Photodamage of the Photosynthetic Apparatus and Its Dependence on the Leaf Developmental Stage in the *npq1* Arabidopsis Mutant Deficient in the Xanthophyll Cycle Enzyme Violaxanthin De-epoxidase

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The *npq1* Arabidopsis mutant is deficient in the violaxanthin de-epoxidase enzyme that converts violaxanthin to zeaxanthin in excess light (xanthophyll cycle). We have compared the behavior of mature leaves (ML) and developing leaves of the mutant and the wild type in various light environments. Thermoluminescence measurements indicated that high photon flux densities (>500 μmol m^{-2} s^{-1}) promoted oxidative stress in the chloroplasts of *npq1* ML, which was associated with a loss of chlorophyll and an inhibition of the photochemical activity. Illuminating leaf discs in the presence of eosin, a generator of singlet oxygen, brought about pronounced lipid peroxidation in *npq1* ML but not in wild-type leaves. No such effects were seen in young leaves (YL) of *npq1*, which were quite tolerant to strong light and eosin-induced singlet oxygen. Non-photochemical energy quenching was strongly inhibited in *npq1* YL and ML and was not improved with high-light acclimation. Our results confirm that the xanthophyll cycle protects chloroplasts from photooxidation by a mechanism distinct from non-photochemical energy quenching and they reveal that the absence of xanthophyll cycle can be compensated by other protective mechanisms. *npq1* YL were observed to accumulate considerable amounts of vitamin E during photoacclimation, suggesting that this lipophilic antioxidant could be involved in the high phototolerance of those leaves.

Violaxanthin (V) de-epoxidase is a chloroplastic enzyme that is localized in the lumen of the thylakoids and converts the diepoxide xanthophyll V to the monoepoxide antheraxanthin (A) and the epoxide-free zeaxanthin (Z) at an optimum pH of about 5 (Rockholm and Yamamoto, 1996; Eskling et al., 1997; Bugos et al., 1998). In vivo, the de-epoxidation reaction is triggered by an increase in the proton gradient across the thylakoid membranes in plants exposed to excessive light. Upon return to limiting light conditions, Z is epoxidized back to V by a Z epoxidase enzyme localized on the stromal side of the thylakoid membranes. These stoichiometric and cyclic conversions of V, A, and Z are called the xanthophyll (or V) cycle and play a major role in controlling the efficiency of light harvesting in plants (Demmig-Adams and Adams, 1996; Horton et al., 1996). In limiting light conditions, the presence of V in the light-harvesting complexes (LHCs) of photosystem II (PSII) is associated with maximum efficiency of light harvesting, whereas the synthesis of Z is correlated with down-regulation of the light-harvesting efficiency caused by an increased dissipation of excitation energy as heat. The latter process, which is measured as non-photochemical quenching (NPQ) of chlorophyll (Chl) fluorescence, is potentially important for the protection of the photosynthetic apparatus from photodamage; it can protect the photosensitive PSII reaction center from overexcitation, and it also can lower the formation of harmful reactive molecules in the LHCs such as triplet Chl and singlet oxygen ($^1O_2$). Accordingly, full conversion of V to Z prior to light stress at chilling temperatures has been observed to prevent irreversible photoinhibition of PSII in the mangrove *Rhizophora mangle* (Demmig-Adams et al., 1989).

Several mutants of Arabidopsis affected in the NPQ process have been isolated by Niyogi et al. (1998). Among those mutants, *npq1* has been shown to be defective in the gene encoding V de-epoxidase. Leaves of this mutant have no functional V de-epoxidase, are unable to convert V to Z, and exhibit strongly inhibited NPQ. However, the *npq1* mutation did not affect the efficiency of electron transport either in low light or in strong, saturating light (Niyogi et al., 1998; Havaux and Niyogi, 1999). Although short-term light stress was observed to damage PSII more severely in the mutant compared to the wild type, this differential PSII-photoinhibition was attenuated in long-term experiments (Niyogi et al.,

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1998; Havaux and Niyogi, 1999), suggesting that photoacclimation of PSII reduced the requirement for an active V cycle. However, the *npq1* mutant was found to suffer from lipid peroxidative damage during prolonged exposure to high light, indicating increased susceptibility to photooxidation in the absence of the V cycle (Havaux and Niyogi, 1999). It was suggested that the antioxidant effect of the V cycle on thylakoid lipids supplements that of the lipophilic antioxidant vitamin E (α-tocopherol). In the present study, we examined further the photosynthetic behavior of *npq1* in different light environments. Different aspects of the photochemical apparatus of the chloroplasts (pigments, photochemical activity, and lipid peroxidation) were analyzed in plants exposed to different photosynthetically active photon flux densities (PPFDs) up to 1,500 μmol m^{-2} s^{-1} and also in leaf discs exposed to $^1O_2$. The results confirm that the V cycle protects the chloroplasts against photooxidation and that this protection directly involves Z in a mechanism distinct from NPQ. Leaf age was found to strongly influence the photoprotection exerted by the V cycle, with young leaves (YL) of *npq1* being apparently unaffected by the lack of V cycle when exposed to PPFDs that photodamaged mature leaves (ML).

RESULTS

Photosynthetic Pigments and Tocopherols in Wild-Type and *npq1* Leaves under Low- and High-Light Conditions

Figure 1A shows the total Chl and carotenoid concentrations in ML of the wild type and the *npq1* mutant of Arabidopsis grown for 10 or 20 d under a wide range of PPFDs from 100 to 1,500 μmol m^{-2} s^{-1}. When the PPFD was increased up to 500 μmol m^{-2} s^{-1}, the levels of both Chls and carotenoids increased in wild-type leaves. Above 500 μmol m^{-2} s^{-1}, the carotenoid content was stable, whereas the Chl level decreased slightly (by approximately 10%). The *npq1* mutant behaved like the wild type in the low/moderate PPFD range (<500 μmol m^{-2} s^{-1}), but the Chl content strongly decreased at high PPFDs. For instance, in *npq1* plants grown at 1,500 μmol m^{-2} s^{-1}, the Chl level fell to about 60% of the level measured in wild-type leaves. The carotenoid concentration also decreased in *npq1* leaves grown at high PPFDs but to a smaller extent compared to the Chl content. However, this decrease was not found for the V-cycle carotenoids, which increased with PPFDs in both genotypes (Fig. 1B). The V-cycle pigments were partially de-epoxidized in wild-type plants at PPFDs higher than 500 μmol m^{-2} s^{-1} (Fig. 1B). At the highest PPFD, almost 50% of the V pool was de-epoxidized in the wild type. As expected, no significant synthesis of A and Z was found in the *npq1* mutant.

