THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 14, Issue of April 2, pp. 13878–13888, 2004
Printed in U.S.A.

The Effect of Zeaxanthin as the Only Xanthophyll on the Structure and Function of the Photosynthetic Apparatus in *Arabidopsis thaliana**

Received for publication, October 10, 2003, and in revised form, January 7, 2004
Published, JBC Papers in Press, January 13, 2004, DOI 10.1074/jbc.M311154200

Michel Havaux‡§, Luca Dall'Osto¶, Stephan Cuiné‡, Giovanni Giuliano‖, and Roberto Bassi¶**‡‡**

From the ‡CEA/Cadarache, DSV, DEVM, Laboratoire d'Ecophysiologie de la Photosynthèse, UMR 163 CEA-CNRS, Université Méditérranée-CEA 1000, F-13108 Saint-Paul-lez-Durance, France, the ¶Dipartimento Scientifico e Tecnologico, Università di Verona, Strada Le Grazie 15, I-37134 Verona, Italy, the ‖ENEA, Casaccia Research Center, P. O. Box 2400, I-00100AD Rome, Italy, and the ‡‡CEA/Cadarache, DSV, DEVM, Laboratoire de Génétique et Biophysique des Plantes, UMR 163 CEA-CNRS-Université Méditérranée, 163 avenue de Luminy, F-13288 Marseille cedex 09, France

In green plants, the xanthophyll carotenoid zeaxanthin is synthesized transiently under conditions of excess light energy and participates in photoprotection. In the *Arabidopsis lut2 npq2* double mutant, all xanthophylls were replaced constitutively by zeaxanthin, the only xanthophyll whose synthesis was not impaired. The relative proportions of the different chlorophyll antenna proteins were strongly affected with respect to the wild-type strain. The major antenna, LHCII, did not form trimers, and its abundance was strongly reduced as was CP26, albeit to a lesser extent. In contrast, CP29, CP24, LHCI proteins, and the PSI and PSII core complexes did not undergo major changes. PSII-LHCII supercomplexes were not detectable while the PSI-LHCI supercomplex remained unaffected. The effect of zeaxanthin accumulation on the stability of the different Lhc proteins was uneven: the LHCII proteins from *lut2 npq2* had a lower melting temperature as compared with the wild-type complex while LHCI showed increased resistance to heat denaturation. Consistent with the loss of LHCII, light-state 1 to state 2 transitions were suppressed, the photochemical efficiency in limiting light was reduced and photosynthesis was saturated at higher light intensities in *lut2 npq2* leaves, resulting in a photosynthetic phenotype resembling that of high light-acclimated leaves. Zeaxanthin functioned *in vivo* as a light-harvesting accessory pigment in *lut2 npq2* chlorophyll antennae. As a whole, the *in vivo* data are consistent with the results obtained by using recombinant Lhc proteins reconstituted *in vitro* with purified zeaxanthin. While PSII photoinhibition was similar in wild type and *lut2 npq2* exposed to high light at low temperature, the double mutant was much more resistant to photooxidative stress and lipid peroxidation than the wild type. The latter observation is consistent with an antioxidant and lipid protective role of zeaxanthin *in vivo*.

When vascular plants or green algae are suddenly exposed to high light, the diepoxide xanthophyll violaxanthin is rapidly converted via the intermediate antheraxanthin to the epoxide-free zeaxanthin under the action of the enzyme violaxanthin deepoxidase (1, 2). The latter enzyme is located in the lumen of the thylakoids and binds to the thylakoid membrane in response to lumen acidification in the light (3). Upon return to light limiting conditions, zeaxanthin is epoxidized back to violaxanthin by a zeaxanthin epoxidase enzyme localized on the stromal side of the thylakoid membranes. These stoichiometric and cyclic conversions of violaxanthin, antheraxanthin, and zeaxanthin are called the violaxanthin cycle and have profound effects on light harvesting and light energy utilization in Photosystem (PS)[1] II. It is well established that zeaxanthin synthesis and lumen acidification in high light act synergistically to convert PSII from a state of maximum efficiency of light harvesting to a state of high energy dissipation in the form of heat (4–6). The latter phenomenon is usually measured as a nonphotochemical quenching of chlorophyll fluorescence (NPQ). NPQ is important for the protection of the photosynthetic apparatus against photodamage; it can protect the photosensitive PSII reaction center from overexcitation, thereby increasing its resistance to photoinhibition, as confirmed in NPQ-deficient *Arabidopsis* mutants (7–9). Additionally, it has been shown that zeaxanthin increases the resistance to thylakoid membrane photodestruction through lipid peroxidation by a mechanism distinct from NPQ, which remains to be clarified (8, 10–12). The xanthophyll cycle and the associated NPQ exert their protective roles mainly under conditions of fluctuating light intensity (9, 13).

Zeaxanthin is virtually absent from green plants grown under optimal conditions, and it accumulates transiently only under excess light energy. However, the constitutive presence of zeaxanthin in the chlorophyll antennae has been reported in a number of mutants of *Arabidopsis thaliana*, which thus provide an interesting source for analyzing the effects of this carotenoid on the organization and function of the photosynthetic apparatus (14–16). In the *Arabidopsis aba* mutants, zeaxanthin epoxidase is not functional, causing accumulation of zeaxanthin at the expense of the epoxy-xanthophylls antheraxanthin, violaxanthin, and neoxanthin (17, 18). For instance, in the *aba1* mutant, zeaxanthin represents ~50% of the xanthophyll pool (14). Although this xanthophyll perturbation had

* 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.

§ To whom correspondence should be addressed: CEA/Cadarache, DEVM, Laboratoire d'Ecophysiologie de la Photosynthèse, F-13108 Saint-Paul-lez-Durance, France. Tel.: 33442257476; Fax: 33442256265; E-mail: michel.havaux@cea.fr.

** Supported by MIUR Projects FIRB N° RBAU01E3CX and FIRB n°RBNE01LACT.

[1] The abbreviations used are: PS, photosystem; LHC (I and II), light-harvesting complex (of PSI and PSII); Lhca and Lhcb, protein component of LHCI and LHCII, respectively; CP24, CP26 and CP29, minor LHCII (Lhcb6, Lhcb5, and Lhcb4, respectively); NPQ, nonphotochemical quenching; WT, wild type; TL, thermoluminescence; Φ, quantum yield of O_2 evolution; *E*, Emerson enhancement; DM, dodecyl-D-maltoside.

limited effects on the photosynthetic performance of the leaves, a decreased stability of the trimeric LHCII complexes (major chlorophyll antenna of PSII) and a weakened association between LHCII trimers and the minor LHCII antennae were noticed (14–16,19). However, the *aba* mutants are also deficient in the hormone abscisic acid (ABA), which is synthesized from neoxanthin through a series of intermediates. Consequently, the *aba* mutants have a number of additional problems: they cannot regulate stomatal opening, they exhibit a dramatic wilty phenotype and they require special growth conditions (water-saturated atmosphere). Niyogi *et al.* (7) identified a new allele of *aba1*, *npq2-1*. Interestingly, *npq2-1* is not a complete loss-of-function mutation: ABA is synthesized in sufficient amounts so that the mutant is not particularly sensitive to wilting (7). Nevertheless, epoxy-xanthophylls are drastically reduced to trace amounts. Thus, as far as xanthophyll pigments are concerned, *npq2-1* is more appropriate for functional studies than the previously isolated *aba* alleles. *Npq2-1* was crossed with the *lut2* mutant, deficient in lutein, and the resulting double mutant, which does not contain any xanthophyll carotenoid except zeaxanthin, was analyzed for its photosynthetic characteristics and high light responses. Substitution of all xanthophylls by zeaxanthin selectively affected the PSII antenna size, resulting in a photosynthetic phenotype resembling, in some aspects, that of high light-acclimated leaves, and enhanced noticeably the tolerance of the leaves to photooxidative stress. In contrast to the case of Lhcb proteins forming the PSII antenna system, the stability of the Lhca proteins of PSI appeared to be enhanced by zeaxanthin.

EXPERIMENTAL PROCEDURES

Plant Material and Stress Treatments—The wild type (WT), the double mutant *lut2 npq2*, and the *ch1* mutant of *Arabidopsis thaliana* (L.) Heynh. were grown under controlled conditions of light (300 μmol of photons m^{-2} s^{-1}; photoperiod, 8 h), temperature (23 °C/20 °C, day/night) and relative air humidity (50–70%). The *ch1-1* and *npq2-1* mutants were obtained from the Nottingham Arabidopsis Stock Center, and a T-DNA insertion allele of *lut2* was isolated through PCR screening from the Versailles T-DNA collection. Detached leaves were exposed to high light (1300 μmol of photons m^{-2} s^{-1}) at 10 °C, as described elsewhere (20).

Pigment Analyses—Photosynthetic pigments extracted in methanol were separated and quantified by HPLC, as described in detail elsewhere (20).

Thylakoid Preparation—Thylakoid membranes were prepared according to Ref. 21 except that a mixture of the proteinase inhibitors (Sigma) was used in the first step, and Tricine was replaced by HEPES in the last step. The protein content of thylakoid membranes was determined with a modified Lowry protein assay using bovine serum albumine as a standard (Sigma). The absorbance and fluorescence-excitation spectra of thylakoid suspensions were measured with an Uvikon 954 spectrophotometer and a PerkinElmer LS50B luminometer, respectively.

Stroma lamellae were isolated from thylakoids disrupted in a French press as described elsewhere (22). Fluorescence emission spectra of the isolated stroma lamellae were measured in liquid nitrogen before and after addition of octyl glucoside (0.5%).

Protein Separation and Quantification—Non-denaturing Deriphat-PAGE was performed following the method developped by Peter and Thornber (23). The composition of the stacking gel was: 3.5% (w/v) acrylamide 48/1.5 (48% acrylamide/1.5% bisacrylamide), 12 mM Tris, 48 mM glycine, pH 8.5. The composition of the resolving gel was: acrylamide (48%/1.5%) gradient from 4% al 8% (w/v) stabilized by a glycerol gradient from 8% to 16%, 12 mM Tris, 48 mM glycine, pH 8.5. Gels were polymerized with 0.02% ammonium persulfate and 0.075% TEMED. For 1.5-mm-thick gels a volume of 40 μ1 of extract with 25 μg of chlorophylls was loaded per lane. The electrophoresis reservoir buffer was 12.4 mM Tris, 96 mM glycine, pH 8.3, and 0.1% Deriphat-160. Purified Deriphat-160 was used. Gels were electrophoresed overnight at 4 °C under a constant voltage of 50 V.

