DIFFERENT ROLES OF ALPHA- AND BETA-BRANCH XANTHOPHYLLS IN PHOTOSYSTEM ASSEMBLY AND PHOTOPROTECTION

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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 non-photochemical 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 an 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 LHCII 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, while carotenoid composition of photosystem II core complex was influential. In-depth analysis of the mutant phenotypes suggests that alpha-branch (lutein) and beta-branch (zeaxanthin, violaxanthin, neoxanthin) xanthophylls have distinct and complementary roles in antenna protein assembly and in the mechanisms of photoprotection.

Carotenoids (car)1 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 (xant) form a sub-group 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, β-carotene binds to reaction center subunits of both photosystem I (PSI) and II.
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(PSII), while xanthophylls are both accessory pigments and structural elements of light-harvesting complexes (Lhc). Together with β-carotene, they act both as chromophores, absorbing light energy which 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 in two distinct branches: hydroxylation of α-carotene gives rise to lutein (Lute), the most abundant xanthophyll in leaves (Figure 1), while hydroxylation of β-carotene gives rise to zeaxanthin (Zea). In normal light conditions zeaxanthin is epoxidized into antheraxanthin (Anthera) and violaxanthin (Viola) (Figure 1) while in excess light de-epoxidation prevails, leading to the accumulation of Zea (1, 2).

LHCII, the major light harvesting complex of photosystem II binds Lute, Viola and neoxanthin (Neo) (Figure 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 proteins and is essential for protein folding and for photoprotection (10-13). Zea exerts several photoprotective roles: (a) through the enhancement of Non Photochemical Quenching (NPQ) of excess light energy (14-17); (b) through the protection of thylakoid lipids from peroxidation (8, 18); (c) through the reduction of PSII antenna size (13, 19). Neo has a role in the protection of photosynthetic apparatus by promoting the scavenging of superoxide anions, a ROS formed in the Mehler reaction (20). The function of Viola is more controversial: it has been suggested to be involved both in the reversion of NPQ (21) and in chlorophyll (Chl) triplet quenching (22). A previous report showed that the over-accumulation of Viola increases photoprotection in vivo (23). Furthermore, cooperative effects on photoprotection by different xanthophyll species have been suggested by early experiments using recombinant Lhc proteins reconstituted with different xanthophylls (24).

These data suggest that different xanthophyll species may have distinct roles in photoprotection. Nevertheless, the role of β-β-xanthophylls (in particular, Neo and Viola) in the photosynthetic process is not completely understood. The recent elucidation of the higher plant β-ring hydroxylation (25, 26) showed that two different classes of enzymes are involved in hydroxylation of the ε- and β-ionone rings of carotenes: ferredoxin-dependent di-iron oxygenases (CHY1 and CHY2), active in β-ring hydroxylation, and cytochromes P450: LUT1, required for ε-ring hydroxylation and LUT5, showing in vivo a major hydroxylation activity on the β-ring of α-carotene as well as minor activity on the β-rings of β-carotene. Plant mutants are now available, specifically lacking xanthophylls in the β-β-branch (Neo, Viola, Zea) and useful for the analysis of xanthophyll function in photosynthesis.

In this work, we compare the photosystem structure and photoprotection properties of two triple mutants containing low levels of xanthophylls, but mutually exclusive in composition: chy1chy2lut5 contains only Lute, while chy1chy2lut2 contains only β-β-xanthophylls (Neo, Viola, Zea). We find that Lute alone is unable to provide sufficient levels of photoprotection even in moderate light, thus conferring to the chy1chy2lut5 mutant the highest sensitivity to photooxidation ever described in a xanthophyll mutant.

Experimental Procedures

Plant material - T-DNA insertion mutants were identified in the Syngenta and Salk collections. Individual mutants were crossed, and F1 seeds were grown and self-fertilized to obtain the F2 generation. The genotype of the F2 individuals was checked by PCR using gene-specific primers and T-DNA primers (26).
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All mutants were in the Columbia background and were grown at 22 °C under a photoperiod of 16 h light (45 µmol m⁻² s⁻¹) and 8 h darkness.

