Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 8762–8767, July 1999
Plant Biology

The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism

MICHEL HAVAUX*† AND KRISHNA K. NIYOGI‡

*Commissariat à l'Energie Atomique/Cadarache, Département d'Ecophysiologie Végétale et de Microbiologie, Laboratoire d'Ecophysiologie de la Photosynthèse, F-13108 Saint-Paul-lez-Durance, France; and ‡Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720-3102

Edited by Olle Bjorkman, Carnegie Institution of Washington, Stanford, CA, and approved May 21, 1999 (received for review February 1, 1999)

ABSTRACT **When light energy absorbed by plants becomes excessive relative to the capacity of photosynthesis, the xanthophyll violaxanthin is reversibly deepoxidized to zeaxanthin (violaxanthin cycle). The protective function of this phenomenon was investigated in a mutant of *Arabidopsis thaliana*, *npq1*, that has no functional violaxanthin deepoxidase. Two major consequences of the *npq1* mutation are the absence of zeaxanthin formation in strong light and the partial inhibition of the quenching of singlet excited chlorophylls in the photosystem II light-harvesting complexes. Prolonged exposure of whole plants to bright light resulted in a limited photoinhibition of photosystem II in both *npq1* and wild-type leaves, although CO_2 fixation and the linear electron transport in *npq1* plants were reduced substantially. Lipid peroxidation was more pronounced in *npq1* compared with the wild type, as measured by chlorophyll thermoluminescence, ethane production, and the total hydroperoxy fatty acids content. Lipid peroxidation was amplified markedly under chilling stress, and photooxidative damage ultimately resulted in leaf bleaching and tissue necrosis in *npq1*. The *npq4* mutant, which possesses a normal violaxanthin cycle but has a limited capacity of quenching singlet excited chlorophylls, was rather tolerant to lipid peroxidation. The double mutant, *npq4 npq1*, which differs from *npq4* only by the absence of the violaxanthin cycle, exhibited an increased susceptibility to photooxidative damage, similar to that of *npq1*. Our results demonstrate that the violaxanthin cycle specifically protects thylakoid membrane lipids against photooxidation. Part of this protection involves a mechanism other than quenching of singlet excited chlorophylls.**

The chloroplast of plants is a remarkable system that converts solar energy into chemical energy with a high efficiency. However, reactive forms of oxygen can be produced during illumination of chloroplasts, especially when the absorption of light energy exceeds the capacity of photosynthesis and the photoprotective mechanisms are overwhelmed (1, 2). Indeed, at high photon flux densities (PFDs), the accumulation of excitation energy in the light-harvesting chlorophyll antennae (LHC) of the photosystems favors the production of triplet excited chlorophyll molecules (^{3}Chl) that can interact with O_2 to generate reactive singlet oxygen ($^1O_2$). Overreduction of the photosynthetic electron carrier chain would also favor the direct reduction of O_2 by photosystem I (PSI) and the subsequent production of damaging reactive oxygen species, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$). In addition, many environmental stress conditions limit the ability of a plant to utilize light energy through photosynthesis so that excessive excitation of the photosystems can occur at moderate light intensities (3). Consequently, photooxidative damage, especially to PSII (4–6), appears to be an unavoidable consequence of the photosynthetic activity and can be a major factor that causes sustained depressions in photosynthetic efficiency (photoinhibition).

Carotenoids play a key role in the protection of photosynthetic organisms against the toxic effects of light. They are able to quench ^{3}Chl and $^1O_2$ (7, 8), and these functions have been demonstrated *in vitro* in PSII complexes (9). Under excess light, there is also a rapid change in the carotenoid composition of the LHCs: the diepoxide xanthophyll violaxanthin (V) is rapidly and reversibly converted via the intermediate antheraxanthin (A) to the epoxide-free zeaxanthin (Z) under the action of the enzyme V deepoxidase (10–12). Although this xanthophyll interconversion (V cycle) has been studied extensively in the recent years, its physiological role is not yet completely understood. The phototransformation of V is involved in the conversion of PSII to a state of high thermal energy dissipation and low Chl fluorescence emission. It has been suggested that Z could quench directly the singlet excited state of chlorophylls (^{1}Chl) (11, 13) or could favor proton-induced aggregation of the LHCs of PSII leading to energy dissipation (14). An increase in thermal deactivation of ^{1}Chl is potentially beneficial because it can protect the PSII reaction centers from overexcitation and subsequent photoinhibition and it also can reduce the probability of ^{3}Chl and $^1O_2$ formation in the LHCs. It must be noted, however, that the protective function of the V cycle is probably not restricted to PSII, because the cycle takes place in both PSII and PSI (15). Inhibition of the V cycle by DTT, a potent (though nonspecific) inhibitor of the V deepoxidase, was associated with only a slight increase in the level of PSII photoinhibition in strong light whereas, concomitantly, pronounced lipid peroxidation monitored by the production of ethane was induced (16). This suggests that the V cycle could be involved in a general protection of the photosynthetic apparatus against photooxidation.

Recently, using a Chl fluorescence video-imaging system, Niyogi and coworkers (17, 18) succeeded to isolate mutants of the green alga *Chlamydomonas reinhardtii* and of the higher plant *Arabidopsis thaliana*, which is defective in the V cycle. The *npq1 Arabidopsis* mutant was observed to be unable to form Z in excessive light, and a detailed genetic analysis demonstrated that the *npq1* mutation affected the structural gene encoding V deepoxidase. Clearly, this mutant provides a unique tool to understand the photoprotective function of the V cycle in higher plants. Rather surprisingly, although detached *npq1* leaves showed a significantly greater sensitivity than did the wild type to a short-term photoinhibitory light treatment during which gas exchange was restricted, growth of *npq1* plants was not inhibited significantly in the natural sunlight of the Californian summer (18). Similarly, photoautotrophic growth of the *npq1* mutant of *Chlamydomonas* was not impaired relative to the wild type in high light (17). In this study, we analyzed the behavior of *npq1*

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: A, antheraxanthin; Chl, chlorophyll; LHC, light-harvesting complex; NPQ, nonphotochemical energy quenching; PFD, photon flux density; PS, photosystem; TL, thermoluminescence; V, violaxanthin; Z, zeaxanthin.
†To whom reprint requests should be addressed. e-mail: michel.havaux@cea.fr.