We have also examined the level of α-tocopherol, the major antioxidant present in the thylakoid membrane lipid matrix (Fryer, 1992). Increasing PPFD caused a marked increase in the concentration of this compound, with maximal accumulation being found in plants grown at 1,000 μmol m^{-2} s^{-1} (Fig. 1C). Both genotypes behaved similarly, although *npq1* seemed to contain slightly more α-tocopherol than the wild type.

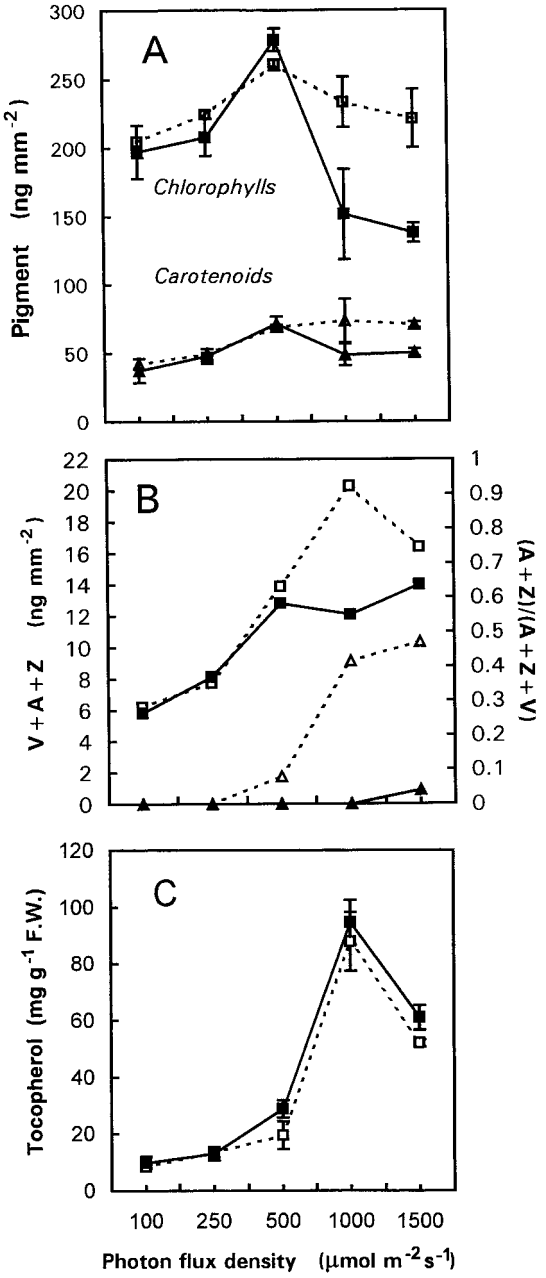

Figure 1. Total Chl (squares) and carotenoid (triangles) contents (A), xanthophyll-cycle pigments pool (V + A + Z, squares) and de-epoxidation status of the xanthophyll cycle (A + Z)/(V + A + Z) (triangles; B), and α-tocopherol content of ML of Arabidopsis plants (white symbols, wild type; black symbols, *npq1*; C) grown at PPFDs from 100 to 1,500 μmol m^{-2} s^{-1}. Duration of the light treatments was 20 d except for the highest PPFD (10 d). Leaves were taken in the morning, approximately 1 h after the beginning of the light phase. Data are the means of four separate experiments ± SD.

Table I presents a detailed analysis of the pigment and tocopherol content in wild-type and *npq1* plants at low and high PPFD. As observed in Figure 1 for longer treatments, exposure of *npq1* plants to a PPFD of 1,500 μmol m^{-2} s^{-1} for 3 d (photoperiod, 15 h) caused a marked decrease in Chl; Chl *b* decreased by 30% and Chl *a* decreased by 15% in well-developed ML of the *npq1* mutant (Table I). In contrast, the carotenoid concentrations remained stable (neoxanthin, lutein, and β-carotene) or increased (V + A + Z, +75%). In the wild type, all pigments except Chl *b* increased during the strong-light treatment, particularly the V + A + Z pool, which increased by 160%.

When developing *npq1* YL in the center of the leaf rosette were examined (Table I), a completely different picture was observed. The strong-light treatment did not provoke loss of pigments as was observed in ML. *npq1* YL were very similar to wild-type YL with respect to the pigment concentration (excluding, of course, A and Z), both in low light and in high light. In low light there was no significant difference between YL and ML of wild type and *npq1* (data not shown). No synthesis of Z or A took place during high-light treatment of *npq1* YL (data not shown). In the wild type, the steady-state epoxidation status of the xanthophyll cycle was roughly similar in YL and ML ([A + Z]/[V + A + Z] $\approx$ 0.45) in the experiment (Table I).

The 3-d light treatment caused a pronounced accumulation of α-tocopherol in both wild-type and *npq1* leaves (Table I). This accumulation was particularly marked in *npq1* YL (92 μg g^{-1} fresh weight versus 79 μg g^{-1} in wild-type YL), whereas *npq1* ML accumulated less α-tocopherol than all other types of leaves (44 μg g^{-1}). γ-Tocopherol was also found in high-light-treated leaves, whereas only traces of this compound were detected in leaves grown at moderate PPFD. Again, accumulation of γ-tocopherol was the most pronounced in *npq1* YL.

Photooxidative Damage and Photosynthesis in YL and ML of *npq1* and Wild Type

Accumulation of the lipophilic antioxidants, α-tocopherol and γ-tocopherol, at high PPFDs suggests the formation of $^1O_2$ in the chloroplasts (Logan et al., 1998). We have previously shown that the absence of xanthophyll cycle is associated with an increased susceptibility to photooxidative damage of the chloroplasts (Havaux and Niyogi, 1999), and this phenomenon is reflected here by the loss of pigments in ML of high-light-grown *npq1* plants. The unsuccessful adaptation of *npq1* to photooxidative conditions was checked by thermoluminescence (TL) measurements (Fig. 2). The high-temperature TL bands emitted by Chl-containing material at temperatures higher than 70°C are independent of pre-illumination and are attributed to lipid peroxidation products (Vavilin and Ducruet, 1998). Light emission is believed to result from interaction of compounds such as oxy- and peroxy-radicals or dioxetanes leading to generation of triplet carbonyls (Cadenas, 1984), which can interact with Chl *a* molecules (Sharov et al., 1996), resulting in luminescence emission. The amplitude of the high-temperature TL bands, usually peaking at 70°C to 90°C and 115°C to 135°C, is well correlated with the amount of lipid peroxides present in the thylakoid membranes, providing a good indicator of the lipid peroxidation status of thylakoid membranes (Hideg and Vass, 1993; Stallaert et al., 1995; Marder et al., 1998; Vavilin and Ducruet, 1998; Havaux and Niyogi, 1999). The relative amplitude of the two lipid peroxide-related TL bands has been shown to depend on the water content of the leaf samples and on the rate of dehydration during TL heating (Ducruet and Vavilin, 1999; see also below). The main advantages of the TL method are that the level of lipid peroxidation is measured in situ and

Table I. *Effects of high-light stress (3 d at 1,500 μmol m^{-2} s^{-1}) on the pigment content (ng mm^{-2}) and the tocopherol content (μg g^{-1} fresh wt) of YL and ML of wild type and npq1 Arabidopsis grown at a PPFD of 250 μmol m^{-2} s^{-1}*

Data are mean values of a minimum of three separate experiments ± SD. For the low-light growth conditions, only data of ML are given because there was no significant difference between YL and ML (wild type and *npq1*) with respect to their pigment and tocopherol content.

