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Chlamydomonas Xanthophyll Cycle Mutants Identified by Video Imaging of Chlorophyll Fluorescence Quenching

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The photosynthetic apparatus in plants is protected against oxidative damage by processes that dissipate excess absorbed light energy as heat within the light-harvesting complexes. This dissipation of excitation energy is measured as nonphotochemical quenching of chlorophyll fluorescence. Nonphotochemical quenching depends primarily on the ΔpH that is generated by photosynthetic electron transport, and it is also correlated with the amounts of zeaxanthin and antheraxanthin that are formed from violaxanthin by the operation of the xanthophyll cycle. To perform a genetic dissection of nonphotochemical quenching, we have isolated *npq* mutants of Chlamydomonas by using a digital video-imaging system. In excessive light, the *npq1* mutant is unable to convert violaxanthin to antheraxanthin and zeaxanthin; this reaction is catalyzed by violaxanthin de-epoxidase. The *npq2* mutant appears to be defective in zeaxanthin epoxidase activity, because it accumulates zeaxanthin and completely lacks antheraxanthin and violaxanthin under all light conditions. Characterization of these mutants demonstrates that a component of nonphotochemical quenching that develops in vivo in Chlamydomonas depends on the accumulation of zeaxanthin and antheraxanthin via the xanthophyll cycle. However, observation of substantial, rapid, ΔpH-dependent nonphotochemical quenching in the *npq1* mutant demonstrates that the formation of zeaxanthin and antheraxanthin via violaxanthin de-epoxidase activity is not required for all ΔpH-dependent nonphotochemical quenching in this alga. Furthermore, the xanthophyll cycle is not required for survival of Chlamydomonas in excessive light.

INTRODUCTION

In photosynthetic membranes of green algae and plants, incident light is absorbed by light-harvesting chlorophyll–carotenoid–protein complexes (LHCs) (reviewed in Jansson, 1994; Grossman et al., 1995; Paulsen, 1995) associated with the reaction centers of photosystem II (PSII) and PSI. When a photon of light is absorbed, chlorophyll in the antenna enters the singlet excited state. This excitation energy can be transferred to the reaction centers to drive electron transport that oxidizes H_2O and generates chemical energy in the forms of ATP and NADPH for the reduction of CO_2. Regulation of light harvesting and electron transport is critical for ensuring that the production and consumption of chemical energy are balanced.

At low incident photon flux density (PFD), it is imperative that the plant capture and utilize light at a very high efficiency, whereas at higher PFDs, excess excitation energy must be dissipated safely to avoid damage to the reaction centers and other cellular constituents. In the natural environment, the absorption of light energy often exceeds the capacity of photosynthesis, causing overreduction of electron carriers and the accumulation of excitation energy in the light-harvesting antennae. Overreduction of electron carriers would favor the direct reduction of O_2 by PSI (Mehler, 1951) and the production of damaging reactive oxygen species (Asada, 1994; Foyer and Harbinson, 1994), such as superoxide (O_2^-), whereas the accumulation of excitation energy in the antennae would favor the production of triplet excited chlorophyll molecules that can interact with O_2 to produce reactive singlet oxygen. Indeed, photooxidative damage, especially to PSII, appears to be an unavoidable consequence of photosynthetic electron transport, necessitating the turnover and repair of damaged reaction center subunits (Barber and Andersson, 1992; Aro et al., 1993).

Several processes most probably contribute to the survival of plants when they are exposed to excess excitation. Carotenoids within the LHC may serve as photoprotectants, because they can directly quench triplet chlorophyll and singlet oxygen (Cogdell and Frank, 1987; Young, 1991; Demmig-Adams et al., 1996). Other processes in the light-harvesting antennae may minimize the production of reactive chlorophyll and oxygen species by promoting the dissipation of excess absorbed light energy harmlessly as heat (reviewed in Demmig-Adams and Adams, 1992; Björkman and Demmig-Adams, 1994; Horton et al., 1994, 1996). This photoprotective energy dissipation is measured as nonphotochemical quenching of chlorophyll fluorescence (NPQ). The yield of room temperature chlorophyll fluorescence, proportional to the average lifetime of the singlet excited state of chlorophyll (Krause and Weis, 1991), is decreased when excitation