Arabidopsis plants during prolonged exposure (several days) to high-light irradiances. Interpretation of the results was aided by the use of another mutant, *npq4*, which has a normal V cycle but exhibits greatly reduced nonphotochemical energy quenching (NPQ) in PSII (19). The results confirm the relative photostability of PSII in the absence of the V cycle and point to a specific involvement of Z in the protection of the chloroplast membranes against photooxidative damage. Our data also suggest that part of the antioxidant activity of Z is not related to quenching of ^{1}Chl in the LHCs.

MATERIALS AND METHODS

Plant Material and Treatments. Wild-type *A. thaliana* (L.) Heynh. (ecotype Columbia) and *npq1–2, npq4–1*, and *npq4–1 npq1–2* mutants were grown on a mixture of sand and sphagnum peat at 22°C/18°C (day/night) under a PFD of 220 μmol of photons·m^{-2}·s^{-1} with a photoperiod of 8 hr and a relative air humidity of 60%.

Strong light stress (1,500–1,700 μmol of photons·m^{-2}·s^{-1}, 15-hr photoperiod) was imposed on whole plants at 23°C by using 150-W metal halide lamps (Osram, Berlin) equipped with two infrared suppressor filters (Schott). Chilling stress was imposed on plants placed in closed growth chambers at 6°C/5°C (day/night) and under a PFD of 900 μmol of photons·m^{-2}·s^{-1} (12-hr photoperiod).

CO_2 exchange by *Arabidopsis* plants was measured in tightly closed glass vessels of 20 liters in volume (two plants per vessel). Temperature, light, air humidity, and gas composition of the atmosphere in the vessels were carefully controlled, and photosynthesis, respiration, and evapotranspiration were monitored continuously as described elsewhere (20). The plants from the phytotron first were adapted in the glass vessels to a PFD of 300 μmol of photons·m^{-2}·s^{-1} (10 hr per day) at 22°C/18°C for 5 days, and then PFD was increased from 300 to 1,500 μmol of photons·m^{-2}·s^{-1}, with the other environmental conditions remaining unchanged.

Photosynthetic Pigments and Vitamin E. Pigments were extracted, separated, and quantified by HPLC, as described previously (21). Vitamin E (α-tocopherol) was determined by HPLC by using the protocol described in ref. 22.

Thermoluminescence. Chl thermoluminescence was measured with a Hamamatsu photomultiplier tube (R376) in a laboratory-made set-up (23). The leaf sample preadapted to darkness for 15 min was heated from 25 to 170°C at a rate of 7°C min^{-1}.

Volatile Alkanes. Leaf samples (fresh weight, 250 mg) were placed into a 16-ml flask and sealed under nitrogen atmosphere. *In situ* decomposition of ω-3 unsaturated hydroperoxy fatty acids into ethane was accelerated by a brief heat treatment (90 s) of the samples in a microwave oven. After cooling the flask, 1 ml of the gas phase was withdrawn and injected in a gas chromatograph for ethane determination (16).

Hydroperoxy Fatty Acids. The hydroperoxy fatty acid content of the leaves was determined by HPLC quantification of their hydroxy derivatives obtained after $NaBH_4$ reduction and hydrolysis of a lipid extract, as described in ref. 24.

Photosynthetic Measurements *in Vivo*. Chl fluorescence was measured with a pulse–amplitude-modulation-type fluorometer (Walz, Effeltrich, Germany) (25) after 15-min dark preadaptation. The maximal quantum yield of PSII photochemistry was calculated as $F_v/F_m = (F_m - F_o)/F_m$, where F_o is the initial fluorescence level excited by a weak red light pulsed at 1.6 kHz, and F_m is the maximal level of Chl fluorescence obtained by applying an 800-ms flash of intense white light (4,500 μmol of photons·m^{-2}·s^{-1}). Leaves were illuminated with white light produced by a Schott KL1500 light source (Schott, Mainz, Germany), and the nonphotochemical quenching of F_m was monitored by sequentially applying intense light pulses. The quantum yield of noncyclic electron transport in the light was calculated as $\Delta F/F_m = (F_m - F)/F_m$, where F is the actual level of Chl fluorescence.

The net rate of photosynthetic oxygen evolution in saturating CO_2 was measured with a Clark-type oxygen electrode (Hansatech LD2/2; Hansatech Instruments, Pentney King's Lynn, U.K.). The maximal quantum yield of gross O_2 evolution (Φ) also was measured with the photoacoustic technique by using a modulated (21-Hz) white light of PFD 12 μmol of photons·m^{-2}·s^{-1}, as described (21).

RESULTS

Photosynthetic Characteristics of *npq1*. As expected (18), *npq1* leaves were unable to convert V to A and Z when the PFD was increased suddenly from 220 μmol of photons·m^{-2}·s^{-1} (growth conditions) to 1,500 μmol of photons·m^{-2}·s^{-1} (Fig. 1*A*). Even prolonged exposure (several days) to strong light did not cause any significant formation of Z and A in *npq1* (see legend of Fig. 2). Upon transfer to bright light, heat losses were enhanced, as reflected by the quenching of the F_m of Chl fluorescence emission (Fig. 1*B*). The extent of this fluorescence quenching was reduced substantially in *npq 1* compared with the wild type, as reported previously (18).