Separation of the pigmented thylakoid complexes was carried out by sucrose density gradient centrifugation (24). Membranes corresponding to 600 μg of chlorophyll were washed with 5 mM EDTA and then solubilized in 1 ml of 0.6% dodecyl-D-maltoside (-DM), 10 mM Hepes, pH 7.5, by vortexing for 1 min. The solubilized samples were centrifuged at 15,000 × *g* for 10 min to eliminate non-solubilized material and then fractionated by ultracentrifugation in a 0.1–1 M sucrose gradient containing 0.06% -DM and 10 mM Hepes, pH 7.5 (22 h at 280,000 × *g* at 4 °C). The green bands of the sucrose gradient were harvested with a syringe and were submitted to SDS-PAGE separation with a Tris-Tricine buffer system as previously described (25). The concentration of the separated proteins was determined from the intensity of the bands stained with Coomassie Blue. The blue color intensity was calibrated with known concentrations of recombinant Lhcb3 protein.

Thylakoid membrane proteins resolved by SDS-PAGE in 13% acrylamide gels were electroblotted onto nitrocellulose membranes. Membranes were incubated with the antibody raised against Lhcb2, CP26 (Lhcb5), CP24 (Lhcb6), or CP29 (Lhcb4) and were revealed with alkaline phosphatase conjugate. Band intensities were analyzed using the Gene Tools software for Sungene (Synoptics Ltd.). Membranes for D1 were revealed by enhanced luminescence as described elsewhere (26).

In Vitro Reconstitution of Lhca Proteins—Lhca 1 and Lhca 4 proteins were overexpressed in bacteria and reconstituted *in vitro* with purified pigments as previously reported (27) by using either a full carotenoid extract from thylakoids or purified zeaxanthin.

Protein Stability—The stability of the minor and major LHCIIs, separated by sucrose density gradient centrifugation, was analyzed by following the decay of the 492-nm circular- dichroism signal induced by increasing temperature from 20 °C to 80 °C with a time slope of 1 °C min^{-1} and a resolution of 0.2 °C (28). The thermal stability of the protein was determined by finding the temperature corresponding to half of the signal decay. All samples were normalized to an optical density of 0.6. The same procedure was used to measure the stability of recombinant Lhca1 and Lhca4 proteins.

Chlorophyll Fluorescence—Chlorophyll fluorescence from intact leaves was measured at 25 °C and 10 °C with a PAM-2000 fluorometer (Walz), as described previously (20). The initial level of chlorophyll fluorescence (F_o) was excited with a dim red light modulated at 600 Hz. The true F_o level was determined after a 2-s pulse of far-red light. Variable fluorescence was induced in diuron-infiltrated leaves with a red light of 6.5 μmol m^{-2} s^{-1} produced by a light emitting diode. Leaf discs were infiltrated for 30 min with 2.5 10^{-5} M diuron. The half-time of the fluorescence rise was taken as a measure of the functional antenna size of PSII (29). The maximal quantum yield of PSII photochemistry was measured in dark-adapted leaves from the maximal fluorescence level (F_m) and the initial level (F_o): $(F_m - F_o)/F_m = F_v/F_m$. F_m was obtained by applying a 800-ms pulse of intense white light. The actual quantum yield of PSII photochemistry was measured in light-adapted leaves by the ratio $\Delta F/F_m'$; where F_m' is the maximal fluorescence level and ΔF is the difference between F_m' and the steady-state fluorescence level. White light was produced by a Schott KL1500 light source. Chlorophyll fluorescence emission spectra from leaf discs or thylakoid suspensions were measured in liquid nitrogen with a PerkinElmer LS50B luminometer equipped with fiber optic light guides (10).

Photoacoustic Spectroscopy—Photosynthetic O_2 evolution was estimated from the photobaric component of the photoacoustic signal generated by leaf discs illuminated with white, green, or red light modulated at 19 Hz. The experimental setup and the procedure used to separate the photobaric signal from the photothermal signal have been described in detail elsewhere (Ref. 9, see also Ref. 30 for review). Green light (37 μmol of photon m^{-2} s^{-1}) was obtained by filtering white light through an Oriel 57558 broadband interference filter (peak wavelength, 510 nm; bandwidth, 60 nm) and red light (57 μmol of photon m^{-2} s^{-1}) was obtained with an Oriel 57610 filter (peak wavelength, 645 nm; bandwidth, 70 nm). The photon flux density of the green and red light beams was adjusted so that the level of light absorption by the leaf samples (estimated by the amplitude of the light-saturated photothermal signal, A_{PT}^{+}) was similar in both spectral domains. The quantum yield of O_2 evolution (Φ) was estimated by the ratio between the amplitude of the photobaric signal (A_{ox}) and the amplitude of the maximal (light-saturated) photothermal signal (A_{pt}): A_{ox}/A_{pt} (30). The Emerson enhancement E of O_2 evolution was determined in state 1 or in state 2 by adding a continuous far-red light (>715 nm, 75 watts m^{-2}) to the modulated blue-green light (obtained with a BG38 Schott filter; photon flux density, 50 μmol m^{-2} s^{-1}). E (%) = $[(\Phi(+FR) - \Phi(-FR))/\Phi(-FR)] \times 100$. State 2 was reached by illuminating the leaves with the blue-green light for min. 10 min, and state 1 was obtained after 10-min illumination with far-red light. Photon flux densities were measured with a Li-Cor quantum meter (Li-185B/Li-190SB).

Thermoluminometry—The thermoluminescence (TL) emitted by leaf

TABLE I
Photosynthetic pigment content of Arabidopsis WT and lut2 npq2 leaves

Data are mean values of 4–5 experiments ± S.D. V+A+Z is the xanthophyll cycle pool size (violaxanthin+antheraxanthin+zeaxanthin). Leaves were harvested from plants exposed to a photon flux density of 300 μmol m^{-2} s^{-1} and were rapidly frozen in liquid nitrogen before pigment analysis.

Pigments	WT	*lut2 npq2*
	ng mm^{-2}	
Xanthophylls		
Neoxanthin	9.4 ± 1.8	n.d.[a]
Violaxanthin	5.3 ± 0.4	n.d.
Antheraxanthin	0.1 ± 0.1	n.d.
Lutein	24.8 ± 4.3	n.d.
Zeaxanthin	0.1 ± 0.1	18.1 ± 0.6
V+A+Z	5.5 ± 0.4	n.d.
β-Carotene	12.2 ± 1.6	12.1 ± 0.7
Chlorophylls		
Chlorophyll *b*	95.4 ± 19.3	47.4 ± 6.2
Chlorophyll *a*	234.0 ± 32.8	154.6 ± 18.2
Chl *a*/chl *b*	2.4	3.3
Car/Chl	0.17	0.15
Xanthophyll/chl	0.12	0.09

[a] n.d., not detected or traces.

discs (diameter, 1 cm) was measured with a laboratory-built apparatus described elsewhere (31). The sample was slowly heated at a rate of 6 °C min^{-1} from 25 to 150 °C. The amplitude of the TL band peaking at ~135 °C was used as an index of lipid peroxidation (30, 31).

Polarography—O_2 exchange by detached leaves was measured at 25 °C with a Clark-type O_2 electrode (Hansatech LD2/2). Leaves were illuminated with white light produced by a LS2 light source (Hansatech). Photon flux density was adjusted using neutral density filters. High CO_2 was generated in the electrode with a carbonate/bicarbonate buffer.

RESULTS

Leaves of the *Arabidopsis lut2 npq2* double mutant are completely deficient in the xanthophylls neoxanthin, violaxanthin, antheraxanthin, and lutein (Table I). The missing xanthophylls are replaced by zeaxanthin, resulting in a carotenoid/chlorophyll ratio (0.15) and a xanthophyll/chlorophyll ratio (0.09) not very different from the wild-type values (0.17 and 0.12, respectively). However, *lut2 npq2* leaves were paler than wild-type leaves, and this was due to a substantial reduction (~40%) of the total chlorophyll and carotenoid content per leaf area unit. This pigment decrease was accompanied by a noticeable increase in the chlorophyll *a* to *b* ratio (from 2.4 to 3.3), as previously reported for the *lut2 aba1* mutant (16, 33). An augmentation of the chlorophyll *a*-to-chlorophyll *b* ratio is usually interpreted as resulting from a decrease in the PSII light-harvesting system (which binds most of the chlorophyll *b* molecules) and/or an increase in the PSI/PSII ratio. However, we cannot exclude that this change was due to alterations of the chlorophyll *a* to chlorophyll *b* ratio within individual chlorophyll proteins in the light-harvesting antenna. Indeed, recombinant LHCII complexes reconstituted with zeaxanthin as the only carotenoid have been shown to have an increased chlorophyll *a*/chlorophyll *b* ratio as compared with native LHCII (34).

Relative PSII and PSI Excitation—Fig. 1*a* shows the chlorophyll fluorescence emission spectra of wild-type and *lut2 npq2* leaves measured at 77 K. As compared with wild type, *lut2 npq2* exhibited a noticeable decrease (~30%) in the PSII bands (at 685 and 695 nm) while the PSI band (at 730 nm) was unchanged (Fig. 1*a*). This selective decrease in PSII fluorescence emission was confirmed in thylakoid suspensions (Fig. 1*b*).

The light energy distribution between PSI and PSII was also determined *in vivo* using the photoacoustic method. Fig. 2 shows the photobaric photoacoustic signal (due to modulated O_2 evolution, see Ref. 30) generated by an *Arabidopsis* leaf disc

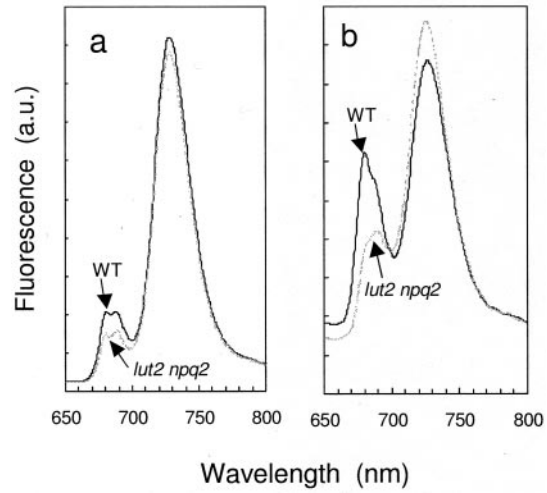

FIG. 1. **Chlorophyll fluorescence emission spectra measured at 77K in (*a*) leaves or (*b*) thylakoids of wild type and *lut2 npq2*.** Excitation wavelength was 440 nm. The measurements were performed three times on different leaves or thylakoid preparations with similar results.