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energy is used for photochemistry (photochemical quenching) or is dissipated as heat (NPQ). A decrease in the lifetime of the singlet excited state of chlorophyll leads to decreased production of triplet chlorophyll and singlet oxygen. Furthermore, by decreasing the efficiency of energy transfer to PSII reaction centers, NPQ prevents overreduction of the reaction centers, which is a condition associated with PSII damage (Aro et al., 1993). Because NPQ competes with photochemistry, it must be strictly controlled to avoid losses in photosynthetic efficiency in limiting light. State transitions and processes in the PSII reaction center may also contribute to NPQ (Weis and Berry, 1987; Krause and Weis, 1991; Johnson and Krieger, 1994); however, the major component of NPQ is the pH-dependent ("energy-dependent" or qE) type of quenching due to dissipation of excess absorbed light energy as heat (Horton et al., 1994).

Although the mechanism by which NPQ quenches singlet excited chlorophyll has not been fully elucidated, NPQ depends primarily on a ΔpH across the thylakoid membrane that is generated by photosynthetic electron transport. When the absorption of light energy exceeds the capacity for its utilization, the magnitude of this ΔpH increases, which results in an elevated proton concentration in the thylakoid lumen and/or in localized membrane domains. An elevated lumenal proton concentration may favor protonation of acidic amino acid side chains on the chlorophyll *a/b* binding polypeptides of PSII (Horton et al., 1996), leading to a conformational change in the LHCs that triggers NPQ. This stimulation of NPQ correlates with a decreased lifetime of chlorophyll fluorescence (Gilmore et al., 1995, 1996).

It has been suggested that carotenoids, specifically xanthophylls, play a critical role in generating NPQ. The extent of NPQ in leaves is correlated with the levels of zeaxanthin and antheraxanthin (Demmig-Adams, 1990; Demmig-Adams and Adams, 1996a), which are formed from violaxanthin through the operation of the xanthophyll cycle (Figures 1A and 1B; reviewed in Yamamoto, 1979; Hager, 1980; Pfündel and Bilger, 1994). In limiting light, zeaxanthin epoxidase converts zeaxanthin to violaxanthin via the intermediate antheraxanthin. In excessive light, an elevated $[H^+]$ in the thylakoid lumen activates violaxanthin de-epoxidase, generating zeaxanthin and antheraxanthin. Furthermore, leaves of sun plants have an increased total xanthophyll cycle pool size relative to leaves of shade plants (Thayer and Björkman, 1990), and plants growing in natural sunlight exhibit diurnal variations in the de-epoxidation state of xanthophyll cycle constituents (high levels of zeaxanthin at midday) that are consistent with zeaxanthin and antheraxanthin being critical for NPQ (Björkman and Demmig-Adams, 1994). In vitro studies using isolated thylakoids have also correlated an increase in the level of zeaxanthin and antheraxanthin with an increase in ΔpH-dependent NPQ (Gilmore and Yamamoto, 1993).

The way in which zeaxanthin and antheraxanthin may promote NPQ is still controversial. Zeaxanthin and antheraxanthin are associated with the LHCs (Thayer and Björkman, 1992; Bassi et al., 1993; Ruban et al., 1994; Lee and Thornber, 1995), and recent biophysical measurements of the energy levels of the lowest singlet excited states of zeaxanthin and antheraxanthin have established the feasibility of direct quenching of singlet excited chlorophyll molecules by these xanthophylls (Frank et al., 1994; Owens, 1994). The energy level of the LHC-associated carotenoids is a key factor for enhancing NPQ in vitro (Phillip et al., 1996); however, these experiments also suggest that zeaxanthin and antheraxanthin may affect NPQ by altering the structure of the LHC (Phillip et al., 1996; Ruban et al., 1996). This has led to the alternative hypothesis that zeaxanthin and antheraxanthin do not directly quench singlet excited chlorophyll molecules but that they facilitate LHC aggregation (Ruban et al., 1997) and "concentration quenching" via chlorophyll dimerization (Horton et al., 1996).

Although physiological, biochemical, and biophysical methods have been widely used to study NPQ, a systematic genetic approach has been lacking. To gain new insights into the mechanism of NPQ and its importance for photoprotection, we have isolated mutants of Chlamydomonas with aberrant NPQ. These *npq* mutants were generated by insertional mutagenesis of the nuclear genome and were identified using a digital video-imaging system. A subset of these mutants is defective in the xanthophyll cycle; these mutants are described in this report.