We also have examined whether the inhibition of the V cycle in *npq1* affected the photosynthetic performance of the leaves. Neither the light-limited nor light-saturated rate of photosynthetic O_2 evolution was affected by the mutation: the net photosynthetic rate at 4,000 μmol of photons·m^{-2}·s^{-1} was about 184 nmol of O_2·cm^{-2}·s^{-1} in both genotypes. Photoacoustic measurements in dim white light confirmed that the maximal quantum yield of gross O_2 evolution (Φ) was not changed in *npq1*: Φ (in relative values) was 3.00 ± 0.44 in the mutant and 2.99 ± 0.31 in the wild type. The net rate of CO_2 fixation by whole plants also was observed to be unaffected by the mutation: on average, a wild-type plant fixed 4,889 ± 361 μl of CO_2/hr and a mutant plant

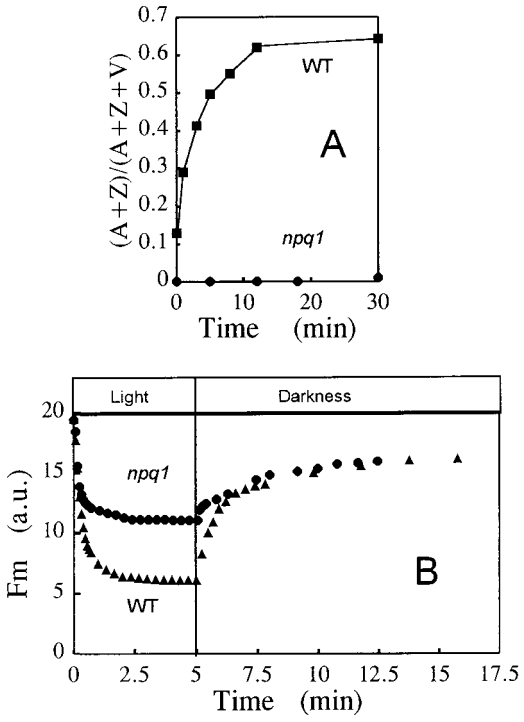

FIG. 1. V cycle and NPQ in the *npq1* mutant of *A. thaliana*. (*A*) Conversion of V to A and Z, as monitored by the (A + Z)/(A + Z + V) ratio, in leaves of the wild type and *npq1* mutant suddenly exposed to a strong, white light of PFD of 1,500 μmol of photons·m^{-2}·s^{-1}. (*B*) Quenching of F_m induced by the strong, white light and subsequent relaxation of the fluorescence quenching in the dark. The F_o and F_m fluorescence levels at time 0 were identical in the wild type and the mutant.

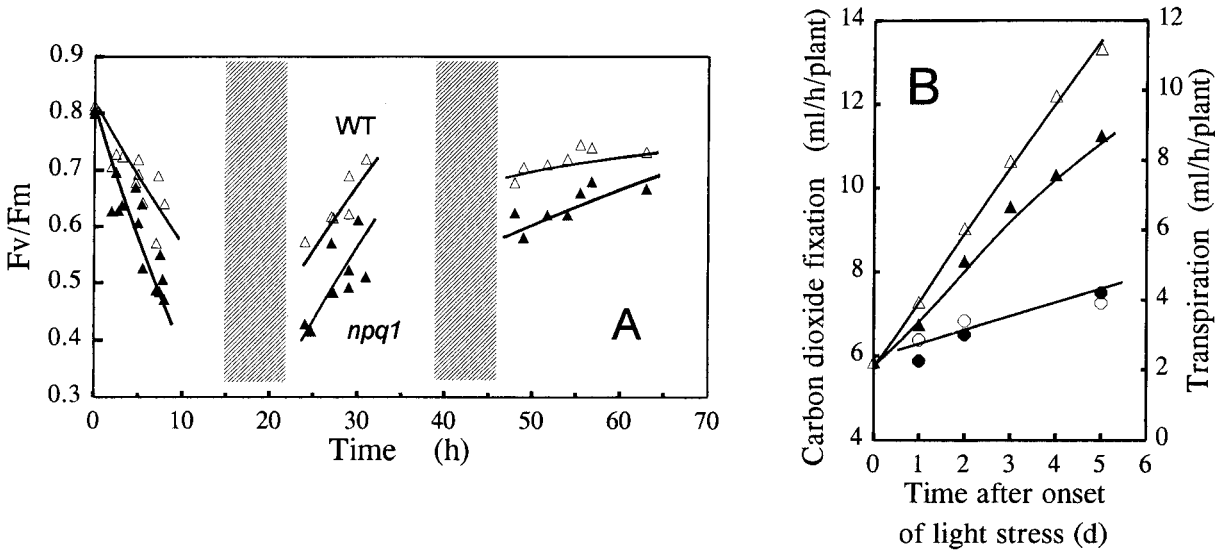

FIG. 2. Inhibition of PSII photochemistry and of photosynthetic CO_2 fixation during strong light stress. (*A*) F_v/F_m during a strong light stress (1,500 μmol of photons·m^{-2}·s^{-1}) of wild-type and *npq1 Arabidopsis* leaves. The hatched areas indicate the night periods. Each experimental point is the mean value of 4–15 measurements. Chl fluorescence was measured only on green leaves or the green parts of partially bleached *npq1* leaves (see Fig. 3). (A + Z)/(V + A + Z) values: wild type, 0.07 and 0.53 before and after light stress (60 hr); *npq1*, 0 and 0.025 before and after light stress (60 hr). (*B*) Net photosynthetic CO_2 fixation by one *Arabidopsis* plant (△, wild type; ▲, *npq1* mutant) exposed to strong, white light. At time 0, plants were shifted from 300 μmol of photons·m^{-2}·s^{-1} to 1,500 μmol of photons·m^{-2}·s^{-1}, and the rate of CO_2 fixation was measured every day during 1 hr from 11:30 a.m. to 12:30 p.m. Transpiration of *npq1* (●) and wild-type (○) plants was comparable, indicating that stomatal closure was not responsible for the lower CO_2 fixation activity of *npq1*.

fixed 5,120 ± 228 μl CO_2/hr under a PFD of 300 μmol of photons·m^{-2}·s^{-1}.