In vivo fluorescence and NPQ measurements – Non-photochemical quenching of chlorophyll fluorescence, qP (photochemical quenching) and PSII yield (ΦPSII) was measured on whole leaves at RT (room temperature, 22°C) with a PAM 101 fluorimeter (Walz, Effeltrich, Germany). Minimum fluorescence (F₀) was measured with a 0.15 µmol m⁻² s⁻¹ beam, maximum fluorescence (Fₘ) was determined with a 0.6 sec light pulse (4500 µmol m⁻² s⁻¹), white continuous light (1200 µmol m⁻² s⁻¹) was supplied by a KL1500 halogen lamp (Schott, Mainz, Germany). NPQ, qP and relative electron transport rate (ETR) were calculated according to the following equation (27): NPQ = (Fₘ - Fₘ')/Fₘ', qP = (Fₘ - Fₛ)/(Fₘ - F₀), rel ETR = ΦPSII · PAR, where Fₘ is the maximum Chl fluorescence from dark-adapted leaves, Fₘ' the maximum Chl fluorescence under actinic light exposition, Fₛ the stationary fluorescence during illumination, PAR the photosynthetic active radiations (white light, measured as µmol m⁻² s⁻¹).

∆pH-dependent component of chlorophyll fluorescence quenching (qE) was performed as described previously (28). Variable fluorescence was induced in leaf discs, infiltrated with DCMU 3.0 10⁻⁵ M, sorbitol 150 mM, with a green light of 7 µmol m⁻² s⁻¹ produced by a light emitting diode. The time corresponding to 2/3 of the fluorescence rise (T₂/₃) was taken as a measure of the functional antenna size of PSII (29). In DCMU-treated leaves, rate of fluorescence rise depends on light intensity and functional antenna size of PSII. Thus, keeping constant the saturating flash intensity, PSII with higher functional antenna size will reduce more rapidly all the available QA pool, and will have a lower T₂/₃ of fluorescence raise.

For measurements of PSII repair process, whole plants were illuminated at RT for 1.5 hours to induce 50 to 60% photoinhibition of PSII (PPFD of 1800 µmol m⁻² s⁻¹ for WT plants, 700 µmol m⁻² s⁻¹ for chy1chy2lut5 plants) and restoration of the Fv/Fm ratios was subsequently followed at irradiances of 30 µmol m⁻² s⁻¹ at RT (30).

Pigment analysis - Pigments were extracted from dark-adapted leaf discs: samples were frozen in liquid nitrogen, ground in 85% acetone buffered with Na₂CO₃, then the supernatant of each sample was recovered after centrifugation (15 min. at 15,000 g, 4° C); separation and quantification of pigments were performed by HPLC (31) and by fitting of the spectrum of the acetone extract with spectra of individual pigments (32).

Thylakoid isolation and thylakoid protein preparation – Unstacked thylakoids were isolated from leaves as previously described (33). Membranes corresponding to 500 µg of chlorophylls were washed with 5 mM EDTA and then solubilised in 1 ml of 0.6% α-DM) 10 mM HEPES pH 7.5. Solubilised samples were then fractionated by ultracentrifugation in a 0.1-1 M sucrose gradient containing 0.06% α-DM, 10 mM HEPES pH 7.5 (22 h at 280,000 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 µg of chlorophylls were loaded for each sample and electobotted 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 (35). Signal amplitude was quantified using the GelPro 3.2 software (BIORAD).

Electron microscopy – Intact leaf fragments from wild-type and mutant 3-weeks-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, USA) at RT. Fluorescence emission spectra were measured at RT using a Jobin-Yvon Fluoromax-3 spectrofluorimeter at RT.

Measure of ΔpH – The kinetic of ΔpH formation across the thylakoid membrane was measured using the method of 9-aminoacridine (9-AA) fluorescence quenching, as previously described (37). Reaction buffer composition: 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.
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**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 hours) in a growth chamber at low temperature (10° C), then immediately frozen in liquid nitrogen. Photoxidative 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 (TBA). The MDA-(TBA)₂ complex was separated from other TBA 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 Probe, Eugene). SOSG is a fluorescent probe highly selective for ¹O₂ that increase its 530 nm emission band in 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 the 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 yield of SOSG (λₑᵥₐ 480 nm, λₑₘᵢₙ 530 nm) were determined before and after light treatment in order to quantify ¹O₂-dependent fluorescence increase.