RESULTS

Xanthophyll Cycle in Wild-Type Chlamydomonas

To establish conditions that would allow for survival of strains defective in the xanthophyll cycle, we determined the composition of the xanthophyll cycle pool in Chlamydomonas cells grown in different media and at different PFDs. As shown in Table 1, the xanthophyll cycle pool of Chlamydomonas cells grown photoautotrophically in HS medium (Harris, 1989) or photoheterotrophically in TAP medium (Harris, 1989) at a PFD of 50 μmol photons m^{-2} sec^{-1} or less was composed almost entirely of violaxanthin. At a PFD of 300 to 350 μmol photons m^{-2} sec^{-1}, approximately half of the pool was composed of the de-epoxidized xanthophylls (zeaxanthin and antheraxanthin). Furthermore, at the higher PFDs, the total xanthophyll cycle pool size (violaxanthin, antheraxanthin, and zeaxanthin) was increased by more than twofold when expressed relative to chlorophyll *a* (Table 1).

The kinetics of de-epoxidation of violaxanthin to form zeaxanthin and antheraxanthin upon exposure of dark-adapted wild-type cells to excessive light is shown in Figure 1C. Cells grown at 100 μmol photons m^{-2} sec^{-1} still maintained a xanthophyll cycle pool composed almost exclusively of violaxanthin, and this composition was not significantly changed after an overnight dark period. However, when the cells were exposed to 800 μmol photons m^{-2} sec^{-1}, approximately

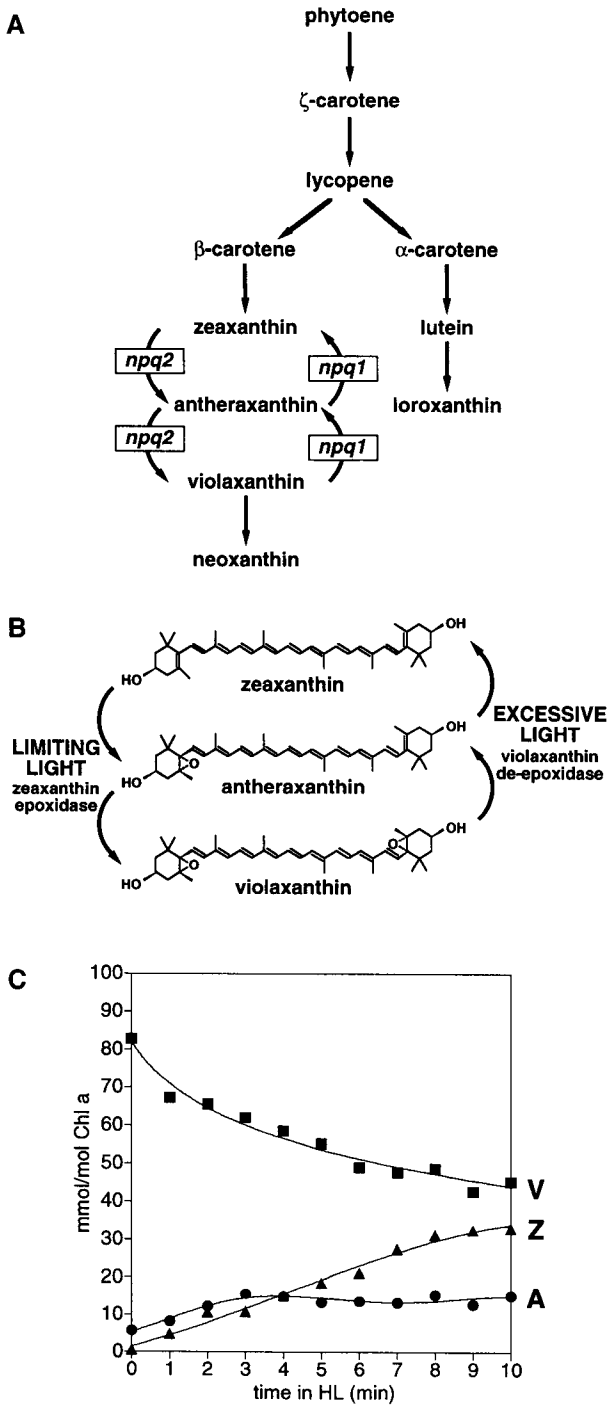

Figure 1. Xanthophyll Metabolism in Chlamydomonas.