Photoinhibition of PSII, Electron Transport, and CO_2 Fixation in *npq1*. Whole *Arabidopsis* plants were exposed to a high PFD of 1,500 μmol of photons·m^{-2}·s^{-1} for several days. The maximal quantum yield of PSII photochemistry (F_v/F_m) markedly decreased during the first day of illumination, and this decrease was significantly more pronounced in *npq1* than in the wild type, indicating that *npq1* was more prone to PSII photoinhibition in this time scale (Fig. 2*A*), as reported previously by Niyogi *et al.* (18). However, during the second day of illumination, PSII partially recovered, as demonstrated by the progressive increase in the F_v/F_m ratio. F_v/F_m recovered further during the third day, reaching, in the wild type, a value close to that measured before the light treatment. Interestingly, the difference between the two genotypes was strongly attenuated with time: at the end of day 3 (i.e., about 60 hr after the beginning of the light treatment), F_v/F_m was reduced by only 12% in *npq1* compared with the F_v/F_m value of the wild type. These results suggest that PSII in both the wild type and *npq1* was able to acclimate to strong light in long-term experiments. However, the difference in NPQ between wild type and *npq1* (Fig. 1*B*) was maintained during light acclimation (data not shown).

When the quantum yield of electron transport ($\Delta F/F_m$) was measured (in red light of PFD 70 μmol of photons·m^{-2}·s^{-1}) rather than the maximal photochemical efficiency of PSII, a noticeable difference was observed between *npq1* and the wild type. After 3 days of light stress, the $\Delta F/F_m$ was reduced from 0.61 ± 0.02 to 0.46 ± 0.03 in the wild type and from 0.60 ± 0.03 to 0.39 ± 0.04 in *npq1*, although the leaves used for those measurements had quite similar F_v/F_m (0.60 ± 0.11 and 0.59 ± 0.01). This suggests that whole-chain electron transport was more affected than PSII in *npq1* leaves.

Similarly, CO_2 fixation by *npq1* plants was noticeably more affected by prolonged exposure to high PFD than CO_2 fixation of wild-type plants, and this difference was not due to differential stomatal closure (Fig. 2*B*). The rate of CO_2 fixation by wild-type plants increased more than that of *npq1* plants during the 5-day light treatment, indicating that the wild type grew larger than the mutant. Taken together, our observations under photoinhibitory light conditions indicate that the absence of the V cycle increased the photosensitivity of the photosynthetic apparatus and that some components other than PSII are likely to be affected.

Lipid Peroxidation in *npq1*. The visible phenotype of the plants exposed to high light intensities (Fig. 3) suggested that *npq1* plants suffered from photooxidative damage, with some leaves exhibiting bleached patches and necrotic areas after long treatments (4 days or more at 1,600 μmol m^{-2}·s^{-1}). Therefore, we examined the lipid peroxidation status of *Arabidopsis* chloroplasts *in vivo* by using the thermoluminescence (TL) technique (26–28). Fig. 4*A* shows the TL signal from a wild-type leaf. Two distinct bands were obtained that were peaking at *ca.* 70°C and 135°C. These "high-temperature" TL bands do not result from charge recombination of PSII redox components. The 135°C band is attributed to energy transfer from lipid peroxidation products to Chl molecules in thylakoid membranes (28, 29). Presumably, the 135°C band is generated in the reaction of thermal decomposition of lipid or fatty acid cycloperoxides that, in turn, leads to formation of carbonyls in triplet state followed by migration of excitation energy toward Chl (28). The amplitude of this band has been correlated in previous studies with the accumulation of conjugated diene and malondialdehyde and has been used to detect lipid peroxidation in plants exposed to various photooxidative stress conditions, including water stress (30), heavy metals

FIG. 3. Wild-type and *npq1* mutant plants after 4 days at 1,600 μmol of photons·m^{-2}·s^{-1}.

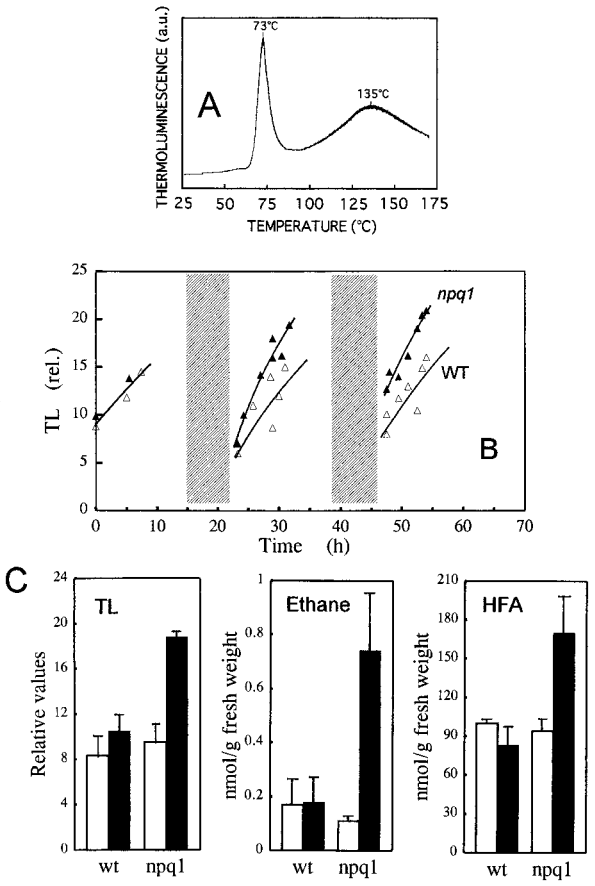

FIG. 4. Lipid peroxidation in wild-type and *npq1* leaves during strong light stress. (*A*) Typical TL curve of an *Arabidopsis* leaf (wild type), showing the lipid-peroxidation-related TL band at *ca.* 135°C. (*B*) Amplitude of the 135°C TL band during a strong light treatment (1,500 μmol of photons·m^{-2}·s^{-1}) of wild-type and *npq1 Arabidopsis* leaves. The gray areas indicate the night periods. Each experimental point corresponds to one TL measurement performed on six discs taken from different leaves. (*C*) Comparison of the effects of a strong light treatment (3 days at 1,500 μmol of photons·m^{-2}·s^{-1}) on the amplitude of the 135°C TL band, the production of ethane, and the accumulation of hydroperoxy fatty acids (HFA) in *npq1* and wild-type *Arabidopsis* leaves. Open bars, before light stress; solid bars, after light stress. Data are mean values of three to four separate experiments. See legend of Fig. 2 for (A + Z)/(V + A + Z) data.