**RESULTS**

**Pigment composition**

Wild-type, single and triple mutants chy1chy2lut2 and chy1chy2lut5, 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, while 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 daily sprayed 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 a lutein-less and increased α-carotene internal controls, while 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 Figure 1A, while Figure 1B and Table 1 show the leaf pigment composition of the mutants analyzed (see additional results, 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, vs 0.77 for chy1chy2lut2. In these conditions, lut2npq1, the most light sensitive xanthophyll mutant so far described (7), had Fᵥ/Fₘ ratio of 0.8, 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 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 (Figure 1B). β-carotene normally binds photosynthetic reaction centers and Lhca, while 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
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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 is the only oxygenated carotenoid in chy1chy2lut5, while chy1chy2lut2 leaves contain Neo, Anthera, Viola and Zea in approximately equal amounts. lut2 and lut5 had similar functional antenna size, measured from kinetics of fluorescence rise in DCMU, while chy1chy2lut5 antenna size was 57% with respect to WT and chy1chy2lut2 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 effect of the different carotenoid composition (Figure 2A). The mutants showed a membrane organization very similar to that of wild-type chloroplasts. All genotypes formed well-defined grana, containing approximately 9.1 ± 3.3 (WT, lut5), 8.9 ± 2.5 (chy1chy2lut5), 10.1 ± 3.6 (lut2), 6.9 ± 2.7 (chy1chy2lut2) stacks. Statistical analysis revealed that only the chloroplasts from chy1chy2lut2 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: while WT and lut2 showed few of these globular structures, respectively 5 ± 3 and 4 ± 2 per chloroplast, the two triple mutants showed a strongly increased incidence of these structure: 14 ± 5 and 18 ± 6, 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 (Figure 2B). The amount of LHCII trimers was found to be roughly proportional to Lute/chlorophyll ratio, in agreement with previous results (44). The chy1chy2lut5 pattern was very similar to that of WT, with abundant LHCII trimers and low monomers level, the major difference consisted into a higher PSII core/Lhc ratio. These results show that even severe changes in β-xanthophyll content, although causing a strong reduction of biochemical antenna size, still do not yield into major qualitative changes in the organization of thylakoid membranes and of the photosynthetic apparatus: the PSI-LHCI complex results unaffected while in PSII-LHCCI, the trimeric organization of LHCCI is disrupted only in the genotypes lacking Lute (Figure 2B). Green bands were harvested from the gradient, analyzed by optical spectroscopy and their pigment composition determined by HPLC (Table 2). The PSI-LHCI 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), while LHCCI from chy1chy2lut5 has only 2.9 bound Lute molecules per 14 Chl; this evidence shows that even severe changes in 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 vs chy1chy2lut5. The pigment composition indicates that in lut2 Lute is substituted predominantly by violaxanthin, while in chy1chy2lut2 a more balanced content of β-β-xanthophylls is observed, including significant levels of Zea and Anthera. Separation of thylakoid proteins on SDS-PAGE, followed by quantitative immunoblotting with specific antibodies (48) revealed comparable reductions
photosynthetic functions of alpha- and beta-xanthophylls of most antenna proteins in the two triple mutants (Figure 2C). A clear difference was observed in CP26, which was completely absent in chy1chy2lut5.

PSII function and excess energy dissipation

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

Wild-type and lut5 plants showed saturation of ETR at approx 600 µmol m⁻² s⁻¹ while 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 (Figure 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 (Figure 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 (Figure 3B). Analysis of 9-aminoacridine quenching, a measure of lumen acidification on isolated chloroplasts, were 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 (Figure 3C).

NPQ was measured on leaves (Figure 4A, B). Wild-type plants, upon illumination for 9 minutes 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 dark recovery (Figure 4A, B). In this analysis we included genotypes previously described in 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, qI), both triple mutants showed very little or no recovery (Figure 4A, B), suggesting that they were photoinhibited. Net qE values are plotted as a function of light intensity (Figure 4C, D) 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: while in chy1chy2lut2 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 that α-carotene 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. Figure 4E shows that PsbS is present in all genotypes in comparable amounts.