(A) The xanthophyll biosynthetic pathway in Chlamydomonas. The defects in two mutants affected in nonphotochemical quenching of chlorophyll fluorescence (*npq1* and *npq2*) are shown.

(B) The xanthophyll cycle.

(C) Kinetics of conversion of xanthophyll cycle pigments in wild-type Chlamydomonas. Strain CC-425 was grown photoautotrophically in HS medium at 100 μmol photons m^{-2} sec^{-1}, dark adapted overnight, and then exposed to high light (HL; 800 μmol photons m^{-2} sec^{-1}) for 10 min. Pigment determination by HPLC was performed with samples taken at each time point. Chl, chlorophyll; A, antheraxanthin; V, violaxanthin; Z, zeaxanthin.

half of the violaxanthin was converted to antheraxanthin and zeaxanthin within 10 min (Figure 1C). Slow re-epoxidation of zeaxanthin and antheraxanthin to violaxanthin occurred during a subsequent period in the dark or in low light (data not shown). These results suggested that mutants defective in the xanthophyll cycle and unable to convert violaxanthin to antheraxanthin and zeaxanthin would not be significantly impaired in growth at a PFD of up to 100 μmol photons m^{-2} sec^{-1}.

NPQ in Wild-Type Chlamydomonas

NPQ was induced during exposure of cells to 800 μmol photons m^{-2} sec^{-1} (high light). As shown in Figures 2A and 2B, the development of NPQ was characterized by a rapid initial increase in NPQ (decrease in fluorescence), followed by a slower further increase. Most of the NPQ that occurred during 10 min of illumination with high light was rapidly reversed during a subsequent period of 5 min in the dark (Figure 2). The addition of 10 μM nigericin to the cells before exposure to high light inhibited NPQ (data not shown). Furthermore, a rapid reversal of NPQ was observed if nigericin was added during illumination (data not shown), suggesting that the observed NPQ depends on the ΔpH.

The slower, second phase of NPQ developed in parallel with formation of zeaxanthin and antheraxanthin (Figure 3). Consistent with results from other laboratories (Gilmore and Yamamoto, 1993; Demmig-Adams and Adams, 1996b), a higher correlation was observed between NPQ and the amount of antheraxanthin and zeaxanthin (Figures 3B and 3C) than between NPQ and the amount of zeaxanthin alone (Figure 3A). These results suggested that the slower second phase but not the rapid initial phase of NPQ was associated with the conversion of violaxanthin to zeaxanthin and antheraxanthin.

Isolation of Mutants with Altered NPQ

To initiate a genetic analysis of NPQ, we isolated Chlamydomonas mutants with altered kinetics and/or extent of NPQ by using a fluorescence video-imaging system, as shown in Figure 4A. Insertional mutagenesis of the CC-425 parental strain (*arg7-8 cw15 mt+ sr-u-2-60*) was performed

Table 1. HPLC Analysis of the Xanthophyll Cycle Pigments in Wild-Type Chlamydomonas Cells under Different Growth Conditions[a]

		mmol/mol Chlorophyll *a*				
Growth Medium	Incident PFD	V	A	Z	V + A + Z	(A + Z)/(V + A + Z)
TAP	5	118	4	0	122	0.03
TAP	50	175	8	3	186	0.06
TAP	300	127	51	109	287	0.56
HS	50	123	3	0	126	0.02
HS	350	148	46	83	277	0.47

[a]Cells of an Arg^+ control strain wt, derived from CC-425, were grown at the indicated PFD (μmol photons m^{-2} sec^{-1}) for 9 days. Cells were grown photoheterotrophically on agar medium containing acetate as a carbon source (TAP) or photoautotrophically on minimal medium lacking acetate (HS). Values are the averages of duplicate determinations. V, violaxanthin; A, antheraxanthin; Z, zeaxanthin.

by transformation with a linearized plasmid containing the wild-type *ARG7* gene. Because integration of transforming plasmid DNA into the nuclear genome of Chlamydomonas occurs primarily by nonhomologous recombination (Debuchy et al., 1989; Kindle et al., 1989), transformation results in random mutagenesis (Tam and Lefebvre, 1993; Davies et al., 1994) due to interruption and sometimes deletion of genes resulting from plasmid DNA integration. This inserted DNA serves as a convenient tag to facilitate eventual isolation of the gene affected in the mutants (Tam and Lefebvre, 1993; Gumpel et al., 1995; Davies et al., 1996).