(28), intense light (28, 29), or temperature stress (28, 29). The origin of the sharp 70°C band found in *Arabidopsis* is unclear and was tentatively ascribed to thermolysis of a (yet unidentified) volatile compound (J.-M. Ducruet, personal communication).

Fig. 4*B* shows the effect of exposing plants to strong light on the amplitude of the lipid-peroxide-related 135°C TL band. During the first day of illumination, the TL band amplitude increased similarly in both genotypes. The 135°C peak amplitude decreased back to the initial value during the night, indicating metabolism of the lipid-peroxidation products detected by the TL method. During the two successive days of light stress, the TL band amplitude increased again, but the increase was much more marked in *npq 1* (+103% on day 3) compared with the wild type (+52% on day 3), indicating enhanced peroxidative damage to lipids in *npq1*.

This differential lipid peroxidation was confirmed by volatile alkane measurements in leaves (Fig. 4*C*). Ethane is the end product of ω-3 unsaturated hydroperoxy fatty acids, and its production occurs via β-scission of the corresponding alkoxy radicals (31). The ethane production after 3-day exposure to strong light was increased dramatically in *npq1* leaves but not in wild-type leaves. Hydroperoxy fatty acids also were quantified by HPLC. The results (Fig. 4*C*) showed that, in contrast to wild-type leaves, *npq1* leaves accumulated high amounts of hydroperoxides during strong light stress, thus confirming the ethane and TL data.

Lipid peroxidation in *npq1* was accompanied by some losses of chlorophyll (approximately −20%) whereas the total carotenoid content increased substantially (Table 1). Particularly, the V content noticeably increased in *npq1* as did the pool of xanthophyll-cycle pigments in the wild type. The concentration of vitamin E (α-tocopherol), the major lipophilic antioxidant present in the thylakoid membrane lipid bilayer (32), also was determined. Vitamin E strongly increased with light stress in both genotypes but the final concentration was higher in the wild type compared with *npq1*. Accumulation of vitamin E confirms that the light stress used in this study increased ^{3}Chl and $^1O_2$ formation (33). The lower level found in *npq1* possibly resulted from vitamin E breakdown associated with scavenging of lipoperoxy radicals (34).

Lipid peroxidation also can occur under low or moderate light when plants are exposed to an additional constraint such as water stress (35) or chilling stress (34). When *Arabidopsis* plants were exposed for 5 days to 6°C in moderate light (PFD, 900 μmol photons m^{-2} s^{-1}), pronounced lipid peroxidation took place, and the difference between the wild type and the *npq1* mutant was amplified: the amplitude of the 135°C TL band increased from 8.5 ± 0.9 to 20.2 ± 2.3 in the wild type and from 9.6 ± 0.9 to 35.6 ± 9.3 in *npq1*. Concomitantly, there was a significant difference in PSII photoinhibition between the two genotypes: F_v/F_m was 0.63 ± 0.15 and 0.44 ± 0.20 in wild type and *npq1*, respectively. Water stress also was observed to cause a dramatic lipid peroxidation in *npq1* compared with the wild type (data not shown).

Antioxidant Activity of Z That Is Unrelated to ^{1}Chl Quenching. To determine whether the protection against photooxidation mediated by the V cycle is solely a result of the involvement of Z in thermal dissipation (^{1}Chl quenching), we examined a new *Arabidopsis* mutant called *npq4* (19). This mutant has normal pigment composition, PSII-mediated electron transport, and V cycle activity but exhibits strongly reduced extent and reversibility of ^{1}Chl quenching (19). Although the *NPQ4* gene has not been identified yet, one can hypothesize that the mutation affects either the binding of A and Z to the LHCs or the conformational changes in the LHCs involved in the engagement of NPQ. From

Table 1. Photosynthetic pigments (total chlorophylls, total carotenoids, V + A + Z) and vitamin E (α-tocopherol) in wild-type and *npq1 Arabidopsis* plants before (LL) and after 3 days at 1,500 μmol of photons·m^{-2} (HL)

	Wild type		*npq1*	
Compound	LL	HL	LL	HL
Pigments, ng·mm^{-2}				
Chls	228.9 ± 13.7	201.3 ± 10.5	213.8 ± 7.6	170.2 ± 15.5
Carotenoids	53.6 ± 2.6	81.1 ± 5.8	55.2 ± 0.9	74.8 ± 9.7
V + A + Z	9.5 ± 0.1	28.2 ± 2.8	11.1 ± 0.3	22.9 ± 1.6
Vitamin E, μg·g^{-1} F.W.	9.2 ± 1.0	29.5 ± 1.6	9.3 ± 2.1	19.9 ± 2.0

Data are mean values of 3–6 separate experiments ± SD. F.W., fresh weight.

the simple mutants *npq1* and *npq4*, a double mutant was constructed: *npq4 npq1* has a limited capacity of quenching ^{1}Chl, similar to that of *npq4*, and has no V cycle. When exposed for 15 min to white light of PFD of 1,500 μmol of photons·m^{-2}·s^{-1}, the steady-state value of the nonphotochemical fluorescence quenching ([F_m in the dark/F_m in the light] − 1) was 0.70 ± 0.01 in *npq4* and 0.72 ± 0.04 in *npq1 npq4* (and about 2 in wild type, cf. Fig. 1*B*). Therefore, by comparing *npq4* and *npq4 npq1*, we were able to examine the photoprotective role of the V cycle with limited thermal deexcitation of ^{1}Chl.