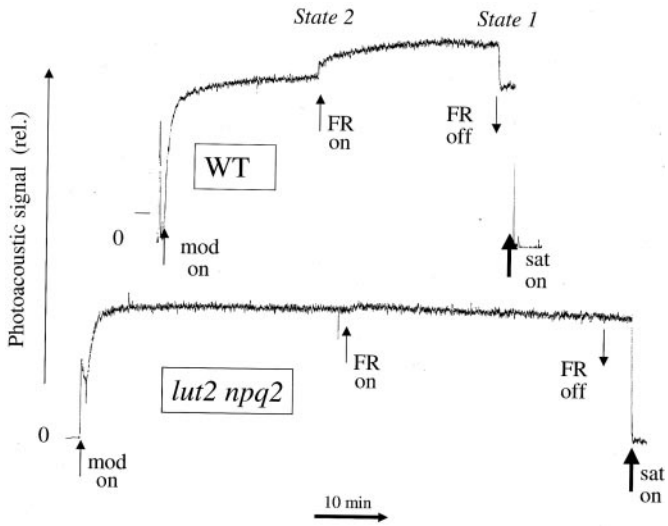

FIG. 2. **Photobaric photoacoustic signal (related to modulated O_2 evolution) generated by wild type and *lut2 npq2 Arabidopsis* leaves in modulated blue-green light (19 Hz, 30 μmol of photons m^{-2} s^{-1}), and its enhancement by continuous far-red light (>715 nm, 15 watts m^{-2}).** *Mod on*, modulated exciting light on; *sat on*, photosynthetically saturating continuous white light on (3500 μmol of photons m^{-2} s^{-1}); *FR on/off*, continuous far-red light on/off. Leaves were first driven to state 2 by illumination with the modulated blue-green light and then to state 1 by adding the far-red light to the modulated light. The trace shown is the quadrature component of the photoacoustic signal which is a pure photobaric (modulated gas exchange) signal.

illuminated with a weak blue-green light beam (350–600 nm) modulated at a low frequency of 19 Hz. The signal amplitude was lower in the double mutant relative to wild type, indicating a reduction (approximately −20%) of photosynthetic O_2 evolution. Because of its spectral characteristics, the measuring light is preferentially absorbed by PSII pigments, and, consequently, linear electron transfer and O_2 evolution are limited by the photon delivery to PSI. When a strong (continuous) far-red light, absorbed almost exclusively by PSI, is added to the measuring light, this limitation is removed and O_2 evolution increases, becoming limited by the photochemical potential (light absorption × quantum yield) of PSII. This effect can be quantified by the so-called Emerson enhancement (*E*), which is the ratio of the O_2 evolution signal measured in the presence

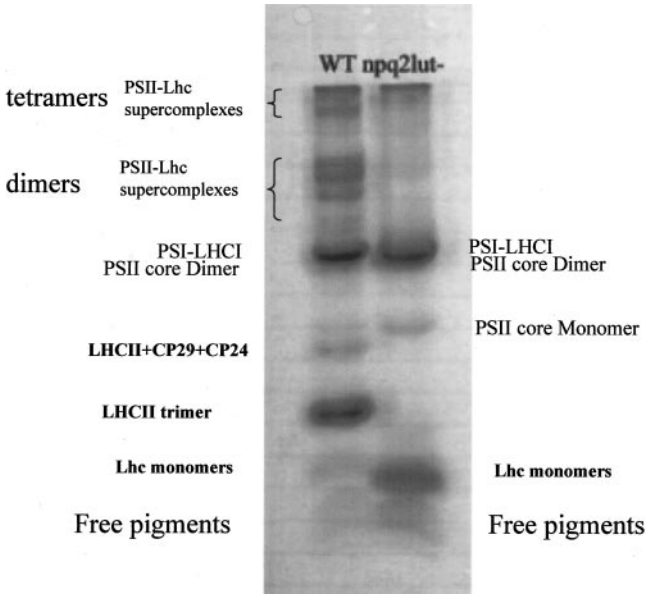

FIG. 3. **Non-denaturing Deriphat-PAGE separation of thylakoid pigmented complexes from wild-type and *lut2 npq2* thylakoids.**

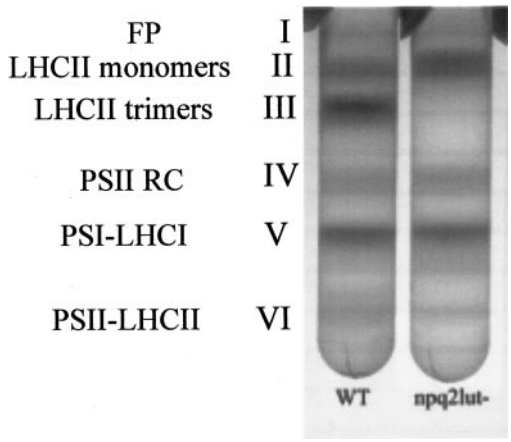

FIG. 4. **Separation of thylakoid pigmented complexes of wild-type and *lut2 npq2* thylakoids by ultracentrifugation on sucrose gradient (*FP*, free pigments).**

of the far-red background light to the O_2 signal measured in its absence (30). 10-min exposure to the blue-green light drove wild-type leaves to the light state 2 characterized by a relatively even distribution of light energy between the two photosystems resulting from the migration of LHCII toward PSI. Consequently, *E* was low (~7%) under those light conditions (Fig. 2, *upper trace*). In state 1 (*i.e.* after prolonged illumination with far-red light), *E* was high, ~20%, indicating a strong imbalance of blue-green light distribution in favor of PSII. Strikingly, *E* was measurable in the double mutant neither in state 1 nor in state 2 (Fig. 2, *lower trace*), indicating that the double mutation removed the PSI limitation of electron transport and that PSII was limiting in all light conditions. Thus, the fluorescence and photoacoustic data presented above indicate a selective decrease in PSII excitation in *lut2 npq2* leaves, which could be due to a decrease in the number of PSII centers and/or a reduction of the PSII antenna system. The strong decrease in the 685-nm fluorescence band emitted by *lut2 npq2* thylakoid suspensions, attributed in part to LHCII-chlorophylls, supports the latter possibility (Fig. 1*b*).

PSII and PSI Antenna Composition and Organization—We estimated the functional antenna size of PSII by *in vivo* chlorophyll fluorescence induction measurements in the presence of the herbicide diuron, as described by Malkin *et al.* (29). The half-time of the induced fluorescence rise in *lut2 npq2* leaves (~110 ± 18 ms) was noticeably higher than that measured in wild type (63 ± 10 ms), clearly indicating smaller PSII antennae in the former plants. This effect was confirmed by the non-denaturing green gels presented in Fig. 3. No LHCII trimer was detected in the double mutant. This loss was associated with an increase in the LHCII monomer band. A similar phenomenon was reported in the *Arabidopsis lut2 aba1* mutant (16). However, it is evident that the accumulation of LHCII monomers in *lut2 npq2* compensated only partially for the loss of LHCII trimer. We also observed that the absence of LHCII trimer in *lut2 npq2* was accompanied by a complete disappearance of the PSII-LHCII supercomplexes as well as by a loss of the LHCII-CP29-CP24 supercomplex. In contrast, the PSI-LHCI band was found in both wild type and *lut2 npq2*, and the PSII reaction center band was marginally affected by the double mutation. This was confirmed by the separation of the thylakoid pigmented complexes by ultracentrifugation on sucrose gradient (Fig. 4): the LHCII trimer band disappeared from the *lut2 npq2* preparation while the PSI-LHCI and the PSII RC bands were not affected appreciably. The latter observation is consistent with the presence of normal concentration of β-carotene in *lut2 npq2* leaves (see Table I) since this carotenoid is present in the LHCI complexes and the PS reaction centers, not in the LHCII complexes (35). The different sucrose bands were harvested and their protein content was analyzed by SDS- PAGE (Fig. 5). It is evident that the relative proportions of the PSII antennae were noticeably changed in *lut2 npq2*: Lhcb1 and Lhcb2 (present mainly in lanes II, III and VI in wild type and lane II in *lut2 npq2*) were strongly reduced. CP26 (Lhcb5) decreased also in the double mutant while CP24 (Lhcb6) and CP29 (Lhcb4) increased. The abundance of the PSII antennae was quantified by converting the intensity of the Coomassie Blue staining into protein concentration, and the data are summarized in Table II. On a chlorophyll basis, CP26 was reduced by 30% in *lut2 npq2*. Lhcb1 + 2 was strongly decreased by a factor of about 2. CP24, CP29, and Lhcb3 were not reduced. All Lhca proteins (see *lane V* in Fig. 5, bands enclosed in a rectangle) were found in *lut2 npq2*, and their relative abundance was not changed significantly with respect to wild type.

A yellowish band (FP) containing free pigments was found at the top of the sucrose gradient (*band I* in Fig. 4), and its pigment content was analyzed by HPLC. It can be seen in Table III that this band was strongly enriched in carotenoids, especially in the *lut2 npq2* preparation. The carotenoid/chlorophyll ratio was 0.83 and 1.03 for wild type and *lut2 npq2*, respectively. These values are considerably higher than the values measured in leaves (~0.16, Table I). This indicates that the carotenoids found in this band did not result exclusively from denaturation of chlorophyll-binding proteins. It is possible that a significant fraction of pigments represent unbound carotenoids that were free in the thylakoid membranes.