Compounds	250 μmol m^{-2} s^{-1}		1,500 μmol m^{-2} s^{-1}			
			WT		*npq1*	
	Wild-type ML	*npq1* ML	ML	YL	ML	YL
Photosynthetic pigments						
Neoxanthin	5.2 ± 0.1	5.0 ± 0.1	6.4 ± 0.7	5.2 ± 0.2	4.5 ± 0.7	5.7 ± 0.4
V + A + Z	7.7 ± 0.3	8.1 ± 1.0	20.0 ± 3.5	22.9 ± 0.8	13.9 ± 1.3	23.5 ± 2.2
Lutein	24.1 ± 0.5	23.6 ± 1.9	35.4 ± 1.8	33.3 ± 1.7	26.5 ± 3.4	35.9 ± 1.4
β-Carotene	12.5 ± 4.8	11.1 ± 2.7	14.9 ± 2.1	14.2 ± 2.2	13.9 ± 1.3	14.6 ± 1.7
Chl *b*	74.6 ± 3.1	67.7 ± 4.9	74.2 ± 7.0	59.6 ± 3.2	48.1 ± 9.6	56.4 ± 3.1
Chl*a*	149.6 ± 5.8	139.8 ± 10.8	167.6 ± 19.1	141.4 ± 4.5	116.6 ± 22.7	138.5 ± 9.9
Chl *a*/Chl *b*	2.01	2.06	2.25	2.37	2.42	2.45
Vitamin E						
α-Tocopherol	23.5 ± 1.7	19.8 ± 0.9	57.2 ± 2.1	78.7 ± 5.1	43.8 ± 3.9	91.9 ± 3.7
γ-Tocopherol	0.5 ± 0.3	0	15.5 ± 1.2	12.6 ± 0.1	12.4 ± 2.2	19.1 ± 2.6

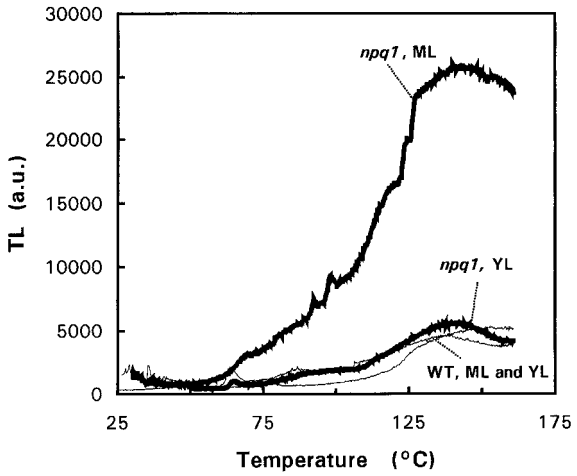

Figure 2. TL curves of Arabidopsis ML and YL (wild type and *npq1* mutant) treated for 3 d at 1,500 μmol m^{-2} s^{-1}. Thick lines, *npq1*; thin lines, wild type.

that the signal reflects peroxidative damage of the thylakoid membranes only.

Figure 2 shows the TL curves of YL and ML from wild-type and *npq1* plants treated at 1,500 μmol m^{-2} s^{-1} for 3 d. As described previously (Havaux and Niyogi, 1999), *npq1* ML exhibited a very strong luminescence band at 135°C, indicating accumulation of lipid hydroperoxides and peroxidative degradation of thylakoid membrane lipids in the absence of the V cycle. However, *npq1* YL had a very low TL signal in the 25°C to 150°C region that was indistinguishable from both YL and ML leaves of the wild type.

Figure 3 shows the 77 K Chl-fluorescence emission spectra of high-light-treated YL and ML. The fluorescence bands at approximately 680 and 690 nm are attributed to PSII, whereas the 730-nm band is due to the photosystem I (PSI) LHC (Krause and Weis, 1984; Govindjee, 1995). *npq1* ML noticeably differed from wild-type YL and ML and from *npq1* YL; the PSII bands were substantially reduced compared to the PSI band. This confirms that the pigment system of *npq1* ML was more sensitive to photodestruction than that of *npq1* YL or wild-type leaves and suggests that pigment destruction in *npq1* leaves preferentially affected PSII.

The results from room-temperature Chl-fluorescence and photoacoustic measurements (Table II) showed that strong-light stress had little or no effect on wild-type leaves and *npq1* YL. In contrast, photochemistry was inhibited in *npq1* ML, as indicated by the lowering of the quantum yield of PSII-mediated electron transport (Φ_{PSII}), the quantum yield of oxygen evolution, and the efficiency of photochemical energy storage. Under control light conditions (250 μmol m^{-2} s^{-1}), the photosynthetic characteristics of YL and ML were identical in both genotypes.

The ability of *npq1* YL to tolerate high light was not due to an intrinsically higher efficiency of PSII electron transport or an improved capacity for NPQ. Figure 4 shows that Φ_{PSII} in *npq1* YL (grown in moderate light) was not significantly different from that of ML when exposed to a wide range of PPFDs. Although the absence of the V cycle in *npq1* resulted in a marked reduction of the NPQ of Chl fluorescence, as previously demonstrated (Niyogi et al., 1998), Table III shows that acclimation to strong white light did not restore NPQ to the wild-type level even in developing *npq1* YL. However, measurements of 77 K Chl fluorescence showed that acclimated *npq1* YL had a slightly different distribution of light energy between PSII and PSI, in favor of PSI (Fig. 3). The ratio of the Chl fluorescence emission at 730 nm to the Chl fluorescence emission at 680 nm (measured at 77 K) was 3.83 ± 0.36 and 4.31 ± 0.45 in wild-type ML and YL, respectively. In acclimated