The effects on PSII photochemistry and lipid peroxidation of 3-day exposure of *npq4* and *npq4 npq1* plants to strong light are shown in Table 2. Clearly, the double mutant suffered more from the light stress than *npq4*: the amplitude of the 135°C TL band and the ethane production were higher in the double mutant compared with the simple mutant. F_v/F_m seemed to be slightly more reduced in *npq1 npq4* but the difference with *npq4* was not statistically significant. The pool of V cycle pigments increased noticeably during light stress in both *npq4* and *npq4 npq1*. In fact, the difference found between *npq4* and *npq4 npq1* in Table 2 resembles that found between the wild type and *npq1* in Fig. 4. However, *npq4* leaves were more peroxidized than wild-type leaves after light treatment (Table 2), and the different genotypes can be ranked with respect to sensitivity to photooxidation as follows (least to most sensitive): wild type < *npq4* < *npq1* = *npq1 npq4*. One then can conclude that, at least for conditions of prolonged illumination with high-intensity light, a significant part of the protective action of Z against photooxidative damage occurs via a mechanism different from ^{1}Chl quenching in the LHCs because inhibition of NPQ in *npq4* did not lead to marked lipid peroxidation.

DISCUSSION

Because of their unique lipid composition characterized by a high proportion of galactolipids containing highly unsaturated fatty acids (C18:3), chloroplast membranes are very sensitive targets for photodestruction by active forms of oxygen. The results of this study demonstrate that the V cycle is involved specifically in the protection of the photosynthetic membranes against lipid peroxidation. Lipid peroxidation in the *npq1* mutant was accompanied by pigment losses (Table 1) and ultimately resulted in leaf bleaching, necrosis, and reduced growth (Figs. 3 and 2*B*). The antioxidant activity of the V cycle may be crucial for the survival of plants in natural conditions when a combination of factors brings about excessive excitation of the Chl antennae and/or favors oxygen photoreduction by the photosynthetic electron transport chain. In this study, such conditions were obtained in moderate light by leaf dehydration and chilling temperature, leading to a strongly increased lipid peroxidation in *npq1* compared with wild type.

Despite the inhibition of the photoregulation of thermal energy dissipation in PSII (Fig. 1*B*), the absence of the V cycle in *npq1* had relatively minor effects on the PSII photochemical efficiency, with the notable exceptions of short-term photoinhibitory light treatment and chilling stress in the light. Possibly, acclimation of *Arabidopsis* plants to high-light irradiance was associated with a stimulation of the repair process of photodamaged PSII reaction center complexes, as reported previously in other plant species (36–39). As a result, increased energy dissipation in the LHCs and reduced energy delivery to the PSII reaction centers probably became of lesser importance. At chilling temperatures, the acclimation process may be less efficient, e.g., because of increased membrane viscosity (40), leading to a differential photoinhibition of PSII in *npq1* and the wild type. On the other hand, it has been suggested that photoinhibition actually requires Z and that sustained photoinhibition represents sustained maintenance of Z-dependent energy dissipation (41, 42). This possibility could provide an alternative explanation to the finding that photoinhibition in Z-deficient *npq1* plants was not amplified strongly compared with wild-type plants.

Our lipid peroxidation data are neutral with respect to the mechanism by which Z exerts its antioxidant activity. However, the differential sensitivity to photooxidation of the *npq1* and *npq4* mutants, which both have a strongly reduced NPQ, indicated that part of the protection by the V cycle occurred by a mechanism different from increased energy dissipation in the LHCs by NPQ. Consequently, one can suggest that an important aspect of the protective function of the V cycle is to scavenge $^1O_2$ and/or free radicals that otherwise would interact with the lipids surrounding the photosystems. This scavenging activity can take place in the LHCs and/or their boundary lipid environment. In fact, because of their higher number of conjugated double bonds, A and Z are supposed to be better photoprotectors than V with a higher efficiency for deexciting $^1O_2$ (43, 44). Conversely, V is a light-harvesting accessory pigment that is able to transfer excitation energy to Chl *a* (45, 46), and there are some data indicating that V could be a better light harvester than Z *in vivo* (47). It is known that singlet–singlet energy transfer from carotenoids to Chls requires a very close contiguity of the pigments (8, 48). The removal of the two epoxides confers a difference in the end-group structure of the xanthophylls (49) that can perturb pigment–pigment or pigment–protein interactions (50) and, hence, the excitation energy transfer to Chls. Moreover, the Z excited states lie below that of V, leading to a less favorable energy transfer to Chl from Z or even to a change in direction of energy transfer between carotenoid and Chl (11, 51). Thus, the xanthophyll cycle can be seen as an elegant mechanism that rapidly converts, within the LHCs, an efficient accessory pigment in weak light (V) to an efficient photoprotector in strong light (Z).

Carotenoids also can trap various types of free radicals (43). When incorporated into liposomes, carotenoids prevent them from being oxidized (52), and Z has been demonstrated to be a very efficient radical scavenger and chain-breaking antioxidant in peroxyl-radical-mediated peroxidation of artificial lipid mem-

Table 2. Effects of strong light stress (3 days at 1,600 μmol photons m^{-2} s^{-1}) on the V cycle pigments, F_v/F_m, and the level of lipid peroxidation, as measured by the amplitude of the 135°CTL band and the ethane production in leaves of the wild type, the *npq4* mutant, and the *npq4 npq1* double mutant of *Arabidopsis*