The major and minor LHCIIs were also quantified by Western blotting (Fig. 6). The major LHCII and CP26 were observed to noticeably decrease while CP29 and CP24 increased, confirming the data of Figs. 3–5. As the electrophoretic separation shown in Fig. 6 was done at constant protein loading, the CP29 and CP24 increase is attributable to the strong decrease in the major LHCII. Immunoblot analysis of the PSII reaction center D1 confirmed that the abundance of reaction center was not substantially affected by the double mutation.

The Deriphat gels were submitted to a second electrophoretic separation by SDS-PAGE (data not shown). These two-dimensional gels confirmed the strong decrease in Lhcb1 + 2 relative

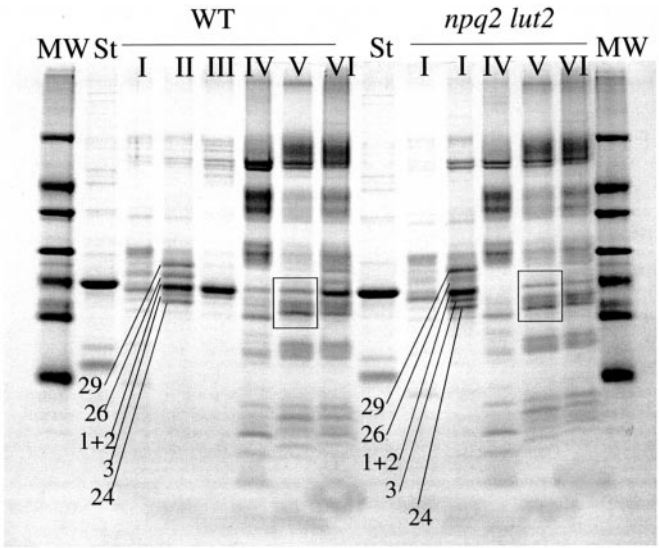

FIG. 5. **Separation of the sucrose bands I-IV (see Fig. 4) by SDS-PAGE.** *St*., standard protein (recombinant Lhcb3) used to quantify the Coomassie Blue-stained bands. The first and last lanes (*MW*) are the molecular mass ladders: 66, 45, 36, 24, 20.1, and 14.2 kDa. *29*, CP29; *26*, CP26; *24*, CP24; *1 + 2*, Lhcb1 + 2; *3*, Lhcb3. The bands in *boxes* are Lhca 1–4.

to the minor LHCII antenna proteins in *lut2 npq2* and that the stoichiometry of the Lhca proteins relative to the PSI reaction center was unchanged in the mutant.

Stroma lamellae were isolated from wild-type and *lut2 npq2* thylakoids. The low temperature (77 K) fluorescence emission spectrum of the isolated stroma lamellae had a single major peak at ~730 nm (Fig. 7), in agreement with previous studies (22). We studied the effect on the fluorescence emission spectra of the detergent octyl glucoside which is known to disconnect LHCII, not LHCI, from the PSI reaction center (22). Disconnection of the highly fluorescent LHCII resulted in an increase in fluorescence at 680 nm relative to the 730-nm fluorescence peak. The fluorescence rise at 680 nm induced by octyl glucoside was not higher in *lut2 npq2* stroma lamellae than in wild-type lamellae, indicating that the monomeric LHCIIs in *lut2 npq2* were not preferentially attached to PSI.

Photosynthetic Electron Transport—Photosynthetic O_2 evolution was measured in leaves with the photoacoustic technique, and the light-dependence curve of the quantum yield of O_2 evolution (Φ) is shown in Fig. 8*a*. In low light (<100 μmol photons m^{-2} s^{-1}), Φ was noticeably lower in *lut2 npq2* relative to wild type. However, increasing light intensity caused a much more rapid decrease in Φ in wild type than in *lut2 npq2*, indicating a more rapid light-saturation of photosynthesis in the former leaves, and this is consistent with the smaller antenna size of PSII in *lut2 npq2* leaves relative to wild-type leaves. However, at 10 °C, the difference between wild type and *lut2 npq2* at high PFDs was strongly attenuated. The same results were observed when photosynthetic electron transport was measured by chlorophyll fluorometry (Fig. 8*b*): the photochemical efficiency of PSII at low photon flux densities was lowered in the mutant while the quantum yield at high photon flux densities was increased relative to wild type. Again, this difference was attenuated at low temperature. We also observed a noticeable inhibition of NPQ in *lut2 npq2* (data not shown), as previously reported for *lut2 aba1* (16).

Photosynthetic O_2 evolution was also measured at 25 °C with a Clark-type electrode. As shown in Table IV, the maximal, light-saturated activity was significantly higher in the double mutant relative to wild type, indicating that the photosynthetic capacities of *lut2 npq2* were not inhibited.

Light-harvesting Function of Zeaxanthin in the lut2 npq2 Antennae—The quantum yield of O_2 evolution (Φ_{510}) in green light absorbed by both chlorophyll and carotenoid molecules was compared with the quantum yield (Φ_{640}) measured in red light absorbed by chlorophylls only (Table IV). The Φ_{510}/Φ_{640} ratio was similar in wild type and *lut2 npq2*. This suggests that the carotenoids of *lut2 npq2* (*i.e.* zeaxanthin and β-carotene) are at least in part functionally coupled to chlorophylls and function as accessory pigments that are able to transfer excitation energy to the chlorophylls.

Fig. 9 shows the light absorption spectra of wild-type and *lut2 npq2* thylakoid suspensions. The major differences between the two genotypes were observed in the 460–520 nm wavelength domain and in the red >660 nm, reflecting the chlorophyll *b* decrease and the carotenoid perturbations in the double mutant. Indeed, as compared with control LHCII, recombinant LHCII containing zeaxanthin exhibits a decreased absorption at 480–500 nm (36), and the absorption peaks of chlorophyll *b* are at 480 nm and 640 nm. A qualitatively similar difference between wild type and *lut2 npq2* was found in the chlorophyll fluorescence excitation spectra (Fig. 9). The similarity of the light-absorption and fluorescence-excitation spectra confirms that zeaxanthin serves as light-harvesting accessory pigment in *lut2 npq2*. As a corollary, this finding indicates also that the fraction of zeaxanthin present in the thylakoid membranes as free pigments must be relatively small.

Stability of the lut2 npq2 Antennae—We examined the thermal stability of LHCIIs prepared from *lut2 npq2* and wild-type leaves (Table V). The samples analyzed were the sucrose bands II and III from wild type and band II from the double mutant. Although band II contains a mixture of monomeric LHCII and minor CPs, their relative contribution to the denaturation curve can be easily distinguished based on the lower melting temperature of minor Lhcs (~58 °C) with respect to major LHCII complex (80 °C), in agreement with previous observations (28, 37). A significant decrease (from 1.5 to 3 °C) in the denaturation temperature of both the minor LHCIIs and Lhcb1 + 2+3 monomers was observed in *lut2 npq2* as compared with wild type, indicating a decreased stability of monomeric zeaxanthin-containing Lhc proteins.

Photostability of lut2 npq2—Detached leaves were exposed to high light stress at low temperature (1300 μmol m^{-2} s^{-1}/10 °C), a condition that leads to photosynthetic saturation in both wild type and *lut2 npq2* (Fig. 8). This treatment caused a rapid decrease in the PSII photochemical efficiency as measured by the chlorophyll fluorescence ratio Fv/Fm (Fig. 10*a*). PSII photoinhibition was similar in wild type and *lut2 npq2*.

The combination of high light and low temperature is very effective for the induction of oxidative stress (38), and photoinhibition of wild type and *lut2 npq2 Arabidopsis* leaves exposed to 1300 μmol m^{-2} s^{-1} at 10 °C was followed by photooxidation (lipid peroxidation) with a time delay of about 15 h (Fig. 10*b*). In this study, lipid peroxidation was monitored *in situ* by a thermoluminescence method (31). Strikingly, wild-type leaves were found to be much more sensitive to photooxidative damage than *lut2 npq2* leaves. The amplitude of the TL band at 135 °C, which is proportional to the amount of lipid hydroperoxides in the thylakoid membranes (31, 32), strongly increased in wild-type leaves after about 15 h in high light whereas TL remained low in *lut2 npq2* leaves even after 25 h in high light. Light stress was also imposed on leaves of the *chlorina1* mutant (*ch1*) deficient in chlorophyll *b* and hence deficient in LHCII (39, 40). Clearly, a strong reduction of the PSII antenna size did not enhance phototolerance. The opposite was actually observed: the lipid peroxidation-related TL signal increased rapidly in *ch1* relative to wild type.

TABLE II

Concentration of the minor and major LHCII apoproteins in WT and lut2 npq2 thylakoids

Proteins were quantified by two different methods: Coomassie Blue staining of proteins separated by SDS-PAGE after ultracentrifugation on sucrose gradient (see Fig. 4) or Western blotting (Fig. 5). Sucrose gradient ultracentrifugation was done with equal chlorophyll concentrations, and Western blots were done with equal protein loading. Data are mean values of three data sets ± S.D.

PSII antenna	Coomassie Blue staining		Immunoblotting	
	WT	*lut2 npq2*	WT	*lut2 npq2*
	μg prot/100μg Chl		*rel. values*	
Lhcb1 + 2	26.1 ± 0.2	14.1 ± 1.6	100	49 ± 8
Lhcb3	3.3 ± 0.2	3.1 ± 0.4	n.d.[a]	n.d.
CP24	2.8 ± 0.2	3.1 ± 0.4	100	171 ± 4
CP29	2.6 ± 0.2	3.2 ± 0.4	100	152 ± 63
CP26	2.7 ± 0.3	1.9 ± 0.2	100	84 ± 1

[a] n.d., not determined.

TABLE III

Pigment content determined by HPLC of the free pigment band in the sucrose gradient of Fig. 4 (band I)

The total amount of chlorophylls loaded in the sucrose gradient was 600 μg. Data are mean values of three experiments ± S.D.

Pigment	WT		*lut npq2*	
	μg	*nmol*	*μg*	*nmol*
Neoxanthin	0.59 ± 0.10	0.98 ± 0.16	n.d.[a]	n.d.
Violaxanthin	0.97 ± 0.01	1.62 ± 0.02	n.d.	n.d.
Lutein	2.46 ± 0.08	4.32 ± 0.15	n.d.	n.d.
Zeaxanthin	n.d.	n.d.	6.33 ± 0.08	11.13 ± 0.14
β-Carotene	0.46 ± 0.02	0.86 ± 0.03	0.71 ± 0.08	1.32 ± 0.14
Total carotenoids	4.84 ± 0.13	7.78 ± 0.22	7.04 ± 0.11	12.45 ± 0.20
Total chlorophylls	5.81 ± 0.49	6.46 ± 0.55	6.85 ± 0.09	7.61 ± 0.10

[a] n.d., not detected.