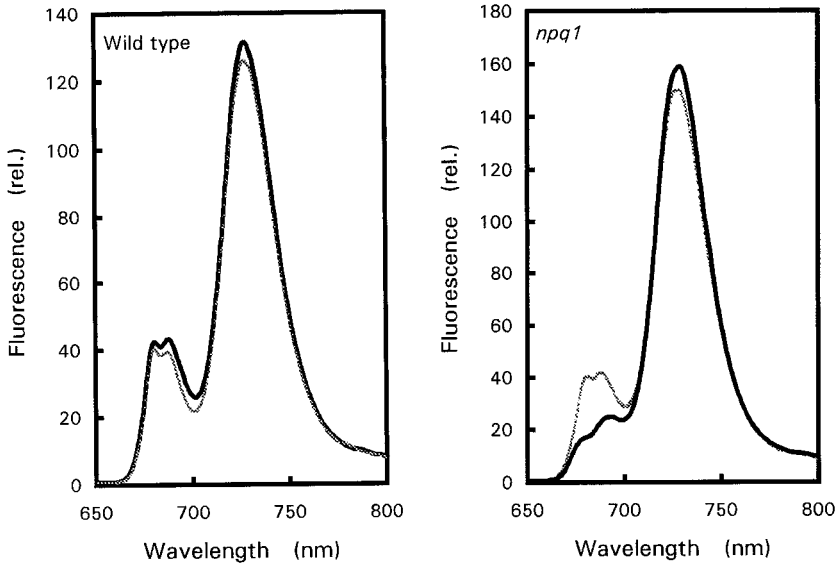

Figure 3. 77 K Chl fluorescence emission spectra of Arabidopsis leaves (YL, thin gray lines; ML, thick black lines) treated for 3 d at a PPFD of 1,500 μmol m^{-2} s^{-1}.

Table II. *Photosynthetic characteristics of YL and ML of wild type and npq1 Arabidopsis determined by Chl fluorometry and photoacoustics before and after high-light stress (3 d at 1,500 μmol m^{-2} s^{-1})*

Data are mean values of more than three separate experiments ± SD. Measurements were performed in white light at a PPFD of 100 μmol m^{-2} s^{-1}.

Treatment	Fluroescence (Φ_{PSII})	Photoacoustics	
		Φ_{O_2} [a]	ES [b]
Control			
WT ML	0.69 ± 0.01	2.39 ± 0	20 ± 3
WT YL	0.69 ± 0.01	2.31 ± 0.15	17 ± 4
npq1 ML	0.68 ± 0.02	2.59 ± 0.12	22 ± 5
npq1 YL	0.68 ± 0.02	1.87 ± 0.25	18 ± 2
Light treated			
WT ML	0.60 ± 0.09	2.09 ± 0.41	16 ± 5
WT YL	0.65 ± 0.02	2.55 ± 0.25	24 ± 3
npq1 ML	0.36 ± 0.18	1.24 ± 0.33	10 ± 4
npq1 YL	0.61 ± 0.07	2.83 ± 0.45	21 ± 10

[a] Φ_{O_2}, Quantum yield of oxygen evolution. [b] ES, Photochemical energy storage.

npq1 YL, the ratio was 4.79 ± 0.65, and it increased to 8.66 ± 2.21 in stressed *npq1* ML.

Leaf Infiltration with Eosin

Arabidopsis YL and ML were exposed to $^1O_2$ toxicity by infiltrating leaf discs with eosin, a well-known generator of $^1O_2$ in the light (Knox and Dodge, 1985). Leaf discs floating on the aqueous solution of eosin were illuminated with white light at a PPFD of 500 μmol m^{-2} s^{-1}. This treatment resulted in a marked lipid peroxidation as reflected by the appearance of a strong TL band at 80°C (Fig. 5A). The 135°C TL band (observed in high-light-treated plants, Fig. 2) was attenuated in this experiment because of the high water content of the leaf discs floating on the aqueous solution of eosin. Indeed Ducruet and Vavilin (1999) have shown that the 80°C/90°C TL band is actually a pseudoband resulting from a competition between thermolysis of peroxides (corresponding to the rising edge of the 130°C/140°C TL band) and a non-radiative hydrolysis below 100°C in wet samples. Consequently, the amount of water retained within the sample during TL warming is a prominent factor governing the TL emission at high temperature. The water content of leaf discs floating on eosin for 5 h was observed to be higher than that of control leaves (92.9% versus 91.4%). When the water content of eosin-treated leaf discs was reduced by leaving the discs on the laboratory bench for 1 h in the dark (resulting in a leaf water content of 86.8%), the 80°C TL band was converted into a 135°/140°C band (Fig. 5B), thus confirming the prominent role of water in the relative amplitude of the two TL bands. The 80°C TL band shown in Figure 5A and the 135°C TL band shown in Figure 2 reflect then similar photooxidative damage in the leaf tissues.

The different types of leaves used in this study took up the same amount of eosin. Eosin-infiltrated leaves illuminated at 390 nm exhibited a strong fluorescence band in the green spectral region peaking at approximately 545 nm, which was not found in control leaves (Fig. 6). The latter wavelength is very close to the maximal fluorescence wavelength of eosin in solution (approximately 543 nm). In addition, the excitation spectrum of this band corresponded to the absorbance spectrum of eosin characterized by a maximum at approximately 390 nm and a shoulder at approximately 430 nm (data not shown). There was no significant difference in the amplitude of the 545-nm band between YL and ML or between wild type and *npq1* leaves (Fig. 6, inset), indicating comparable concentrations of eosin in the leaf tissues.

As expected from the similar pigment content of *npq1* and wild type (Table I), the rate of eosin exci-

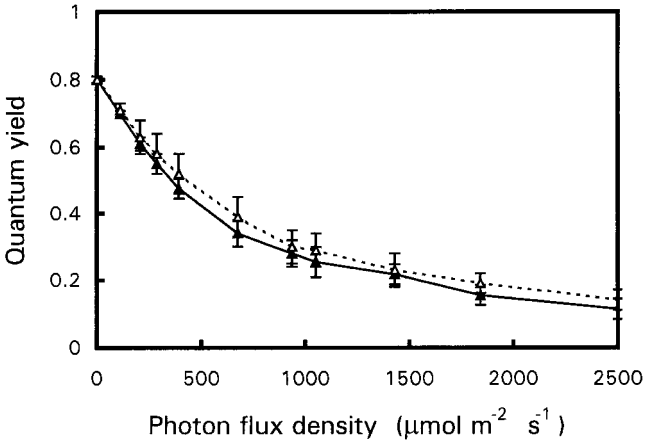

Figure 4. Φ_{PSII} in YL (△) and ML (▲) of *npq1* (grown at 250 μmol m^{-2} s^{-1}) at different PPFDs. Data are mean values of four separate experiments ± SD.

Table III. *NPQ in wild-type and npq1 Arabidopsis leaves before and after 3 d at a PPFD of 1,500 μmol m^{-2} s^{-1}*

NPQ was measured in the steady state after 15 min of illumination with a white light at a PPFD of 1,600 μmol m^{-2} s^{-1}. Data are mean values of four separate experiments ± SD.