Besides NPQ, regulation of PSII excess energy is achieved by state1-state2 transitions. State transitions consist in the reversible movement of LHCl 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
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mechanism, using the well-established chlorophyll fluorescence methods (53) with either PSI-II or PSI-exciting light and saturation pulses. This function was measured in the five genotypes (Additional Figure a1). In WT and lut5 the transition from PSI to PSII light yielded a 7% decrease in Fm while 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 Fs 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 Fs decrease (Figure a1), implying that 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 photooxidative 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 by-product 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 hours, during which a time course experiment of MDA accumulation was performed (Figure 5A). Peroxidation levels were similar for all genotypes during the first 90 minutes of treatment, after which the two triple mutants underwent a dramatic increase after 3 hrs and 5.5 hours. A wider analysis of MDA production, including single and double mutants, is reported as additional results (Additional Figure a2). During the whole period of high light (HL) treatment, WT, lut2 and lut5 did not show increase of lipid peroxidation, while 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 (Figure 5A). The sensitivity to light stress was also assessed on whole plants, by exposing wild-type and mutant genotypes to 2 different light intensities, 750 and 150 µmol m⁻² s⁻¹, and measuring the time-course of PSII photoinhibition. At 750 µmol m⁻² s⁻¹ (Figure 5B) the half time for complete photoinhibition of the triple mutants was less than one hour vs 3 hours for lut5, lut2 and WT. At 150 µmol m⁻² s⁻¹ an intensity that was never described as photoinhibitory for a xanthophyll biosynthesis mutant, chy1chy2lut5 plants still showed a decline of Fv/Fm (Figure 5C) implying an unprecedented level of photosensitivity in this genotype. Measurements of Fv/Fm recovery after a photoinhibitory treatment clearly showed that WT and chy1chy2lut5 leaves have the same kinetic of PSII quantum efficiency recovery (Figure 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 (1O₂) to yield singlet oxygen (1O₂), particularly in PSII (55). In order to compare the capacity for photoprotection of WT and mutants, we evaluated their ability in preventing 1O₂ production of either thylakoids and isolated pigment-protein complexes using SOSG, a highly selective probe for 1O₂ (See methods for details). After illumination of isolated thylakoids with increasing light intensity, both triple mutants showed, at each light intensity, higher 1O₂ production with respect to wild type, lut5 or lut2. The two triple mutants showed a different dependence of 1O₂ production on light intensity: at low light, 1O₂ release was significantly higher in chy1chy2lut5 (Figure 6A). When 1O₂ 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
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fractions showed clear differences, with complexes from chy1chy2lut5 and chy1chy2lut2 yielding twice as much singlet oxygen as those from WT (Figure 6B). lut2 complexes showed an intermediate behavior between wild-type and triple mutants, consistent with previous results (13). Light-harvesting complexes form lut5 exhibited a significantly higher $^1$O$_2$ production with respect to WT Lhcb, consistent with their lower $\beta$$\beta$-xanthophyll content (Table 2). It should be noticed that at low light intensity the difference was even higher, with lut2 and lut5 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 LHCII from chy1chy2lut5 are clearly more sensitive to photobleaching than the corresponding complexes isolated from WT and lut5 (Figure 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 Lhc, where chy1chy2lut2 genotype proved more sensitive to photooxidation than lut2 (Figure 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 by both carotenes, bound to PSII core complexes, and by xanthophylls, mainly bound to Lhcb 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 from 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 into 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 to 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 noticed that only Lhcb proteins, particularly LHCII, are affected while 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 (Fig. 2B). As a result of the higher stability of Lhca vs Lhcb proteins, the PSI antenna function is maintained while PSI antenna function is impaired, as shown by the more oxidized redox state of plastoquinone under low continuous light, in chy1chy2lut2 and chy1chy2lut5 vs WT, lut5 and lut2 (Figure 3B). These results strongly suggest that photoinhibition of chy1chy2lut2 and chy1chy2lut5 does not derive from over-reduction of 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 on Figure 6: thylakoids of WT and lut2 produce far less
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singlet oxygen at all light intensities with respect to both chy1chy2lut2 and chy1chy2lut5; yet, \( O_2 \) production from purified PSII core complexes is independent from the genotype, suggesting that Lhc proteins, in PSII-LHCII supercomplexes, might act in preventing \( O_2 \) production 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 chl mutation, impairing Chl \( b \) synthesis (Additional 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 (Figure a3). Thus, chl and chy1chy2lut2 plants (with a biochemical antenna size even smaller than chy1chy2lut5) can survive without any photoinhibition to the same light intensity (150 \( \mu m \)ol \( m^{-2} s^{-1} \)) that results deleterious for chy1chy2lut5. We conclude that reduced Lhc content cannot be the only reason for the extreme light sensitivity of chy1chy2lut5.