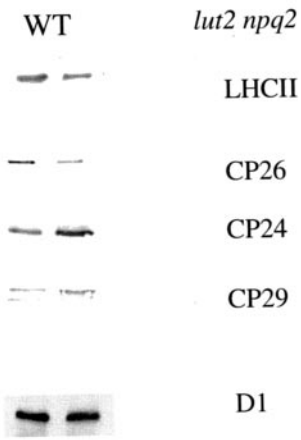

FIG. 6. **Western blots of D1, the major LHCII (Lhcb2), CP26, CP24, and CP29 in wild-type and *lut2 npq2* leaves.** Membrane protein loading: CP29, 7 μg; CP24, 6 μg; LHCII, 2 μg; CP26, 1.5 μg; D1, 20 μg. We checked with different concentration of the protein extracts that the reactions were below saturation. Coomassie Blue-stained gels were used to check equal loading of wild-type and *lut2 npq2* membrane proteins.

DISCUSSION

The Xanthophyll Substitutions in lut2 npq2 Selectively Affect the Major LHCII—The normal carotenoid composition of the PSII antenna complexes is lutein-neoxanthinviolaxanthin which are present in variable proportions depending on the antenna (35). However, *Arabidopsis* mutants with perturbed xanthophyll content have revealed that the xanthophyll composition of LHCIIs is flexible (14–16,41). Moreover, *in vitro* studies of recombinant Lhc proteins refolded with purified pigments have shown that it is possible to reconstitute LHCII complexes with one xanthophyll only, *e.g.* zeaxanthin (34, 42). The present study has shown that zeaxanthin-LHCII can occur also *in vivo* in a xanthophyll mutant since all minor and major LHCIIs were found in *lut2 npq2*. Similarly, all LHCIs were present in *lut2 npq2* leaves. However, the relative proportions of the different LHC forms were strongly affected, indicating that the different LHCs do not have the same sensitivity to the perturbation in xanthophyll composition. The abundance of the major LHCII was selectively reduced, resulting into a noticeable decrease in the PSII antenna size in the mutant, and this effect was due mainly to a loss of Lhcb1 and Lhcb2. Lhcb3 was much less affected by the xanthophyll substitutions (Fig. 5, Table II). Similarly, zeaxanthin had little effect on the minor LHCII (except CP26), the reaction centers and the LHCIs. We also found that the supramolecular organization of LHCII was strongly perturbed by the presence of zeaxanthin. No LHCII trimer and no PSII supercomplexes involving LHCII and the minor LHCIIs was found in the double mutant. Loss of LHCII trimers was also reported in other xanthophyll mutants of *Arabidopsis* such as the *aba* mutants (14, 15) or the *lut2* mutant (16), indicating that the ability to form trimers is lost whenever the normal xanthophyll composition is changed.

In an antisense *Arabidopsis* strain lacking the proteins that form LHCII trimers (Lhcb1 and Lhcb2), the place of LHCII was taken by CP26, which was organized into trimers, possibly together with Lhcb3 (43). Obviously, this was not the case in *lut2 npq2*. Although the Lhcb3 abundance was not affected by the double mutation, CP26 was substantially reduced. It is possible that, like zeaxanthin-containing LHCII, zeaxanthin-containing CP26 was unable to form trimers. A similar phenomenon was observed in the *npq2 lor1* mutant of *Chlamydomonas reinhartii*, the algal counterpart of the *lut 2 npq2 Arabidopsis* mutant (44): LHCII and CP26 decreased whereas CP24, CP29, and PSI antenna proteins did not. The trimeric organization of LHCII was unfortunately not examined in this study. CP26 is located at the tip of the LHCII-PSII supercomplex (45, 46), and it is possible that loss of LHCII trimers in *lut2 npq2* also induced loss of CP26. In contrast, CP24 and CP29 are located in close proximity, between the reaction center and the LHCII trimers (45, 46) and therefore they may be less sensitive to the loss in LHCII. Their stability was, however, decreased in the double mutant (Table V). Fig. 11 shows, as a working hypothesis, the organization and size of the PSII antenna system of the *lut2 npq2* mutant.

The assembly of PSII into supercomplexes and the macro-

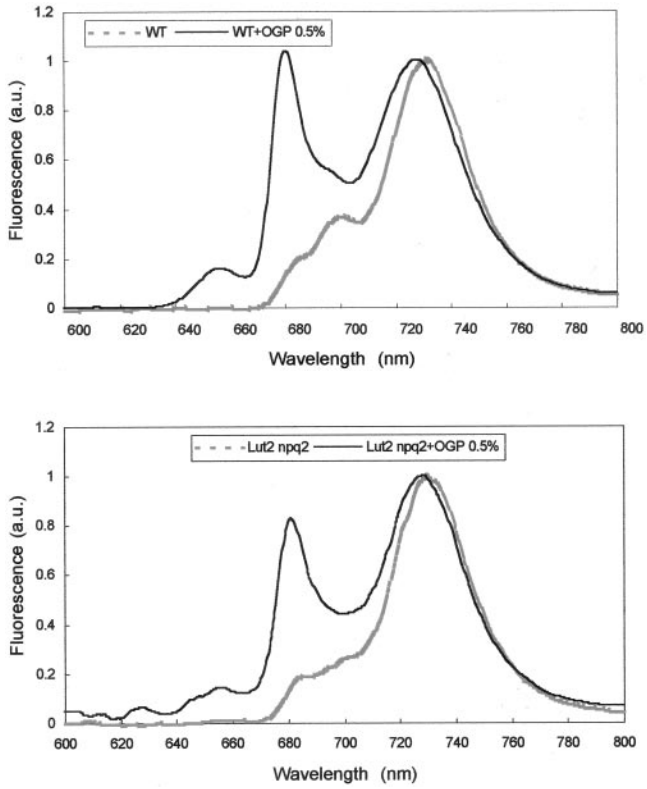

FIG. 7. **Chlorophyll fluorescence emission spectra of stroma lamellae isolated from wild-type and *lut2 npq2* thylakoids.** The spectra were recorded before and after addition of 0.5% octyl glucoside (*OGP*). Excitation light wavelength: 475 nm.

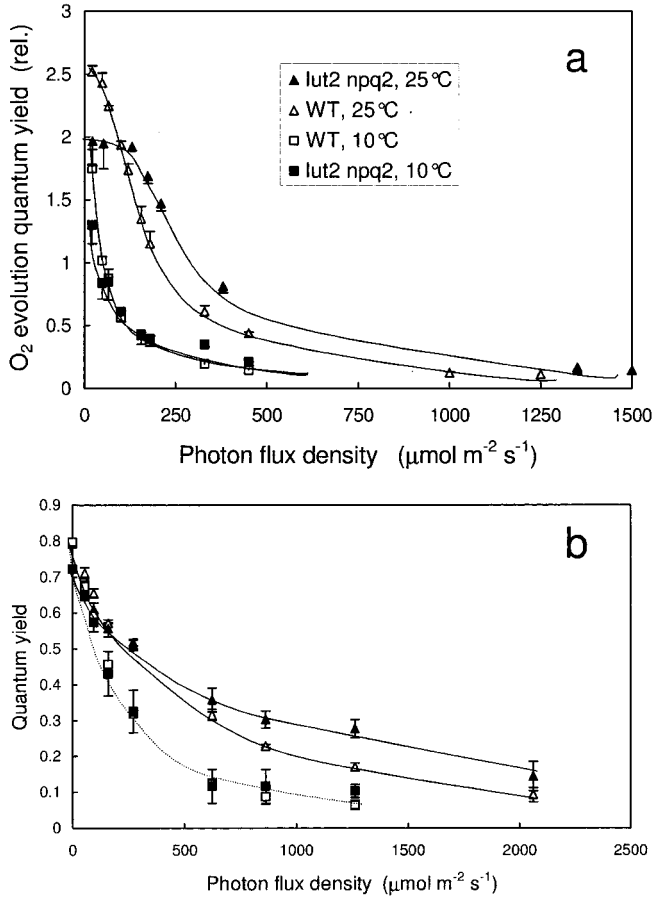

FIG. 8. **Photosynthetic characteristics of wild-type and *lut2 npq2* leaves.** *a*, O_2 evolution quantum yield measured (in relative values) by photoacoustic spectroscopy at 25 or 10 °C. *b*, quantum yield of electron transport measured by chlorophyll fluorometry at 25 and 10 °C. *Open triangle*, wild type at 25 °C; *closed triangle*, *lut2 npq2* at 25 °C; *open square*, wild type at 10 °C; *closed square*, *lut2 npq2* at 10 °C. Data are mean values + S.D. of three separate experiments.

organization of supercomplexes in the thylakoid membrane determine the quantum efficiency of PSII. Therefore, the reduction of the LHCII and CP26 abundance as well as the loss of LHCII supercomplexes in *lut2 npq2* could explain the decreased quantum efficiency of PSII (F_v/F_m) as well as the reduction of NPQ. Alternatively, the constitutive presence of zeaxanthin in the chlorophyll antennae could engage PSII in a permanent state of thermal energy dissipation of the type reported during zeaxanthin retention in some stressed leaves (*e.g.* Ref. 47).