Arabidopsis	TL, a.u.	Ethane, pmol·g^{-1} F.W.	F_v/F_m	V + A + Z, ng·mm^{-2}	(A + Z)/V + A + Z)
Wild type					
Control	8.4 ± 1.6	248 ± 25	0.81 ± 0.01	10.0 ± 0.3	0.11 ± 0.02
Light-treated	10.5 ± 1.5	261 ± 21	0.72 ± 0.06	27.3 ± 2.1	0.51 ± 0.10
npq4					
Control	8.2 ± 2.0	227 ± 82	0.79 ± 0.01	10.8 ± 0.9	0.20 ± 0.02
Light-treated	12.1 ± 1.9	324 ± 26	0.69 ± 0.08	23.1 ± 3.3	0.43 ± 0.09
npq4 npq1					
Control	6.3 ± 0.8	186 ± 31	0.78 ± 0.01	10.8 ± 0.5	0
Light-treated	19.6 ± 1.2	610 ± 155	0.60 ± 0.14	29.7 ± 7.4	0.02 ± 0.01

Data are mean values of 3–10 experiments ± SD. F.W., fresh weight.

branes (53, 54). In this context, it has been suggested (but not yet proven) that the V-to-Z conversion *in vivo* takes place from a free pool of V, in equilibrium with bound V, at the interface between the LHCs and a particular lipid environment (monogalactosyldiacylglycerol) (55) and that Z formed in strong light interacts with the lipid phase of the thylakoid membrane (56–59). Thus, the Z-related reduction of lipid peroxidation observed in this study possibly could be the manifestation of the transitory presence of Z in the thylakoid membrane lipid phase, where it acts as a terminator of peroxy-radical chain reactions. This possibility is still hypothetical and remains to be established in further studies.

The antioxidant effect of Z on thylakoid lipids likely supplements that of the lipophilic antioxidant vitamin E. Interestingly, it has been demonstrated in artificial membranes that carotenoids and vitamin E act synergistically as radical scavengers (60, 61). Thanks to this cooperativity, the presence of relatively little amounts of Z in the thylakoid membrane lipid matrix may have a substantial effect on the photoprotective activity of vitamin E. Presumably, in the presence of ascorbate, carotenoids are able to repair the α-tocopheryl radical, which is produced when vitamin E scavenges an oxy-radical (60). It is tempting to suggest that the absence of Z in *npq1* impaired α-tocopherol recycling and progressively reduced the vitamin E level in thylakoid membrane (as found in this study; Table 1), leading to enhanced lipid peroxidation. This possibility will be examined in future research.

Although the exact molecular mechanism(s) involved in the photoprotective action of Z remain to be elucidated, our data reveal the efficacy of a light-controlled antioxidant system in which a simple chemical substitution in a xanthophyll molecule elicits rather profound changes in the chloroplast membrane photostability. That the V cycle is a ubiquitous system that is found in almost all photosynthetic eukaryotes (10) is probably an indication of its important role in preventing oxidative damage. This is also consistent with the fact that acclimation of plants to high PFD is usually accompanied by a preferential accumulation of the xanthophyll-cycle pigments (refs. 3 and 10; see also Table 1). The light-regulated dynamic interconversion of light-harvesting and photoprotective pigments in the LHCs during the operation of the xanthophyll cycle may confer a selective advantage in many natural environments, particularly those characterized by rapid changes in light intensity and/or by the combination of high light and additional environmental constraints. The protective role of Z against photooxidative damage shown here makes the xanthophyll cycle a potential target of genetic engineering for increasing plant resistance to harsh environments.

We are grateful to J.-L. Montillet and C. Triantaphyllidès for help with lipid peroxide determinations, J.-M. Ducruet for advice on thermoluminescence, M. Péan and the members of the GRAP Laboratory (Commissariat à l'Energie Atomique/Cadarache) for help in growing plants under stress conditions, and C. Lütz for vitamin E determinations. Critical reading of the manuscript by S. Malkin is also acknowledged.

