduction in ETR by 30% and trans-thylakoid ΔpH formation (50%) lead to a much higher level of growth rate inhibition (>90%) (26). This is in contrast with the recent report of tight correlation between growth rate and ETR in a PSI mutant (62). Reduced growth and photosensitivity as a consequence of singlet oxygen production have been recently described in the flu mutant accumulating the photosensitizer protochlorophyllide during dark periods, which act as a signal modulating gene expression (63). It is possible that part of growth reduction observed in chy1chy2lut5 is due to an inhibiting signal from O₂.

Chilling temperatures were shown to induce PSI photoinhibition in some plant species (64). However, previous results in Arabidopsis demonstrated that, using more severe stress conditions than those used in the present work, neither WT nor npq1 plants showed PSI photoinhibition (65). This is consistent with the extreme plasticity of PSI-LHCl regarding xanthophyll composition; in all mutants analyzed, changes in xanthophyll composition strongly affect the antenna size of PSII, whereas Lhca protein level as well as their assembly into PSI-LHCl supercomplexes is not disturbed, as shown by the identical migration rate of PSI-LHCl supercomplexes in sucrose gradients. Nevertheless, since Lhcas bind a significant level of Viola, we cannot completely exclude the possibility that part of the photosensitivity of lutein-only plants was related to the PSI antenna system.

Xanthophyll Species Accomplish Distinct Roles in Photoprotection of Lhc Proteins—We have shown above that the presence of only Lute in Lhc proteins causes photoinhibition in vivo, in isolated thylakoids, and in purified Lhc proteins. This is somehow surprising, since it has been reported that luteinless mutants show increased photosensitivity (13), particularly in the absence of zeaxanthin (7). However, previous work with recombinant Lhc proteins reconstituted in vitro with different xanthophylls has shown that the resistance to photobleaching is maximal when the complexes are reconstituted with more than a single xanthophyll species, particularly Lute and a β-β-xanthophyll (violaxanthin and/or neoxanthin), whereas Lute-only complexes are more sensitive and Viola-only complexes even more so (24, 32). These reports, fully consistent with the results obtained with chy1chy2lut2 and chy1chy2lut5, suggest that, although all are involved in photoprotection, the role of each xanthophyll species is distinct in Lhc proteins. Recently, we have reported a specific activity for neoxanthin in scavenging superoxide anions (20). Lute has been shown to be a better

5 L. Dall’Osto, A. Fiore, S. Cazzaniga, G. Giuliano, and R. Bassi, unpublished results.
quencher for triplet chlorophyll (3Chl*) than violaxanthin in vivo and in vitro (13). Here, we show, both in vivo and in vitro, that the resistance of plants, thylakoid, and purified Lhc proteins to excess illumination is better obtained when α- and β-xanthophylls are available together. Violaxanthin is active in 3Chl* quenching, although to a lower extent with respect to Lute (13). We now show that Lute alone is unable to sustain single oxygen scavenging (Fig. 6, A and B), thus causing rapid photo bleaching of Chl bound to Lhc proteins (Fig. 7, A and B).

We conclude that photoprotection of Lhc proteins requires the cooperative action of xanthophyll species specialized in 3Chl* quenching and ROS scavenging during normal photosynthetic activity. Imbalance between these activities, as a result of a simplified xanthophyll composition, yields enhanced photosensitivity.

Quantitative Limitation in β-β-Xanthophyll Availability Increases Singlet Oxygen Production—Adaptation to high light conditions includes overaccumulation of the β-β-xanthophylls belonging to the xanthophyll cycle (45, 66, 67), whereas overexpression of β-hydroxylase increases the Viola-Anthera-Zea pool and resistance to light stress (23). Mutations in carotenoid hydroxylation limit flow toward final products of xanthophyll biosynthesis pathways (25, 26). As a result, the xanthophyll/chlorophyll ratio is decreased and limits Lhc protein assembly and steady state levels (Fig. 2, B and C). In these conditions, moreover, we can observe an increased level of light sensitivity and singlet oxygen production. The effect of xanthophyll availability for binding to Lhc proteins can be better appreciated by comparing lut2 with chy1chy2lut2, since their Lhc both have a similar xanthophyll composition. Nevertheless, production of singlet oxygen from thylakoids and isolated Lhc proteins is higher in the latter (Fig. 6, A and B). An obvious difference between the Lhc preparation in chy1chy2lut2 and lut2 is the relative abundance in the different gene products (Fig. 2C), showing that Lhcb1 to -3, components of LHCII and Lhcb4 (CP29), are substantially reduced, whereas Lhcb5 (CP26) and Lhcb6 (CP24) are not affected. We conclude that a major site of singlet oxygen scavenging is LHCII.