What’s the origin of the extreme photosensitivity in lutein-only plants?

Although the reduction in Lhc proteins is likely to account for sensitivity of the triple mutants to strong light treatments, the extreme behavior of chy1chy2lut5 can hardly be 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 \( \mu m \)ol \( m^{-2} s^{-1} \) to moderate light conditions (150 \( \mu m \)ol \( m^{-2} s^{-1} \)) 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 \( \alpha \)-carotene in both PSI and PSII core complexes of chy1chy2lut5, partially replacing \( \beta \)-carotene (25, Dall’Osto et al. unpublished results). 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 \( \alpha/\beta \)-carotene ratio as chy1chy2lut5 and yet no increase in lipid peroxidation under stress was observed (Additional Figure a2) nor isolated PSII core complexes show differences in \( O_2 \) production (Figure 6C). Measurements in vivo (Figure a2) and with isolated pigment proteins (Figure 6C) consistently show that accumulation of \( \alpha \)-carotene in lut5 mutant does not affect photoprotection. Additional measurements, performed upon high light stress at both 10\( \degree \) C and 25\( \degree \) 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 neoxanthin-less 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 \( \beta \)-\( \beta \)-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 PSII repair process was comparable in WT vs lutein-only plants (Figure 5D), thus suggesting that \( \beta \)-\( \beta \)-xanthophylls do not affect the recovery of PSII 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) while 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 \( \mu m \)ol \( m^{-2} s^{-1} \), Fig. 6A), suggesting that an impaired function within Lhc proteins is responsible for increased
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oxidative stress. The experiments in Figures 6 and 7 clarify this point: while 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 1O2 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%) leads 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 has 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 1O2.

Chilling temperatures were shown to induce PSI photoinhibition in some plant species (64). However, previous results in Arabidopsis demonstrated that, using stress conditions ever more severe to those used in the present work, neither WT nor npq1 plants showed PSI photoinhibition (65). This is consistent with the extreme plasticity of PSI-LHCI regarding xanthophyll composition: in all mutants analyzed, changes in xanthophyll composition strongly affect antenna size of PSII, while 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.

Nevertheless, since Lhcas bind significant level of Viola, we cannot completely exclude that part of the photosensitivity of lutein-only plants was related to the PSI antenna system as effect of β-β-xanthophylls lacks.

Xanthophyll species accomplish distinct roles in photoprotection of Lhcb proteins.

We have shown above that the presence of only Lute in Lhc proteins causes photooxidation in vivo, in isolated thylakoids and in purified Lhc proteins. This is somehow surprising since it has been reported that lutein-less mutants show increased photosensitivity (13) particularly in the absence of zeaxanth in (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) while 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 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 quencher for triplets 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 alpha and beta 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 (Figure 6A, B) thus causing rapid photobleaching of Chl bound to Lhc proteins (Figure 7A, 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. Unbalance between these activities, as a result of a simplified xanthophyll composition, yields enhanced photosensitivity.
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Quantitative limitation in β-β-xanthophyll availability increases singlet oxygen production.