Treatment	NPQ
Control	
Wild type	
YL	2.36 ± 0.24
ML	2.40 ± 0.03
Npq1	
YL	0.74 ± 0.08
ML	0.49 ± 0.08
Light acclimated	
Wild type	
YL	3.01 ± 0.24
ML	1.94 ± 0.44
Npq1	
YL	0.69 ± 0.07
ML	0.41 ± 0.11

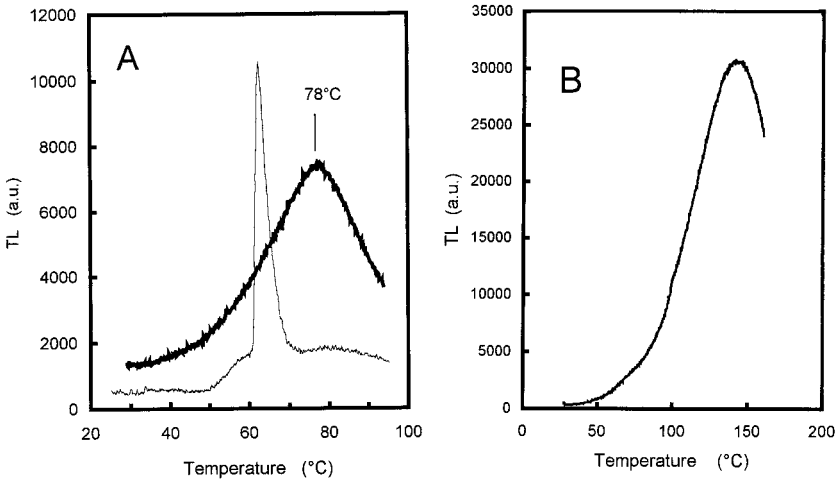

Figure 5. Eosin-induced lipid peroxidation in Arabidopsis leaf discs. A, TL curve of a wild-type Arabidopsis leaf disc before (thin line) and after (thick line) illumination (500 μmol m^{-2} s^{-1}, 375 min) in the presence of eosin. The sharp 65°C TL band in control leaves is typical of Arabidopsis (and was not observed in other plant species such as tobacco, potato, or barley). The origin of this band is unknown; it is not related to lipid peroxidation and could be due to thermolysis of a (yet unidentified) volatile compound (Ducruet and Vavilin, 1998). B, TL was measured in a leaf disc kept for 1 h on filter paper in the dark after light stress (500 μmol m^{-2} s^{-1}, 375 min) in the presence of eosin. The leaf water content decreased from 92.8% to 86.8% during this treatment.

tation in the blue region did not differ between the different samples. This was determined by measuring light absorption in the blue spectral region (400–490 nm) using photoacoustics. The amplitude of the light-saturated photothermal signal (A_{pt}) is proportional to the light absorption of the sample (Malkin and Canaani, 1994) and can thus be used as a measure of leaf absorbance. Average values of A_{pt} (in arbitrary units) were similar in all leaves examined: 6.12 ± 0.75 and 6.18 ± 0.25 in wild-type ML and YL, respectively, and 6.03 ± 0.19 and 6.35 ± 0.14 in *npq1* ML and YL, respectively. From the latter results and the control experiments shown in Figures 5 and 6, the rate of $^1O_2$ formation from eosin excitation appeared to be similar in the different types of leaves examined in this study. Therefore, different responses to the eosin treatment can be interpreted in terms of differential tolerance to $^1O_2$ toxicity.

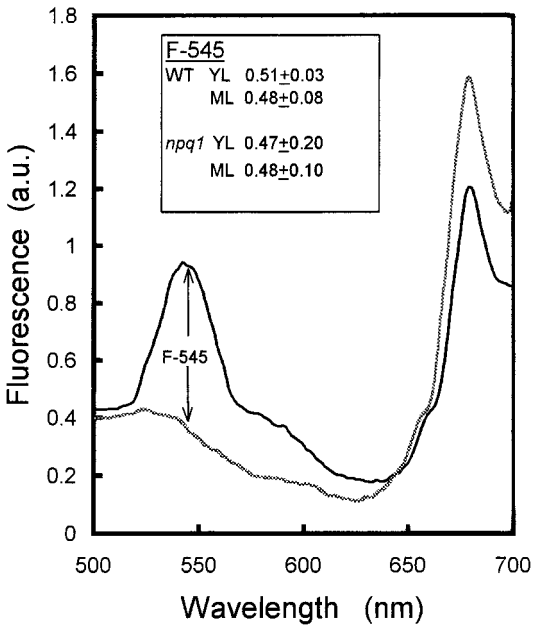

Figure 6. In vivo fluorescence emission of Arabidopsis leaves (wild type) excited at 390 nm. Thick black line, Leaf disc infiltrated with eosin for 5 h; thin gray line, control leaf disc. Inset, Amplitude of eosin fluorescence at 545 nm in wild-type and *npq1* leaf discs. The excitation spectrum of the fluorescence at 545 nm (not shown) exhibited a peak at approximately 390 nm and a shoulder at around 430 nm.

Oxidative Damage in YL and ML of *npq1* and Wild Type Exposed to Eosin-Generated $^1O_2$

Figure 7A shows that $^1O_2$ generated by eosin caused PSII photoinhibition. However, the degree of photoinhibition was similar in the wild type and the *npq1* mutant. In contrast Figure 7B shows that lipid peroxidation was more pronounced in *npq1* ML samples than in wild-type samples. After 400 min in the light, the amplitude of the 80°C TL band was 2-fold higher in *npq1* compared to wild type. When leaves were irradiated through a filter of eosin to prevent excitation of the dye, no lipid peroxidation was observed in *npq1* or wild-type leaves, indicating that lipid peroxidation resulted from $^1O_2$ generation by eosin and not via Chl excitation. Consequently, results shown in Figure 7B show that *npq1* chloroplasts are more vulnerable to $^1O_2$-induced damage than wild-type chloroplasts. The same phenomenon was observed with YL (Fig. 7C), although the difference between *npq1* and the wild type was less marked than that observed with ML. When YL acclimated to the strong white light (3 d at 1,500 μmol m^{-2} s^{-1}) were illuminated with white light at a PPFD of 500 μmol m^{-2} s^{-1} in the presence of eosin (Fig. 7D), lipid peroxidation in *npq1* was reduced compared with nonacclimated *npq1* leaves (compare with Fig. 7C), and no difference was found between *npq1* and the wild type. Qualitatively similar results were obtained when light-acclimated leaves were exposed to a greater light stress (1,500 μmol m^{-2} s^{-1}). One can conclude that *npq1* YL became resistant to $^1O_2$ during light acclimation.