Involvement of Zeaxanthin in Long Term Photoacclimation?—LHCII monomers, not LHCII trimers, are the target of regulated proteolytic degradation during photoacclimation (48). It is believed that degradation is triggered by the migration of peripheral LHCIIs (Lhcb1 + 2) in their monomeric form to the nonappressed thylakoid regions where the protease is located (49). Consequently, it is possible that, in addition to a decreased stability of LHCIIs, LHCII monomerization in *lut2 npq2* favored degradation, thus resulting into a low LHCII level. The differential reduction of Lhcb1 + 2 and Lhcb3 in *lut2 npq2* relative to wild type (Table II) confirms that the peripheral pool of LHCII was more affected by the double mutation than the inner pool. However, LHCII monomerization alone does not seem to be sufficient to enhance degradation. Indeed, in the *lut2* mutant, lutein deficiency is accompanied by a loss of LHCII trimers (16) but the stability of monomers is not affected.[2] In contrast, in the *aba1* mutant, violaxanthin and neoxanthin are replaced by zeaxanthin and the concentration of LHCII (mainly in monomeric form) is substantially reduced (14–16). However, a different picture was found in the *aba3* mutant since the major effect was found in CP26, with the LHCII level being less affected (19).[2] We do not know why

TABLE IV

Photosynthetic O_2 evolution measured with the photoacoustic technique or with the polarographic technique (Clark-type electrode)

Φ_{510} and Φ_{640} are the quantum yield of oxygen evolution (in relative values) in green light (~510 nm) and in red light (~640 nm). Photoacoustic data are mean values ± S.D. of six experiments. Polarographic measurements were performed in the dark and at two different PFDs of white light (100 and 2000 μmol photon m^{-2} s^{-1}). Data are mean values ± S.D. of 3–4 experiments. F.W., fresh weight.

	WT	*lut2 npq2*
Photoacoustic measurements		
Φ_{510} (a.u.)	2.47 ± 0.38	1.54 ± 0.35[a]
Φ_{510}/Φ_{640}	0.69 ± 0.06	0.64 ± 0.09[b]
Polarographic measurements		
	μmol O_2 min^{-1} g^{-1} F.W.	
0 μmol photon m^{-2} s^{-1}	-0.40 ± 0.02	-0.42 ± 0.18[b]
100 μmol photon m^{-2} s^{-1}	1.83 ± 0.43	1.30 ± 0.26[b]
2000 μmol photon m^{-2} s^{-1}	3.49 ± 0.80	5.69 ± 0.28[a]

[a] Significantly different ($p < 0.05$) from the wild type.
[b] Not significantly different from the wild type.

LHCII was differently affected in different *aba* mutants. Nevertheless, zeaxanthin appears to be an important factor in the LHCII/CP26 destabilization and degradation.

Interestingly, it has been shown that illumination also brings about the conversion of LHCII trimers into monomers (50). The changes in the chiral macroorganization of the chromophores associated with the photoinduced LHCII monomerization (51) have been shown to be sensitive to dithiothreitol, a

[2] L. Dall'Osto, G. Giuliano, and R. Bassi, unpublished data.

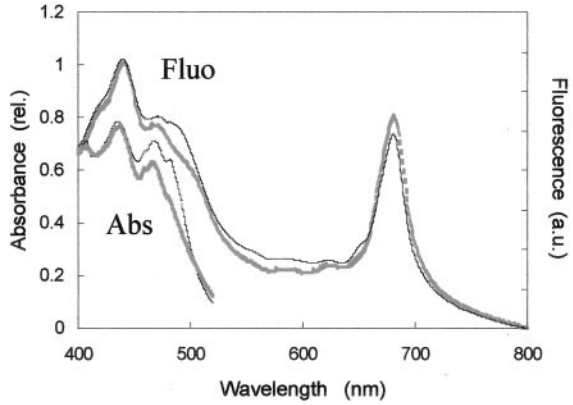

FIG. 9. **Absorption spectra (*continuous line*, *Abs*) and fluorescence excitation spectra (*dashed line*, *Fluo*) at room temperature of thylakoids isolated from wild-type (*black*) and *lut2 npq2* leaves (*gray*).** Fluorescence emission wavelength, 680 nm. The measurements were done on two different thylakoid preparations with similar results.

potent inhibitor of the violaxanthin cycle, suggesting the involvement of zeaxanthin in the monomerization process (52). It is therefore tempting to see the photoinduced synthesis of zeaxanthin, not only as a system that rapidly regulate light harvesting (4), but also as a mechanism that triggers long term acclimation of the PSII antenna size to high light conditions by favoring LHCII monomerization, decreasing LHCII stability and promoting LHCII degradation. In other words, the xanthophyll cycle could function as a signal transduction system. This idea is supported by the similarities between the photosynthetic characteristics of *lut2 npq2* leaves and high light-acclimated wild-type leaves. Acclimation of vascular plants to high light conditions is typically accompanied by a decrease in the major LHCII and an increase in the photosynthetic capacity (53). Those adaptive changes were also observed in *lut2 npq2* leaves (Tables II and IV). Moreover, in *Arabidopsis*, high light was shown to cause a decrease in Lhcb1, Lhcb2, and CP26 while the Lhcb3 and CP29 levels were found to remain constant (54). Again, this differential behavior of the PSII antenna proteins was also observed in *lut2 npq2 versus* wild type. Thus, many features of the photosynthetic apparatus of *lut2 npq2* resemble those of high light acclimated *Arabidopsis* plants, raising the interesting possibility that zeaxanthin is somehow involved in the long term adjustment of the PSII antenna size to the light environment. This possibility is being investigated by further experiments, particularly with zeaxanthin-deficient mutants. Preliminary experiments with the zeaxanthin-deficient *npq1* mutant grown at low and moderate light intensities have shown that the trimeric and monomeric LHCIIs are more abundant in this mutant as compared with wild type (data not shown), in agreement with our hypothesis.

In Vitro properties of Zeaxanthin-LHCII Are Confirmed in Vivo—Zeaxanthin-LHCII complexes reconstituted *in vitro* are very similar to wild type LHCII with respect to energy transfer and photostability (28). In particular, no major alteration of the overall function as antenna was observed in zeaxanthin-LHCII compared with wild-type LHCII (36). This is confirmed here *in vivo* by photoacoustic measurements on leaves and by spectrophotometric/fluorometric analyses of thylakoids. The high photochemical activity of *lut2 npq2* leaves in green light (Table IV) and the similarity between the absorption and chlorophyll-fluorescence excitation spectra (Fig. 9) showed that zeaxanthin functioned as a light-harvesting accessory pigment that was able to transfer excitation energy to the chlorophylls. This result indicates also that zeaxanthin was predominantly bound to antenna proteins and that relatively little zeaxanthin was

TABLE V
Thermostability of minor and/or major LHCIIs prepared from WT and lut2 npq2 thylakoids

Data are mean values of three separate experiments ± S.D.

	Temperature of denaturation		
	CP26 + 29 + 24	Monomeric Lhcb1 + 2+3	LHCII trimer
		°C	
WT	58.1 ± 0.7	71.4 ± 0.1	79.7 ± 0.8
lut2 npq2	55.0 ± 0.2	69.9 ± 0.3	

present as free pigments in the membrane lipid phase. On the other hand, monomeric *lut2 npq2* LHCII were observed to be less stable than wild-type LHCII (Table V), and this is also consistent with *in vitro* measurements of the thermal stability of reconstituted LHCIIs with zeaxanthin (28).

In contrast to PSII, the antenna system of PSI was not affected by the *lut2 npq2* double mutation, suggesting that the stability of the LHCIs was not decreased by zeaxanthin. We have analyzed the thermal stability of recombinant LHCI proteins (Lhca1 and Lhca4) refolded *in vitro* with zeaxanthin or violaxanthin, and we have compared the denaturation temperatures with those of control LHCI complexes determined in previous works (27, 55). As shown in Table VI, zeaxanthin increased noticeably the thermostability of both Lhca1 and Lhca4 (+4 °C). This phenomenon was not observed when violaxanthin was used. Thus, both *in vitro* and *in vivo* experiments show that the PSI antenna system is very robust to the replacement of all xanthophylls by zeaxanthin and may suggest that Lhcb and Lhca proteins differ for some aspects of their overall conformation thus accommodating preferentially violaxanthin *versus* zeaxanthin, respectively. This is consistent with the finding that LHCI exchanges violaxanthin with zeaxanthin both *in vivo* and *in vitro* with high efficiency (56).

lut2 npq2 Leaves Are Tolerant to Photooxidative Stress—In vitro photobleaching experiments have shown that LHCII reconstituted with zeaxanthin alone is almost as tolerant to photodestruction as wild-type LHCII (28). The photostability of zeaxanthin-containing PSII was confirmed *in vivo* in this study. When exposed to excess light energy induced by the combination of high light and low temperature, the extent of PSII photoinhibition, as measured by the F_v/F_m chlorophyll-fluorescence ratio, was similar in wild-type and mutant leaves (Fig. 10*a*). Photoinhibition was followed with a time delay of several hours by photooxidative destruction of thylakoid membranes as measured with a TL index of lipid peroxidation (Fig. 10*b*). However, lipid peroxidation occurred much more rapidly in wild type than in *lut2 npq2*, indicating a strongly increased tolerance of the double mutant to oxidative stress.

The enhanced phototolerance of the double mutant cannot be explained easily by its photosynthetic characteristics. Electron transport in high light at low temperature was similar in wild-type and *lut2 npq2* leaves (Fig. 8), and photoprotective thermal energy dissipation via the NPQ process is lowered in *lut2 npq2* as it is in the *lut2 aba1* mutant (16, 33). Also, it is very unlikely that the high phototolerance of the mutant can be ascribed to the reduction of the PSII antenna size. In contrast with green algae (57, 58), small antenna size does not protect vascular plants from photodamage (59, 60). Accordingly, a chlorophyll *b*-less barley mutant was not found more tolerant to high light stress than wild-type barley (61). As shown in this study, tolerance to photooxidation was not improved in the chlorophyll b-less *ch1 Arabidopsis* mutant that is deficient in LHCII complexes. More probably, the high phototolerance of *lut2 npq2* is related directly to the presence of high amounts of zeaxanthin in the chloroplast. Indeed, studies of *Arabidopsis npq* mutants have shown that, beside its role in the NPQ