Adaptation to high light conditions includes over-accumulation of the β-β-xanthophylls belonging to the xanthophyll cycle (45, 66, 67) while over-expression of β-hydroxylase increases VAZ and resistance to light stress (23). Mutations in carotenoid hydroxylation limit flow towards final products of xanthophyll biosynthesis pathways (25, 26). As a result, the xanthophyll/chlorophyll ratio is decreased and limits Lhcb protein assembly and steady state levels (Figure 2B, 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 to 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 (Figure 6A, B). An obvious difference between the Lhcb preparation in chy1chy2lut2 and lut2 is the relative abundance in the different gene products (Figure 2C) showing that Lhcb1-3, components of LHCII and Lhcb4 (CP29) are substantially reduced while 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 zero (Figure 4 and Additional Table a1), thus ruling out 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 capacity to build up a transmembrane pH gradient is 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 A. thaliana, the antisense β-hydroxylation lines, when crossed into the lut2 background, yielded into a ~32% reduction in xanthophylls 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 beta-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 reduction in ETR and thylakoid trans-membrane ΔpH (Figure 3A, B). This, in principle, could contribute to qE reduction. However, WT at light intensity corresponding to a 50% reduction in transmembrane pH gradient (Figure 3B) develops qE ~ 1, implying that lutein-only plants have a substantially reduced qE, irrespectively of lumen acidification.

Conclusions

The triple mutants here described proves 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 are respectively specialized in triplet quenching and ROS scavenging. Impairing of one of these functions leads to photo damage. We show that β-xanthophylls are also indispensable for the activation of qE.
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Furthermore, in the two triple mutants, limitation of the xanthophyll availability leads to limitation in the accumulation of \( Lhcb \) proteins, particularly LHCII, while Lhca protein level, as well as their assembly into PSI-LHCl supercomplexes, are not affected. This is probably due to the presence of carotene binding sites in LHCl (70, 71).
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FOOTNOTES

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1The abbreviations used are: \(^1\)O\(_2\), singlet oxygen; 9-AA, 9-aminoacridine; ABA, abscisic acid; \(\alpha(\beta)\)-DM: \(n\)-dodecyl-\(\alpha(\beta)\)-D-maltoside; Antera, antheraxanthin; Car, Carotenoid; Chl a,b, chlorophyll a,b; \(^2\)Chl\(^*\), triplet excited state of chlorophyll; DCMU (3-(3,4-dichloro-phenyl)-1,1-dimethylurea); ETR, electron transport rate; \(F_o\), chl fluorescence intensity with all PSII traps open in non-energized, dark-adapted conditions; \(F_m\), chl fluorescence intensity with all PSII traps closed in non-energized conditions; \(F_m'\), chl fluorescence intensity with PSII traps closed in energized conditions; \(F_o'\), chl fluorescence intensity with PSII traps open in energized conditions; \(F_o, F_o-F_o'\), HL, high light; \(Lhca(b)\), light harvesting complex of Photosystem I(II); LHCI, antenna complex of Photosystem I; LHCII, major light-harvesting complex of PSII; Lute, lutein; MDA, malondialdehyde; Neo, neoxanthin; NPQ, Non-photochemical quenching; qE, \(\Delta\)pH-dependent component of NPQ; qI, photoinhibition quenching; qP, photochemical quenching; PSI (II), Photosystem I (II); ROS, reactive oxygen species; RT, room temperature; SOSG, singlet oxygen sensor green; TBA, thiobarbituric acid adduct; VAZ, Viola-Anthera-Zea pool; VDE, violaxanthin de-epoxidase; Viola, violaxanthin; WT, wild type; Xant, xanthophylls; Zea, zeaxanthin; ZEP, zeaxanthin epoxidase.
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FIGURE LEGENDS

Figure 1. Pigment composition. (A) Biosynthetic pathway of carotenoids in *Arabidopsis thaliana*; names of the enzymes controlling each step are indicated. When different from enzyme, the mutant names are given within brackets. (B) Carotenoid composition of dark-adapted leaf from wild type and mutant genotypes. Data are expressed as mean, n=3.

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 thylakoids membranes. Solubilization was performed with 0.6\% α-DM. For each gradient, fractions harvested are indicated. Fractionations were repeated 2 times. (C) Quantitative Western blotting of thylakoids proteins, separated on SDS-PAGE. CP47 (PsbB) specific signal was used as internal standard for normalization to photosystem II reaction centres. Values significantly different from the wild type (P<0.05) (*) or the lut2 (§) are marked as indicated. Data are expressed as means ± SD, n=3.