1. Asada, K. & Takahashi, M. (1987) in *Photoinhibition*, eds. Kyle, D. J., Osmond, C. B. & Arntzen, C. J. (Elsevier, Amsterdam), pp. 227–287.
2. Niyogi, K. K. (1999) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, in press.
3. Demmig-Adams, B. & Adams, W. W., III (1992) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43,** 599–626.
4. Barber, J. & Andersson, B. (1992) *Trends Biochem. Sci.* **17,** 61–66.
5. Prasil, O., Adir, N. & Ohad, I. (1992) in *The Photosystems: Structure, Function and Molecular Biology*, ed. Barber, J. (Elsevier, Amsterdam), pp. 295–348.
6. Aro, E.-M., Virgin, I. & Andersson, B. (1993) *Biochim. Biophys. Acta* **1143,** 113–134.
7. Siefermann-Harms, D. (1987) *Physiol. Plant.* **69,** 561–568.
8. Frank, H. A. & Cogdell, R. J. (1996) *Photochem. Photobiol.* **63,** 257–264.
9. Telfer, A., Dhami, S., Bishop, S. M., Phillips, D. & Barber, J. (1994) *Biochemistry* **33,** 14469–14474.
10. Pfündel, E. & Bilger, W. (1994) *Photosynth. Res.* **42,** 89–109.
11. Demmig-Adams, B. & Adams, W. W., III (1996) *Trends Plant Sci.* **1,** 21–26.
12. Eskling, M., Arvidsson, P.-O. & Akerlund, H.-E. (1997) *Physiol. Plant.* **100,** 806–816.
13. Gilmore, A. M. (1997) *Physiol. Plant.* **99,** 197–209.
14. Horton, P., Ruban, A. V. & Walters, R. G. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47,** 655–684.
15. Thayer, S. S. & Björkman, O. (1992) *Photosynth. Res.* **33,** 213–225.
16. Sarry, J.-E., Montillet, J.-L., Sauvaire, Y. & Havaux, M. (1994) *FEBS Lett.* **353,** 147–150.
17. Niyogi, K. K., Björkman, O. & Grossman, A. R. (1997) *Plant Cell* **9,** 1369–1380.
18. Niyogi, K. K., Grossman, A. R. & Björkman, O. (1998) *Plant Cell* **10,** 1121–1134.
19. Björkman, O. & Niyogi, K. K. (1998) *in Photosynthesis: Mechanisms and Effects Vol. III*, ed. Garab, G. (Kluwer, Dordrecht, The Netherlands), pp. 2085–2090.
20. Fabreguettes, V., Gibiat, F., Pintena, J., Vidal, D. & André, M. (1994) in *Proceedings of the 24th International Conference on Environmental Systems*, ed. The Engineering Society For Advancing Mobility Land Sea Air and Space (Warrendale, PA), Paper no. 941544.
21. Havaux, M. & Tardy, F. (1996) *Planta* **198,** 324–333.
22. Wildi, B. & Lütz, C. (1996) *Plant Cell Environ.* **19,** 138–146.
23. Havaux, M. (1998) *Israel J. Chem.* **38,** 246–256.
24. Degousée, N., Triantaphylidès, C. & Montillet, J.-L. (1994) *Plant Physiol.* **104,** 945–952.
25. Schreiber, U., Schliwa, U. & Bilger, B. (1986) *Photosynth. Res.* **10,** 51–62.
26. Hideg, E. & Vass, I. (1993) *Photochem. Photobiol.* **58,** 280–283.
27. Stallaert, V. M., Ducruet, J.-M., Tavernier, E. & Blein, J.-P. (1995) *Biochim. Biophys. Acta* **1229,** 290–295.
28. Vavilin, D. V., Ducruet, J.-M., Matorin, D. N., Venediktov, P. S. & Rubin, A. B. (1998) *J. Photochem. Photobiol. B* **42,** 233–239.
29. Vavilin, D. V. & Ducruet, J.-M. (1998) *Photochem. Photobiol.* **68,** 191–198.
30. Merzlyak, M. N., Pavlov, V. K. & Zhigalova, T. V. (1992) *J. Plant Physiol.* **139,** 629–631.
31. Frankel, E. N. (1982) *Prog. Lipid Res.* **22,** 1–33.
32. Fryer, M. J. (1992) *Plant Cell Environ.* **15,** 381–392.
33. Logan, B. A., Demmig-Adams, B., Adams W. W., III, & Grace, S. C. (1998) *J. Exp. Bot.* **49,** 1869–1879.
34. Wise, R. R. & Naylor, A. W. (1987) *Plant Physiol.* **83,** 278–282.
35. Smirnoff, N. (1993) *New Phytol.* **125,** 27–58.
36. Kim, J. H., Nemson, J. A. & Melis, A. (1993) *Plant Physiol.* **103,** 181–189.
37. Aro, E.-M., McCaffery, S. & Anderson, J. (1994) *Plant Physiol.* **104,** 1033–1041.
38. Rintamaki, E., Salo, R. & Aro, E.-M. (1994) *Planta* **193,** 520–529.
39. Tyystjarvi, E., Ali-Yrkko, K., Kettunen, R. & Aro, E.-M. (1992) *Plant Physiol.* **100,** 1310–1317.
40. Kanervo, E., Aro, E.-M. & Murata, N. (1995) *FEBS Lett.* **364,** 239–242.
41. Demmig-Adams, B., Moeller, D. L., Logan, B. A. & Adams, W. W., III (1998) *Planta* **205,** 367–374.
42. Verhoeven, A. S., Adams, W. W., III, & Demmig-Adams, B. (1996) *Physiol. Plant.* **96,** 567–576.
43. Krinsky, N. I. (1979) *Pure Appl. Chem.* **51,** 649–660.
44. Edge, R., McGarvey, D. J. & Truscott, T. G. (1997) *J. Photochem. Photobiol. B* **41,** 189–200.
45. Owens, T. G., Gallagher, J. C. & Alberte, R. S. (1987) *J. Phycol.* **23,** 79–85.
46. Peterman, E. J. G., Gradinaru, C. C., Calkoen, F., Borst, J. C., van Grondelle, R. & van Amerongen, H. (1997) *Biochemistry* **36,** 12208–12215.
47. Havaux, M., Gruszecki, W. I., Dupont, I. & Leblanc, R. M. (1991) *J. Photochem. Photobiol. B* **8,** 361–370.
48. Koyama, Y. (1991) *J. Photochem. Photobiol. B* **9,** 265–280.
49. Ruban, A. V., Horton, P. & Young, A. J. (1993) *J. Photochem. Photobiol. B* **21,** 229–234.
50. Ruban, A. V., Phillip, D., Young, A. J. & Horton, P. (1997) *Biochemistry* **36,** 7855–7859.
51. Frank, H. A., Cua, A., Chynwat, V., Young, A., Gosztola, D. & Wasielewski, M. R. (1994) *Photosynth. Res.* **41,** 389–395.
52. Anderson, S. M. & Krinsky, N. I. (1973) *Photochem. Photobiol.* **18,** 403–408.
53. Lim, B. P., Nagao, A., Terao, J., Tanaka, K., Suzuki, T. & Takama, K. (1992) *Biochim. Biophys. Acta* **1120,** 178–184.
54. Sielewiesiuk, J., Matula, M. & Gruszecki, W. I. (1997) *Cell Mol. Biol. Lett.* **2,** 59–68.
55. Rockholm, D. C. & Yamamoto, H. Y. (1996) *Plant Physiol.* **110,** 697–703.
56. Havaux, M. (1998) *Trends Plant Sci.* **3,** 146–151.
57. Tardy, F. & Havaux, M. (1997) *Biochim. Biophys. Acta* **1330,** 179–193.
58. Gruszecki, W. I. & Strzalka, K. (1991) *Biochim. Biophys. Acta* **1060,** 310–314.
59. Havaux, M., Tardy, F., Ravenel, J., Chanu, D. & Parot, P. (1996) *Plant Cell Environ.* **19**, 1359–1368.
60. Böhm, F., Edge, R., Land, E. J., McGarvey, D. J. & Truscott, T. G. (1997) *J. Am. Chem. Soc.* **119**, 621–622.
61. Stahl, W., Junghans, A., de Boer, B., Driomina, E. S., Briviba, K. & Sies, H. (1998) *FEBS Lett.* **427,** 305–308.