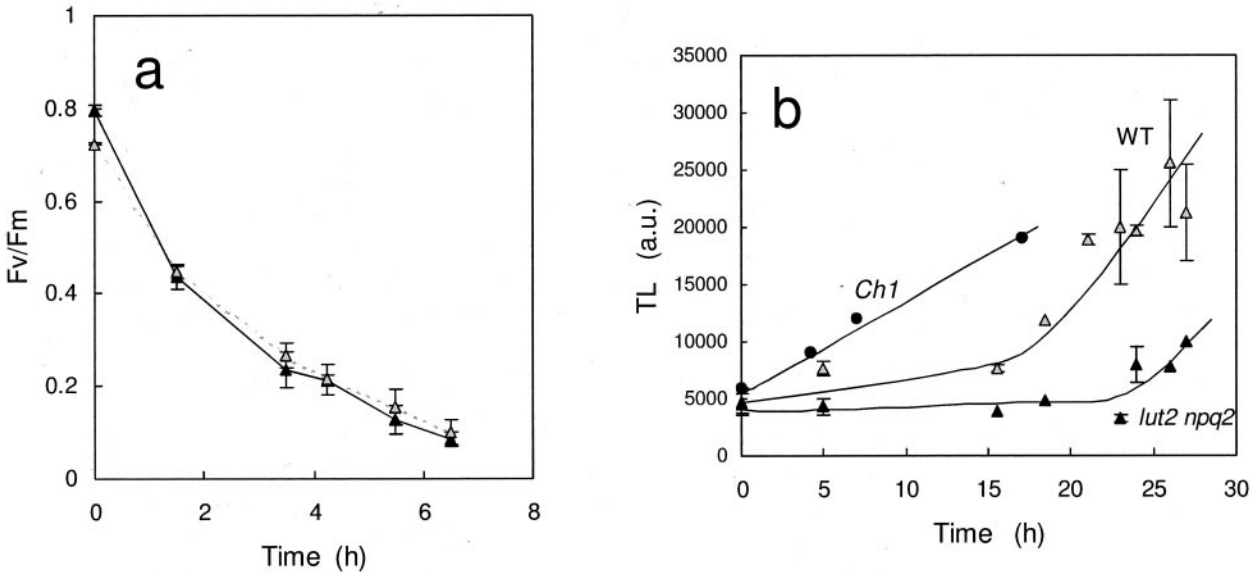

FIG. 10. **Tolerance of wild-type and *lut2 npq2* leaves to high light stress.** *a*, photoinhibition (as measured by the F_v/F_m chlorophyll-fluorescence ratio) and *b*, photooxidation (as measured by the amplitude of the 135 °C-TL band) of wild-type and *lut2 npq2* leaf discs exposed to high light stress (1300 μmol m^{-2} s^{-1} at 10 °C). For comparison purposes, photooxidative stress was also measured in the *ch1* chlorina mutant (*circles*). Data are mean values + S.D. of 2-four separate experiments.

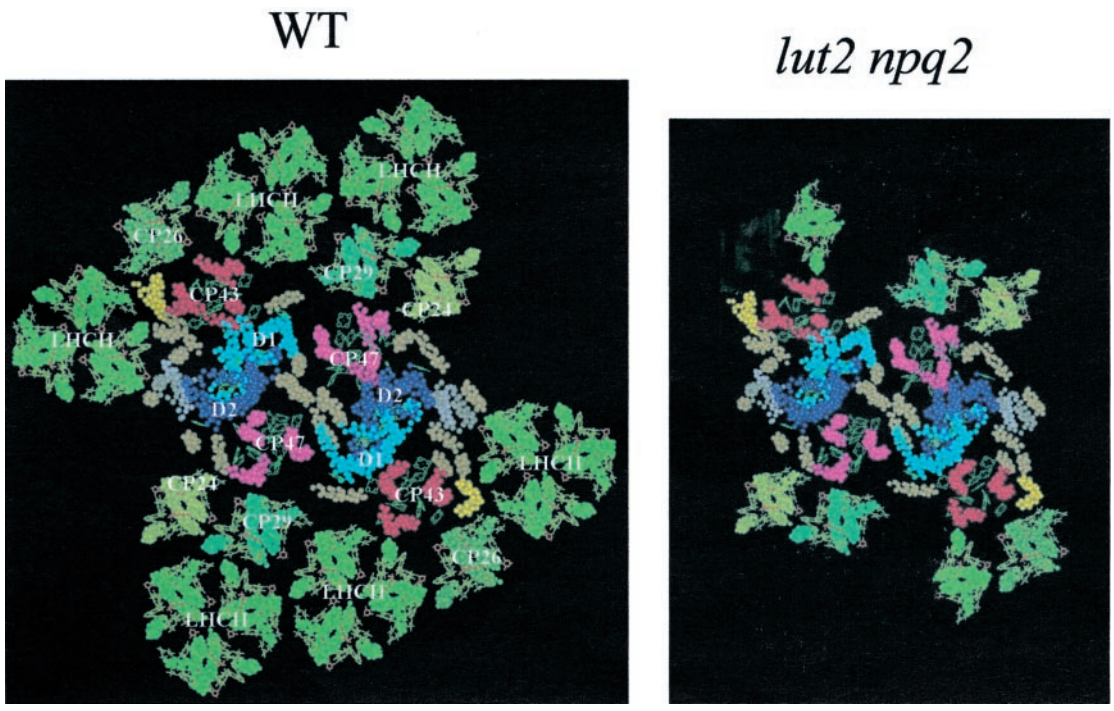

FIG. 11. **Model of the PSII light-harvesting antenna organization in wild-type and *lut2 npq2* mutant.**

mechanism used by plants to dissipate excess light energy (4, 6), zeaxanthin prevents oxidative damage of membrane through lipid peroxidation (8, 9). This function was recently confirmed by Muller-Moulé *et al.* (11) who showed that zeaxanthin deficiency enhances the sensitivity of an ascorbate-deficient Arabidopsis mutant to photooxidative stress. Conversely, transgenic plants overexpressing the β-carotene hydroxylase enzyme accumulated high amount of zeaxanthin and exhibited an increased tolerance to photooxidative stress (62, 63). Our finding that a zeaxanthin-accumulating mutant is highly tolerant to photooxidative stress is consistent with the antioxidant and lipid stabilizing function of zeaxanthin. It is not known whether zeaxanthin synthesized during the operation of the xanthophyll cycle inhibits reactive O_2 species and lipid peroxidation as solubilized pigments in the lipid matrix or bound to proteins. There are some experimental data that are compatible with a direct interaction between zeaxanthin and thylakoid membrane lipids (9, 12, 20). Although zeaxanthin is predominantly located in the light-harvesting system of *lut2 npq2* and is energetically coupled to chlorophylls (Fig. 9 and Table IV), a small fraction of zeaxanthin seems to occur as solubilized pigments in the lipid matrix. Indeed, the free pigment band (band I) in the sucrose gradient of *lut2 npq2* was enriched in zeaxanthin, with a molar zeaxanthin/chlorophyll ratio of 1.47 (Table III). The latter value is much higher than the expected ratio if the zeaxanthin molecules were derived from LHC denaturation. Minor LHCIIs have two carotenoid-binding sites, and the major LHCII has 3 or 4 binding sites in

TABLE VI
Thermal stability of recombinant LHCI proteins with different carotenoid contents

Sample	Temperature of denaturation
	°C
Lhca1 control	45.0[a]
Violaxanthin-Lhca1	44.6
Zeaxanthin-Lhca1	49.0
Lhca4 control	54.1[b]
Violaxanthin-Lhca4	53.9
Zeaxanthin- Lhca4	58.0

[a] Ref. 27.
[b] Ref. 55.

lut2 npq2 or wild type, respectively due to the fact that zeaxanthin cannot bind to the N1 site (34). Consequently, the amount of zeaxanthin from this origin would be of 3 zeaxanthin molecules per 10 chlorophylls (average between CP26, CP29, CP24, and LHCII in their relative amounts). This would yield a molar ratio zeaxanthin/chlorophyll of 0.3 and a zeaxanthin amount of 2.28 nmol while we found 11.13 nmol. Consequently, up to 80% of zeaxanthin in band I of *lut2 npq2* can be putatively considered as free *in vivo*. Similar considerations for wild type fall within a lower figure of 70%. Considering the amount of carotenoids loaded in the sucrose gradient and the amount of carotenoids found in the FP band, putatively free carotenoids in *lut2 npq2* (mostly zeaxanthin) are around 20% of total carotenoids. In wild type, the calculated fraction of free carotenoids is much lower, <10%. These estimates are, of course, approximate; in addition, we cannot exclude the release of zeaxanthin from a still unknown carotenoid-specific binding site with low affinity. Therefore, the free carotenoid pool in the sucrose band I can be both carotenoids dissolved in the membrane lipid matrix and carotenoids weakly bound to the protein phase in equilibrium with the carotenoids dissolved in the lipid matrix. However, a recent study has shown that small amounts of zeaxanthin can provide efficient protection against photooxidative stress in green algae (12). The free zeaxanthin pool in *lut2 npq2* thylakoid membranes could protect lipids by two different mechanisms: either by acting as a lipid peroxidation terminator (64) or by interacting with tocopherols (65). Future studies will have to determine whether the antioxidant activity of zeaxanthin under natural conditions (*i.e.* during the operation of the xanthophyll cycle) occurs in the protein phase, the lipid phase or both.

CONCLUSIONS

The photoinduced conversion of violaxanthin to zeaxanthin is involved in the protection of the thylakoid membranes against photooxidation (8), and, accordingly, this study has shown that the constitutive presence of high amounts of zeaxanthin in the *lut2 npq2* mutant increased substantially the thylakoid tolerance to photooxidative stress. Although the photosynthetic performance of *Arabidopsis* leaves was not dramatically affected by the substitution of all xanthophylls by zeaxanthin, this study has shown that the counterpart of the zeaxanthin-associated phototolerance is a reduced adaptation to low light intensities: *lut2 npq2* had a small antenna system that was unable to perform state transitions, had a reduced photochemical efficiency in low light and an enhanced photosynthetic capacity in high light. These photosynthetic characteristics are related to a selective destabilization and degradation of the major LHCII.

Zeaxanthin accumulation compromised severely the antenna size and stability. Therefore, the strong selection for maintenance of a specific xanthophyll composition (lutein, neoxanthin, violaxanthin) and a light-dependent interconversion of xanthophyll pigments (the violaxanthin cycle) in plant thylakoids during evolution is understandable. As suggested here and in previous studies (16, 33, 44), the wild-type xanthophyll composition is probably the best combination to provide the full range of structural and functional flexibility for photosynthesis under rapidly fluctuating environmental conditions. Although substitution of all xanthophylls by zeaxanthin resulted in viable and phototolerant plants, at least in a controlled environment, optimal fitness in the natural environments requires the wild-type complement of xanthophylls, which probably represents the best compromise between high efficiency of light harvesting in limiting light conditions and efficient protection in high light. As shown by the drastic reduction of the PSII antenna size in the *lut2 npq2* mutant, an active xanthophyll cycle confers advantage over a constitutive expression of zeaxanthin under natural conditions when light intensity can fluctuate considerably from photosynthetically limiting level to light saturating conditions. Our data are also consistent with the idea that zeaxanthin is a messenger acting as a signal transducer of persistent stress in thylakoids (66). Accumulation of zeaxanthin could participate in the long-term down-regulation of the PSII antenna size while it reinforces the PSI antenna stability. The benefit of this differential response of the PSI and PSII antennae to zeaxanthin accumulation is obvious: preferential excitation of PSI relative to PSII can avoid overreduction of the plastoquinone pool and the resulting photoinhibition of the PSII reaction centers.