Figure 3. Analysis of PSII function during photosynthesis. (A) Rate of electron transport. This was measured on whole leaves during illumination with increasing levels of white light. (B) PSII redox state (1 – qP) during steady-state photosynthesis. Leaf discs were given 23 min of illumination in the presence of saturating CO₂. (C) Kinetic of 9-aminocarboxylic quenching as a measure of ΔpH across the thylakoid membranes. Lumen acidification was induced in intact chloroplast after illumination for 2 min with red light (λ>600 nm) at increasingly light intensities. Symbols and error bars show means ± SD (n = 3). Significantly different values (P<0.05) than the wild type are marked by an asterisk. PFD, photon flux density; ETR, electron transport rate; qP, photochemical quenching; 9-AA, 9-aminocarboxylic. WT (solid line), lut5 (dash), chylchy2lut5 (dot), lut2 (dash-dot), chylchy2lut2 (dash-dot-dot).

Figure 4. NPQ analysis of WT and mutant genotypes. Kinetic of non-photochemical quenching formation and relaxation, measured on (A) wild type, lut2, npq1lut2 and chylchy2lut2, (B) wild type, lut5, npq1, chylchy2lut5. Chlorophyll fluorescence was measured during 9 min of illumination at 1100 μmol m⁻² s⁻¹ followed by 9 min of dark relaxation. (C, D) qE, the ΔpH-dependent component of non-photochemical quenching (NPQ), was monitored in intact, dark-adapted leaves. Leaves were given 20 minutes of illumination followed by 20 min of dark relaxation. (E) Immunoblotting of thylakoid membranes with anti-PsbS antibody. Additional genotypes, whose NPQ has already been described in previous report, have been measured and reported here in order to value effects of growth in low light. Symbols and error bars shown means ± SD (n = 4). See Experimental Procedures for details.

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 hours, and kinetic of malondialdehyde (MDA) formation were recorded. (B, C) Fv/Fm 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 hours; C, 7 days). (D) PSII repair efficiency was quantified by measuring Fv/Fm 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 chylchy2lut5 plants for 1.5 hours). Data are expressed as means ± SD (n > 4). Significantly different values (P<0.05) than the wild type (*) or the lut2 (§) are marked as indicated.

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 (A) thylakoids, (B) isolated Lhcb, (C) isolated monomeric PSII core complex. Symbols and error bars
photosynthetic functions of alpha- and beta-xanthophylls show means ± SD (n = 3). In panels 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, while significantly different values (P<0.05) between triple mutants are marked by an asterisk. See Methods for details. SOSG, singlet oxygen sensor green.

**Figure 7. Photobleaching of isolated Lhcb.** (A) Monomeric Lhcb isolated from solubilized thylakoids of WT, lut2, lut5 and triple mutants and (B) trimeric LHCII from WT, lut5 and chylchy2lut5 were analysed by following the Qy-transition absorbance decay during strong illumination, as described in 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 ± SD, n=3.
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Tables

Table 1. Pigment content and photosynthetic parameters Fv/Fm and T2/3 of dark-adapted leaf tissue from wild type and mutant genotypes. Parameters were obtained from measurements on the same samples described in Figure 1B. The time of the fluorescence rise (T2/3) was measured in DCMU-infiltrated leaves, and was taken as a measure of the functional antenna size of photosystem II. Values significantly different from the wild type (P<0.05) (*) or the lut2 (§) are marked as indicated. Data are expressed as means ± SD, n=3. Abbreviations: chl, chlorophyll; car, carotenoid; xant, xanthophyll.

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Table 2. Pigment composition of pigment-protein complexes isolated from thylakoid of wild type, lut2 and triple mutants. Data are normalized to 100 Chl a+b molecules. See Experimental Procedures for details. Abbreviations: alfa/beta-car, α/β-carotene. In all cases error was lower than 2%.