Deficiency in β-β-Xanthophylls Results in Reduction of qE—Both chy1chy2lut2 and chy1chy2lut5 are strongly depleted in NPQ, whose amplitude is close to 0 (Fig. 4 and supplemental Table a1), thus ruling out the possibility that this photoprotection mechanism as a source for their differential photosensitivity. Nevertheless, NPQ and, in particular, qE is strongly dependent on xanthophyll composition (16). The strong reduction of qE in chy1chy2lut2 can be explained by its xanthophyll composition, similar to that of npq1lut2 mutant, a qE null mutant, because of its lack of both Lute and Zea (7). We can observe that chy1chy2lut2 has a somehow higher Zea content with respect to npq1lut2. Since both PsbS levels and the capacity to build up a transmembrane pH gradient are maintained, we conclude that in chy1chy2lut2, Zea is bound to a site unable to induce qE. NPQ values measured in chy1chy2lut2 plants are in agreement with a previous report (68) showing the correlation between xanthophyll content and amplitude of qE; in Arabidopsis thaliana, the antisense β-hydroxylation lines, when crossed into the lut2 background, yielded a ∼32% reduction in xanthophyll content and a ∼35% reduction in NPQ value with respect to lut2. The case of chy1chy2lut5 is different, since Lute is required for qE (7). Nevertheless, antisense inhibition of β-hydroxylase (69) yielded a 50% reduction in Neo and Viola and a significant reduction in qE. Consistently, we show that the lutein-only mutant cannot operate qE. We conclude that a β-β-xanthophyll is needed for operation of qE. Since the aba4 mutant, depleted in neoxanthin, has unaffected qE (20), this xanthophyll can be violaxanthin, when associated to Lute or Zea that sustains qE as the only xanthophyll (19, 44). It can be noticed that chy1chy2lut5 exhibits a reduction in ETR and thylakoid trans-membrane ΔpH (Fig. 3, A and B). This, in principle, could contribute to qE reduction. However, WT at light intensity corresponding to a 50% reduction in transmembrane pH gradient (Fig. 3B) develops qE of ∼1, implying that lutein-only plants have a substantially reduced qE, irrespective of lumen acidification.

Conclusions—The triple mutants here described prove that Arabidopsis can survive, under low light conditions, with only 25% of its leaf carotenoids being represented by xanthophylls and with almost null levels of qE, thus confirming the very high plasticity of its photosynthetic apparatus. Lute as the only xanthophyll induces an extreme photosensitivity due to loss of singlet oxygen-scavenging capacity in Lhc proteins. This result implies that light harvesting by antenna proteins constitutively causes the formation of singlet oxygen, which is scavenged by β-β-xanthophylls before it exits the Lhc pigment-protein complexes. α- and β-xanthophylls specialize in triplet quenching and ROS scavenging, respectively. Impairing of one of these functions leads to photodamage. We show that β-xanthophylls are also indispensable for the activation of qE. Furthermore, in the two triple mutants, limitation of the xanthophyll availability leads to limitation in the accumulation of Lhcb proteins, particularly LHCII, whereas Lhca protein level as well as their assembly into PSI-LHCI supercomplexes are not affected. This is probably due to the presence of carotene binding sites in LHCI (70, 71).

Acknowledgment—We thank Paolo Bernardi for help in sample preparation for electron microscopy.

REFERENCES

1. Yamamoto, H. Y., Nakayama, T. O., and Chichester, C. O. (1962) Arch. Biochem. Biophys. 97, 168–173
2. Demmig-Adams, B., and Adams, W. W. (1992) Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 599–626
3. Liu, Z., Yan, H., Wang, X., Kuang, T., Zhang, J., Gui, L., An, X., and Chang, W. (2004) Nature 428, 287–292
4. Bassi, R., Pineau, B., Daïnese, P., and Marquardt, I. (1993) Eur. J. Biochem. 212, 297–303
5. Morosinotto, T., Caffarrì, S., Dall’Osto, L., and Bassi, R. (2003) Physiol. Plant. 119, 347–354
6. Gilmore, A. M. (2001) Photosynth. Res. 67, 89–101
7. Niyogi, K. K., Shih, C., Chow, W. S., Pogson, B. J., DellaPenna, D., and Bjorkman, O. (2001) Photosynth. Res. 67, 139–145
8. Baroli, I., Do, A. D., Yamane, T., and Niyogi, K. K. (2003) Plant Cell 15, 992–1008
9. Baroli, I., Gutman, B. L., Ledford, H. K., Shin, J. W., Chin, B. L., Havaux, M., and Niyogi, K. K. (2004) J. Biol. Chem. 279, 6337–6344
10. Niyogi, K. K., Bjorkman, O., and Grossman, A. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14162–14167