Acknowledgments—We thank K. Meierhoff and S. Bossmann (Dusseldorf, Germany) for help with D1 quantification, C. Lico (Rome, Italy) for help with *lut 2* isolation, and T. Morosinotto (Verona, Italy) for reconstitution of Lhca proteins and helpful discussion.

REFERENCES

1. Yamamoto, H. J. (1979) *Pure Appl. Chem.* **51,** 639–648
2. Eskling, M., Arvidsson, P.-O., and Akerlund, H. E. (1997) *Physiol. Plant.* **100,** 806–816
3. Hager, A., and Holocher, K. (1994) *Planta* **192,** 581–589
4. Horton, P., Ruban, A. V., and Walters, R. G. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47,** 655–684
5. Demming-Adams, B., and Adams, W. W., III (2000) *Nature* **403,** 371–374
6. Müller, P., Li, X.-P., and Niyogi, K. K. (2001) *Plant Physiol.* **125,** 1558–1566
7. Niyogi, K. K., Grossman, A. R., and Björkman, O. (1998) *Plant Cell* **10,** 1121–1134
8. Havaux, M., and Niyogi, K. K. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 8762–8767
9. Havaux, M., and Kloppstech, K. (2001) *Planta* **213,** 953–966
10. Havaux, M., Bonfils, J.-M., Lütz, C., and Niyogi, K. K. (2000) *Plant Physiol.* **124,** 273–284
11. Müller-Moulé, P., Havaux, M., and Niyogi, K. K. (2003) *Plant Physiol.* 10.1104/pp 103.025320
12. Baroli, I., Do, A. D., Yamane, T., and Niyogi, K. K. (2003) *Plant Cell* **15,** 1–18
13. Külheim, C., Agren, J., and Jansson, S. (2002) *Science* **297,** 91–93
14. Tardy, F., and Havaux, M. (1996) *J. Photochem. Photobiol. B:Biol.* **34,** 87–94
15. Hurry, V., Anderson, J. M., Chow, W. S., and Osmond, C. B. (1997) *Plant Physiol.* **113,** 639–648
16. Lokstein, H., Tian, L., Polle, J. E. W., and DellaPenna, D. (2002) *Biochim. Biophys. Acta* **1553,** 309–319
17. Duckham, S. C., Linforth, R. S. T., and Taylor, I. (1991) *Plant Cell Environ.* **14,** 601–606
18. Rock, C. D., and Zeevaart, J. A. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7496–7499
19. Pesaresi, P., Morales, F., Moya, I., and Bassi, R. (1995) in *Photosynthesis: from Light to Biosphere* (Mathis, P., ed) Vol. IV, Kluwer Acad Publishers, 95–98
20. Havaux, M., Lütz, C., and Grimm, B. (2003) *Plant Physiol.* **132,** 300–310
21. Robinson, H. H., and Yocum, C. F. (1980) *Biochim. Biophys. Acta* **590,** 97–106
22. Bassi, R., Giacometti, G. M., and Simpson, D. J. (1988) *Biochim. Biophys. Acta* **935,** 152–165
23. Peter, G. F., and Thornber, J. P. (1991) *J. Biol. Chem.* **266,** 16745–16754
24. Caffarri, S., Croce, R., Breton, J., and Bassi, R. (2001) *J. Biol. Chem.* **276,** 35924–35933
25. Schägger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166,** 368–379
26. Meurer, J., Meierhoff, K., and Westhoff, P. (1996) *Planta* **198,** 385–396
27. Croce, R., Morosinotto, T., Castelletti, S., Breton, J., and Bassi, R. (2002) *Biochim. Biophys. Acta* **1556,** 29–40
28. Formaggio, E., Cinque, G., and Bassi, R. (2001) *J. Mol. Biol.* **314,** 1157–1166
29. Malkin, S., Armond, P. A., Mooney, H. A., and Fork, D. C. (1981) *Plant Physiol.* **67,** 570–579
30. Malkin, S., and Canaani, O. (1994) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **45,** 493–526
31. Havaux, M. (2003) *Trends Plant Sci.* **8,** 409–413
32. Vavilin, D. V., and Ducruet, J.-M. (1998) *Photochem. Photobiol.* **68,** 191–198
33. Pogson, B. J., Niyogi, K. K., Björkman, O., and DellaPenna, D. (1998) *Proc.*

Natl. Acad. Sci. U. S. A. **95,** 13324–13329
34. Croce, R., Weiss, S., and Bassi, R. (1999) *J. Biol. Chem.* **274,** 29613–29623
35. Bassi, R., and Caffarri, S. (2000) *Photosynth. Res.* **64,** 243–256
36. Polivka, T., Zigmantas, D., Sundstrom, V., Formaggio, E., Cinque, G., and Bassi, R. (2002) *Biochemistry* **41,** 439–450
37. Gastaldelli, M., Canino, G., Croce, R., and Bassi, R. (2003) *J. Biol. Chem.* **278,** 19190–19198
38. Wise, R. R. (1995) *Photosynth. Res.* **45,** 79–97
39. Murray, D. L., and Kohorn, B. D. (1991) *Plant Mol. Biol.* **16,** 71–79
40. Espineda, C. E., Linford, A. S., Devine, D., and Brusslan, J. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 10507–10511
41. Pogson, B., McDonald, K. A., Truong, M., Britton, G., and DellaPenna, D. (1996) *Plant Cell* **8,** 1627–1639
42. Frank, H. A., Das, S. K., Bautista, J. A., Bruce, D., Vasil'ev, S., Crimi, M., Croce, R., and Bassi, R. (2001) *Biochemistry* **40,** 1220–1225
43. Ruban, A. V., Wentworth, M., Yakushevska, A. E., Andersson, J., Lee, P. J., Keegstra, W., Dekker, J. P., Boekema, E. J., Jansson, S., and Horton, P. (2003) *Nature* **421,** 648–652
44. Polle, J. E. W., Niyogi, K. K., and Melis, A. (2001) *Plant Cell Physiol.* **42,** 482–491
45. Harrer, R., Bassi, R., Testi, M.-G., and Shaefer, C. (1998) *Eur. J. Biochem.* **255,** 196–205
46. Yakushevska, A. E., Keegstra, W., Boekema, E. J., Dekker, J. P., Andersson, J., Jansson, S., Ruban, A. V., and Horton, P. (2003) *Biochemistry* **42,** 608–613
47. Adams, W. W. III, Hoehn, A., and Demmig-Adams, B. (1995) *Austr. J. Plant Physiol.* **22,** 75–85
48. Yang, D.-H., Paulsen, H., and Andersson, B. (2000) *FEBS Lett.* **466,** 385–388
49. Yang, D.-H., Webster, J., Adam, Z., Lindahl, M., and Andersson, B. (1998) *Plant Physiol.* **118,** 827–834
50. Garab, G., Cseh, Z., Kovacs, L., Rajagopal, S., Varkonyi, Z., Wentworth, M., Mustardy, L., Der, A., Ruban, A. V., Papp, E., Holzenburg, A., and Horton, P. (2002) *Biochemistry* **41,** 15121–15129
51. Bazda, V., Istokovics, A., Simidjiev, I., and Garab, G. (1996) *Biochemistry* **35,** 8981–8985
52. Gussakovssky, E. E., Barzda, V., Shahak, Y., and Garab, G. (1997) *Photosynth. Res.* **51,** 119–126
53. Walters, R. G., and Horton, P. (1994) *Planta* **195,** 248–256
54. Bailey, S., Walters, R. G., Jansson, S., and Horton, P. (2001) *Planta* **213,** 794–801
55. Morosinotto, T., Castelletti, S., Breton, J., Bassi, R., and Croce, R. (2002) *J. Biol. Chem.* **277,** 36253–36261
56. Morosinotto, T., Baronio, R., and Bassi, R. (2002) *J. Biol. Chem.* **277,** 26913
57. Lee, J. W., Mets, L., and Greenbaum, E. (2002) *Appl. Biochem. Biotechnol.* **98,** 37–48
58. Baroli, I., and Melis, A. (1998) *Planta* **205,** 288–296
59. Tyystjarvi, E., Koivuniemi, A., Kettunen, R., and Aro, E. M. (1991) *Plant Physiol.* **97,** 477–483
60. Tyystjarvi, E., Kettunen, R., and Aro, E. M. (1994) *Biochim. Biophys. Acta* **1186,** 177–185
61. Havaux, M., and Tardy, F. (1997) *Plant Physiol.* **113,** 913–923
62. Davison, P. A., Hunter, C. N., and Horton, P. (2002) *Nature* **418,** 203–206
63. Götz, T., Sandmann, G., and Römer, S. (2002) *Plant Mol. Biol.* **50,** 129–142
64. Lim, B. P., Nagao, A., Terao, J., Tanaka, K., Suzuki, T., and Takama, K. (1992) *Biochim. Biophys. Acta* **1120,** 178–184
65. Palozza, P., and Krinsky, N. I. (1992) *Arch. Biochem. Biophys.* **297,** 184–187
66. Morosinotto, T., Caffarri, S., Dall'Osto, L., and Bassi, R. (2003) *Physiol. Plant.* **119,** 347–354

The Effect of Zeaxanthin as the Only Xanthophyll on the Structure and Function of the Photosynthetic Apparatus in *Arabidopsis thaliana*

Michel Havaux, Luca Dall'Osto, Stephan Cuiné, Giovanni Giuliano and Roberto Bassi

J. Biol. Chem. 2004, 279:13878-13888.
doi: 10.1074/jbc.M311154200 originally published online January 13, 2004