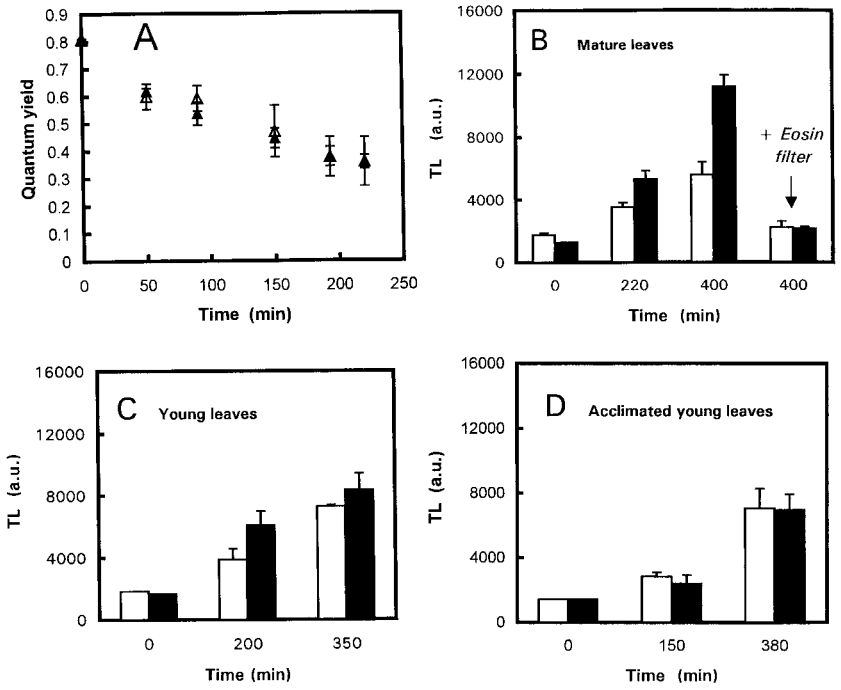

Figure 7. A, PSII photoinhibition (estimated by the decrease in the maximal quantum yield of PSII photochemistry F_v/F_m) in mature Arabidopsis leaf discs (wild type, white symbols; *npq1*, black symbols) during light stress (500 μmol m^{-2} s^{-1}) in the presence of eosin. B through E, Change in the amplitude of the 80°C TL band in wild-type (white bars) and *npq1* (black bars) Arabidopsis leaves illuminated in the presence of eosin. B, ML exposed to a PFD of 500 μmol m^{-2} s^{-1} (white light or light filtered through eosin) + eosin. (A + Z)/(A + Z + V) = 0 for *npq1* and 0.50 for wild type. C, YL exposed to 500 μmol m^{-2} s^{-1} + eosin. D, YL taken from plants treated for 3 d at 1,500 μmol m^{-2} s^{-1} illuminated in the presence of eosin with a white light of PPFD 500 μmol photons m^{-2} s^{-1}. (A + Z)/(A + Z + V) = 0.04 for *npq1* and 0.71 for wild type. Data are the means of a minimum of three separate experiments ± SD.

DISCUSSION

The photosensitivity of the photosynthetic apparatus of Arabidopsis plants increases markedly in the absence of the xanthophyll cycle. Indeed we observed a substantial loss of photosynthetic pigments (Fig. 1; Table I) and an inhibition of the photochemical activity of the chloroplasts (Table II) when *npq1* plants were transferred to high PPFD in the range 1,000 to 1,500 μmol m^{-2} s^{-1}. Both phenomena, which were not observed in wild-type plants, probably resulted from photooxidative damage as revealed by in vivo TL measurements. The strong 135°C TL band recorded in *npq1* ML pre-exposed to high light (Fig. 2) indicated peroxidative damage of thylakoid membrane lipids (Vavilin and Ducruet, 1998). Thus, the presence of Z and A during strong illumination noticeably reinforced the tolerance of the photosynthetic membranes to photooxidation, in agreement with previous work (Havaux and Niyogi, 1999). In this study, we observed that a mutant of Arabidopsis (*npq4*) characterized by a strongly diminished NPQ (but normal V cycle; see Li et al., 2000) was much less photosensitive than *npq1* (with both NPQ and V cycle inhibited), indicating that photoprotection requires not only NPQ but also the physical presence of de-epoxidized xanthophylls.

The photoprotective action of the V cycle seems to be particularly important to preserve the PSII function in high light (Fig. 3; Table II). Loss of PSII activity has been reported to be closely related to thylakoid membrane lipid photodestruction under certain light stress conditions (Sharma and Singhal, 1992; Hideg et al., 1994). Oxidative stress induced by exogenous compounds, such as eosin or the fungal elicitor cryptogein, was also reported to cause a preferential destruction of PSII (Knox and Dodge, 1985; Stallaert et al., 1995). However, it is difficult to decide from our results whether PSII destruction occurred after lipid peroxidation or if lipid peroxidation was induced by PSII photoinhibition.

Experiments with the photosensitizing dye eosin (Fig. 7) showed that photooxidation of *npq1* ML could result from an increase in the intrinsic sensitivity of the thylakoid membranes to active forms of oxygen. Generation of $^1O_2$ during illumination of the dye caused lipid peroxidation, as expected (Knox and Dodge, 1985), and this phenomenon was significantly amplified in *npq1* leaf discs deficient in A and Z compared to wild-type leaf samples containing high levels of Z (Fig. 7, B and C). Thus, the absence of de-epoxidized xanthophylls formed in the V cycle was associated with an increased sensitivity of thylakoid membrane lipids toward the toxicity of $^1O_2$ and perhaps of other active forms of oxygen. This cannot be attributed to different NPQ activities since, in our experiments, $^1O_2$ was generated by direct excitation of eosin. When excitation of the dye was prevented by an appropriate light filter, no lipid peroxidation was noticed, although the PPFD of the (red) light reaching the leaf samples and the level of photoinduced NPQ were unchanged (Fig. 7B). Moreover, the enhanced lipid peroxidation in eosin-treated *npq1* leaves was not accompanied by an increase in PSII inhibition relative to wild-type leaves (Fig. 7A).

Chl excitation can result in $^1O_2$ formation in the LHCs or PSII reaction centers, whereas exogenously added eosin is expected to generate $^1O_2$ throughout

the thylakoid membrane. Because the *npq1* mutant was more susceptible to eosin-induced lipid peroxidation, it seems that the protective effect of Z and A is not restricted to the LHCs but may also involve Z and A that are located in the thylakoid lipid matrix (Havaux, 1998a). This does not exclude, of course, that the V-cycle-dependent NPQ also protects thylakoid from photodestruction by deactivating singlet-excited Chl and by reducing the probability of triplet Chl and $^1O_2$ formation in the LHCs.