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photosynthetic functions of alpha- and beta-xanthophylls

Figures

Figure 1

A

B

mol carotenoid / 100 mol chlorophylls

WT lls ayyayayl5 lls ayyayayl5
photosynthetic functions of alpha- and beta-xanthophylls

Figure 2

A

B

- WT
- chy1chy2lut5
- lut2
- chy1chy2lut2

1. free pigments
2. monomeric Lheb
3. trimeric LHCII
4. PSII core complex
5. PSI-LHCI
photosynthetic functions of alpha- and beta-xanthophylls
photosynthetic functions of alpha- and beta-xanthophylls

Figure 3

A

![Relative ETR vs. PFD (µmol m$^{-2}$ s$^{-1}$)]

B

![1-qP vs. PFD (µmol m$^{-2}$ s$^{-1}$)]

C

![9-AA quenching vs. PFD (µmol m$^{-2}$ s$^{-1}$)]
photosynthetic functions of alpha- and beta-xanthophylls

Figure 4

A

B

C

D

E

WT npq1 lut2 npq1lut2 lut5 chy1chy2lut2 WT npq1 lut2 npq1lut2

0 200 400 600 800 1000 1200

0,0

0,5

1,0

1,5

2,0

2,5

NPQ

Time (min)

0,0

0,5

1,0

1,5

2,0

2,5

Time (min)

light intensity (µmol m\(^{-2}\) s\(^{-1}\))

light intensity (µmol m\(^{-2}\) s\(^{-1}\))

anti-PsbS

127±11 124±12 156±19 121±12 130±13 142±12 127±11 153±18 -
photosynthetic functions of alpha- and beta-xanthophylls

Figure 5

A

B

C

by guest on November 19, 2017 http://www.jbc.org/ Downloaded from
photosynthetic functions of alpha- and beta-xanthophylls

![Graph showing the photosynthetic functions](image-url)
photosynthetic functions of alpha- and beta-xanthophylls

Figure 6

A

B

C

Thylakoids

WT

lut5

chy1chy2lut5

lut2

chy1chy2lut2

Fraction: Lhcb

WT

lut5

chy1chy2lut5

lut2

chy1chy2lut2

Fraction: PSII core complex

WT

lut5

chy1chy2lut5

lut2

chy1chy2lut2
photosynthetic functions of alpha- and beta-xanthophylls

Figure 7

A

B
SUPPLEMENTAL DATA

Figure a1. Measurement of state 1-state 2 transitions. Plants were dark-adapted for 2 hours, then single leaves were illuminated with blue light (40 µmol m\(^{-2}\) s\(^{-1}\), \(\lambda<500\) nm) for 15 min in order to reach state II. Far-red light source was used to induce transition to state I. Value of Fm, Fm' and Fm" were determined by light saturation pulses (4500 µmol m\(^{-2}\) s\(^{-1}\), 0.6 s).
photosynthetic functions of alpha- and beta-xanthophylls

**Figure a2. Lipid peroxidation.** Detached leaves from wild-type and mutant genotypes were treated with strong light (1000 µmol m$^{-2}$ s$^{-1}$) for 5 hrs, after which the amount of MDA was determined by HPLC analysis. Data shown are the means ± SD from at least three independent experiments. Data sets with a significance level of $P<0.05$ (according to Student’s $t$ test) with respect to $t_0$ level are marked by an asterisk.

![Figure a2: Lipid peroxidation](image)

**Figure a3. Photoinhibition of the antenna-less mutant chl under photoxidative stress.** (A, B) Fv/Fm ratio was measured on whole plants WT, triple mutants and chl at two different light intensities (A, 750 µmol m$^{-2}$ s$^{-1}$; B, 150 µmol m$^{-2}$ s$^{-1}$) for a different time interval (A, 5 hours; B, 7 days). Data are expressed as means ± SD (n > 4).
photosynthetic functions of alpha- and beta-xanthophylls


photosynthetic functions of alpha- and beta-xanthophylls

Table a1. Pigment composition, maximal photochemical yield and qE values of dark-adapted leaf tissue from WT and mutant genotypes. Plants grown at 15 µmol m⁻² s⁻¹ for 3 weeks were dark-adapted for 1 hour before pigment extraction and Fv/Fm measurement. Pigments amount are normalized to 100 Chl a+b molecules. qE was measured on whole leaves during 9 min of illumination at 1100 µmol m⁻² s⁻¹ followed by 9 min of dark relaxation. Data are expressed as mean ± SD, n=3. See Experimental Procedures for details.

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Luca Dall’Osto, Alessia Fiore, Stefano Cazzaniga, Giovanni Giuliano and Roberto Bassi
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