Because of their higher number of conjugated double bonds, Z and A are better photoprotectors than V, with a higher efficiency for de-exciting $^1O_2$ (Mathews-Roth et al., 1974) and, consequently, the appearance of Z in the LHCs and/or in their immediate surroundings can enhance the resistance of the photosystems to oxygen toxicity. Carotenoids can also trap various types of free radicals and, when incorporated into artificial lipid membranes, they protect them from being oxidized (Krinsky, 1979). The latter effect was very obvious in liposomes incorporated with Z (Lim et al., 1992; Sielewiesiuk et al., 1997; Woodall et al., 1997). This high protection efficiency of Z could be related to the location of the xanthophyll molecules in the lipid bilayer (Oshima et al., 1993); Z is oriented to the parallel to the hydrocarbon chain of lipids (Gruszecki and Sielewiesiuk, 1990) whereas V, particularly in the cis configuration (Yamamoto and Bangham, 1978; Gruszecki et al., 1999), and non-polar carotenes (such as β-carotene; Van de Ven et al., 1984; Gabrielska and Gruszecki, 1996) orient their long isoprenoid chain perpendicular to the lipid acyl group. Several authors have provided data suggesting that the vertical orientation of Z is very favorable for protection against oxidation at all depths in the hydrophobic lipid phase (Subczynski et al., 1991; Woodall et al., 1997; Berglund et al., 1999).

Although the exact localization and orientation of the xanthophyll-cycle pigments in native thylakoid membranes are not known yet, there is support for a dynamic equilibrium between protein complex-associated xanthophylls and lipid matrix-localized xanthophylls, at least during strong light treatment (Hager and Holocher, 1994; Rockholm and Yamamoto, 1996; Tardy and Havaux, 1997; Bugos et al., 1998; Havaux, 1998a; Ruban et al., 1999; Gruszecki et al., 1999). If this is the case (and considering the in vitro data reported above), the transient presence of Z in the thylakoid membrane lipid matrix could be an efficient system for protecting membrane lipids from destruction by active forms of oxygen generated in strong light (or via eosin).

Another important observation of this study is that developing *npq1* YL were much less affected by the absence of the V cycle than well-developed ML. Previous studies of *npq* mutants did not take into account the influence of leaf age on the responses of the photosynthetic apparatus to light stress (Niyogi et al., 1998; Havaux and Niyogi, 1999). Eosin caused less damage to YL (Fig. 7, B and C), and high-light stress of whole *npq1* plants did not lead to significant photosynthetic perturbations and photooxidation in YL in the center of the leaf rosette, in contrast to ML, which were severely damaged (Figs. 2 and 3; Tables I and II). Very recently, Bugos et al. (1999) reported that V de-epoxidase mRNA, protein, and activity levels are developmentally regulated in tobacco. V de-epoxidase protein and activity were very low in YL, but the levels were much higher in ML. This could indicate that YL have some special features that decrease the need for an active V cycle and, if this is the case in Arabidopsis too, this could be a possible reason why YL of *npq1* were quite similar to wild-type YL. However, in this study, the steady-state epoxidation status of the V-cycle pigments did not differ much between wild-type YL and ML, although no extensive analysis of the characteristics of the V cycle (rate of V photoconversion, light dependence of Z accumulation, etc.) was performed.

It was also observed that acclimation to high light for 3 d increased the phototolerance of *npq1* YL to the wild-type level (Fig. 7D). Again, the differential phototolerance of *npq1* YL and ML cannot be explained by different NPQ activities; NPQ was drastically inhibited in both *npq1* YL and ML, and growth of *npq1* plants in strong light was not associated with an increase in NPQ in either YL or ML (Table III). Moreover, it is unlikely that the higher phototolerance of *npq1* YL compared to ML is due to a higher photosynthetic activity, as shown by the results of Figure 4. Developing leaves were able to acclimate to high-light irradiance in such a way that the V cycle was not required to prevent photooxidative damage, and this acclimation did not involve NPQ. Thus, photoprotection by the xanthophyll cycle seems to be relevant mainly to environmental situations where rapid changes in PFD can occur and leaves can be transiently exposed to bright light.

Acclimation of *npq1* YL to excess light may involve a lowering of the rate of generation of active oxygen species in the chloroplasts and/or a stimulation of photoprotective and repair mechanisms (Melis, 1991; Demmig-Adams and Adams, 1992). Photosynthetic adaptation to high-light irradiance usually involves a selective decrease in the Chl *b*-containing LHCs of PSII, and this probably occurred in our Arabidopsis plants as indicated by the increase in the Chl *a* to Chl *b* ratio (Table I). However, the latter ratio did not differ significantly between YL of light-acclimated *npq1* and wild-type plants. We also observed that acclimated *npq1* YL had a slightly higher ratio of the Chl fluorescence emission at 730 nm to the Chl fluorescence emission at 680 nm (measured at 77K; Fig. 3), suggesting a change in the light distribution between the two photosystems in favor of PSI. Growth of Arabidopsis plants at high-light intensities also induced accumulation of carotenoids, particularly

those involved in the V cycle (Table I), but *npq1* YL did not differ significantly from wild-type leaves with respect to the carotenoid level.

In contrast *npq1* YL exposed to strong light accumulated considerable amounts of vitamin E (α- and γ-tocopherol) to a level significantly higher than that found in wild-type leaves. This increase in vitamin E may represent an acclimative response of the *npq1* mutant to compensate for the lack of Z and A in the photosynthetic membranes. Both α- and γ-tocopherol have an antioxidant and stabilizing action in biomembranes (Di Mascio et al., 1990; Fryer, 1992). Accumulation of vitamin E in *npq1* leaves indicates that membrane lipids are one of the chloroplast components that are the most affected by the lack of V cycle, since vitamin E is located exclusively in the thylakoid membrane lipid matrix. One should note, however, that nonacclimated *npq1* YL were observed to be more tolerant to $^1O_2$ toxicity than older leaves (Fig. 7), although their tocopherol content was identical (approximately 19 $\mu g\ g^{-1}$ fresh weight for both types of leaves), indicating that factors different from the vitamin E content are also involved in the differential phototolerance of *npq1* YL and ML. It is known that the lipid composition of leaves can change substantially with maturity (Liljenberg and Von Arnold, 1987) and, consequently, one cannot exclude that chloroplast lipids are less sensitive to photooxidative damage when leaves are in an early stage of development. Carlsson et al. (1996) reported that pea and wheat YL were less sensitive to oxidation by ozone than older leaves, with marked changes in lipid composition being observed in the latter leaves.

In summary, this study has shown that the protective action of the V cycle against photodestruction of the chloroplast was obvious only in well-developed ML of Arabidopsis. Loss of the V cycle had no such effect in developing YL due to some compensatory mechanisms or some special features of the leaves. Therefore, the absence of an active V cycle does not necessarily result in leaf photooxidative damage at high-light intensities. This study has also confirmed that part of the photoprotective action of the V cycle involves a site of action that is distinct from the LHCs where NPQ occurs: The presence of Z itself in the thylakoid membranes enhances the tolerance of thylakoids to lipid peroxidation. The molecular mechanism by which Z exerts its "direct" antioxidant action in the thylakoid membrane remains to be established.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The *npq1-2* mutant of Arabidopsis, affected in the structural gene of V de-epoxidase (Niyogi et al., 1998), and the wild type (ecotype Columbia) were grown under controlled conditions of temperature (22°C/18°C, day/night), air humidity (60%), and light (100, 250, 500, 1,000, and 1,500 $\mu mol\ m^{-2}\ s^{-1}$). Plants were first grown from seeds for 4 weeks at a PPFD of 250 μmol photons $m^{-2}\ s^{-1}$ and were subsequently transferred to the final PPFD where they were kept for 3, 10, or 21 d, depending on the experiments. Photoperiod was 8 h except when plants were exposed to the highest PPFD (1,500 $\mu mol\ m^{-2}\ s^{-1}$) for 3 d (photoperiod, 15 h). PPFDs were measured with a LI-COR quantum meter (Li-185B/Li-190SB, LI-COR, Lincoln, NE).

Two types of leaves were examined in this study: fully developed ML and developing YL. The so-called YL were the leaves in the center of the leaf rosette with 1.5- to 2-cm length. The average length of the ML in the rosette was 4.1 ± 0.3 cm. We did not characterize the metabolic status of the leaves, but the transition from sink to source leaves in Arabidopsis is known to occur at a relatively early stage of development (Truernit and Sauer, 1995). Little growth occurred during light stress (3 d at 1,500 $\mu mol\ m^{-2}\ s^{-1}$).

Eosin Treatments

Eosin was used to generate $^1O_2$ in the light (Knox and Dodge, 1985). Leaf discs of 1-cm diameter were floated on eosin (aqueous solution of eosin Y 5% [w/v], Sigma, St. Louis) and were illuminated with white light at a PPFD of 500 $\mu mol\ m^{-2}\ s^{-1}$ produced by a 150-W metal halide lamp (Osram, Munich, Germany). The temperature of the eosin solution was maintained constant at 22°C. In one experiment, a 1-cm thickness of eosin was used as a filter to allow irradiation of the samples without excitation of the dye. A PPFD of 500 $\mu mol\ m^{-2}\ s^{-1}$ was transmitted by this filter. We checked that white light and the filtered light at a PPFD of 500 $\mu mol\ m^{-2}\ s^{-1}$ induced the same level of NPQ. Eosin concentration in the leaf discs was estimated from the in vivo fluorescence emission of eosin (see below).

Photosynthetic Pigments and Tocopherols

Photosynthetic pigments were extracted in methanol. After centrifugation and filtration, pigments were separated and quantified by HPLC as previously described (Havaux and Tardy, 1996). Tocopherols were extracted in *N*,*N*-dimethylformamide and were quantified by HPLC as described elsewhere (Wildi and Lütz, 1996), except that the HPLC system was from Thermo Separations (Egelsbach, Germany). Tocopherols alternatively were extracted in methanol and were quantified by HPLC using a liquid chromatograph (model 5000, Varian, Palo Alto, CA) with a C-18 column (Alltima Peek, 5 μm, Alltech, Deerfield, IL) in methanol. Tocopherol was detected by fluorescence (excitation at 290 nm; emission at 330 nm) with an RF-530 system (Shimadzu, Tokyo).

Chl Thermoluminometry

The TL emitted by leaf discs (five discs of 6-mm diameter) was measured with a laboratory-built apparatus (Havaux, 1998b). The samples were slowly heated at a rate of $6°C\ min^{-1}$ from 25°C to 150°C. Temperature was monitored with a K-type thermocouple. The luminescence emission was recorded with a R376 photomultiplier tube

(Hamamatsu Photonics, Hamamatsu City, Japan). The current from the photomultiplier was amplified by a 70710 transimpedance preamplifier (Oriel, Stratford, CT) and was recorded by a computer equipped with a DAQPad-1200 acquisition card (National Instruments, Austin, TX) and software written by J.-M. Ducruet (Commissariat à l'Energie Atomique/Saclay, France).

Chl Fluorometry

In vivo Chl fluorescence from the upper surface of the leaves was measured with a PAM-101 fluorometer (Walz, Effeltrich, Germany; Bolhar-Nordenkampf et al., 1989), as previously described (Havaux and Tardy, 1996). The initial level F_o of Chl fluorescence was excited by a dim red light modulated at 1.6 kHz. The maximal level of Chl fluorescence was induced by an 800-ms pulse of intense white light (4,500 μmol photons m^{-2} s^{-1}). From the maximal fluorescence level (F_m') and the steady-state fluorescence level (F_s) emitted by illuminated leaves, the actual Φ_{PSII} was calculated as $(F_m' - F_s)/F_m'$. The maximal quantum yield of PSII photochemistry was measured in dark-adapted leaves as $(F_m - F_o)/F_m = F_v/F_m$, where F_m is the maximal fluorescence level in the dark. NPQ was determined as $(F_m/F_m') - 1$ (Bilger and Björkman, 1994). Chl fluorescence emission spectra were measured in liquid nitrogen (77K) using an LS50B luminescence spectrometer (Perkin-Elmer, Beaconsfield, UK) equipped with fiber optics. Fluorescence was excited at 440 nm. Fluorescence of eosin infiltrated in leaf discs (for 5 h) was measured at room temperature with a 390-nm exciting light. Leaf discs were carefully rinsed with distilled water before fluorescence measurements.

Photoacoustic Spectroscopy

The photoacoustic signals generated by leaf discs of 1-cm diameter were measured with a laboratory-built photoacoustic spectrometer that has been described (Havaux and Tardy, 1996). The leaf sample placed in the hermetically closed photoacoustic cell was illuminated with white light (<700 nm, 100 μmol m^{-2} s^{-1}) modulated at 19 Hz. Photochemistry was saturated with a strong background light of 4,500 μmol m^{-2} s^{-1}. The photobaric signal (due to modulated oxygen evolution; amplitude, A_{ox}) was separated from the photothermal signal using a well-documented procedure involving light saturation of photosynthesis and phase adjustment in the lock-in amplifier (Malkin and Canaani, 1994). The relative difference between the actual photothermal signal (A_{pt}') and the maximal thermal signal (recorded in the presence of the strong background light; amplitude, A_{pt}) was estimated at a high frequency of 370 Hz, where the photobaric signal is completely damped out. Photochemical energy storage was calculated from the actual and maximal levels of the high-frequency photothermal signal (Malkin and Canaani, 1994) as follows: $(A_{pt} - A_{pt}')/A_{pt}$. The quantum yield of oxygen evolution was estimated (in relative values) by the ratio of A_{ox} to A_{pt}, taking into account the energy storage (Malkin and Canaani, 1994). A_{pt} was also measured in modulated blue light (19 Hz, 400–490 nm, 6 μmol m^{-2} s^{-1}) using an Oriel 57530 interference filter.

ACKNOWLEDGMENTS

Many thanks to J.-M. Ducruet (Commissariat à l'Energie Atomique/Saclay, France) for help with Chl TL and to R. Strasser (University of Geneva) for the loan of fluorescence accessories and helpful discussion.

Received January 27, 2000; accepted May 16, 2000.

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