Arg^+ transformants were grown photoautotrophically on agar medium at 50 μmol photons m^{-2} sec^{-1} and illuminated in the video-imaging system with high light (1200 μmol photons m^{-2} sec^{-1}), which both excited chlorophyll fluorescence and induced NPQ. This high light was nearly sufficient to saturate photochemistry; therefore, most of the quenching of fluorescence that occurred was due to NPQ. At various times during the illumination, fluorescence images (480 × 640 pixels) were captured by the charge-coupled device (CCD) camera and digitized by a computer. NPQ was calculated for each pixel as $(F - F')/F'$, where F is the fluorescence image captured within the first second of illumination and F' is the fluorescence image captured after several minutes of illumination. The NPQ data were displayed as false-color images, allowing identification of putative mutants (Figure 4B).

Approximately 15,000 mutagenized colonies were screened by video imaging, and putative mutants were rescreened by PAM fluorescence analysis. Figure 5 shows the kinetics of NPQ development for representative mutants. Four classes of *npq* mutants were identified: (1) mutants defective in the first phase of NPQ (i.e., *npq4*), (2) mutants defective in the second phase of NPQ (i.e., *npq1*), (3) mutants with an apparent defect in both phases of NPQ (i.e., *npq2* [see the following section] and *npq11*), and (4) mutants with increased NPQ compared with wild-type cells (data not shown). The third class was the most abundant; most of these mutants exhibited a reduced PSII quantum yield (F_v/F_m, where F_v is dark-adapted variable fluorescence and F_m is dark-adapted maximum fluorescence) (data not shown), suggesting that many of these may be partial loss-of-function mutations affecting photosynthetic electron transport.

Identification of Xanthophyll Cycle Mutants

Determination of the pigment composition of the *npq* mutants before and after exposure to high light identified xanthophyll cycle mutants as a subset of the *npq* mutants. As shown in Figure 6, the *npq1* mutant had normal levels of violaxanthin but was unable to convert violaxanthin to antheraxanthin and zeaxanthin during exposure to excessive light, suggesting a defect in the enzyme violaxanthin de-epoxidase. In contrast, the xanthophyll cycle pool of the *npq2* mutant consisted entirely of zeaxanthin under all conditions, even after overnight dark adaptation. In addition to the absence of violaxanthin and antheraxanthin, neoxanthin was completely lacking in the *npq2* mutant (data not shown), suggesting a defect in the enzyme zeaxanthin epoxidase. The positions of the defects in the xanthophyll biosynthetic pathway are shown in Figure 1A.

The *npq1* mutant was defective specifically in the second phase of NPQ development when exposed to excessive light (Figure 5). The *npq2* mutant appeared defective in both phases of NPQ (Figure 5). However, the measured defect in the rapid initial phase was due to a decreased value of F_m, probably resulting from high levels of zeaxanthin already in the cell (NPQ was occurring during the 1-sec saturating pulse of light used to determine the F_m [data not shown]). The absence of the second phase of NPQ in *npq2* reflected the fact that the already high levels of zeaxanthin were not increased further during the 10-min illumination in high light (data not shown).

Genetic Characterization of *npq1* and *npq2*

To determine the genetic basis for the mutant phenotypes, we performed crosses with *npq1* and *npq2*. After crosses to

wild-type strains, the Npq$^-$ phenotypes of both *npq1* (Figures 7A and 7B) and *npq2* (Figure 7C) exhibited a 2:2 segregation ratio in tetrads resulting from meiosis, demonstrating that the Npq$^-$ phenotypes of the mutants result from mutations in single nuclear genes. HPLC analyses of meiotic progeny showed that the Npq$^-$ phenotype of *npq1* cosegregated with the lack of conversion of violaxanthin to antheraxanthin and zeaxanthin when exposed to excessive light (data not shown). Similarly, the Npq$^-$ phenotype of *npq2* cosegregated with the accumulation of zeaxanthin and absence of antheraxanthin, violaxanthin, and neoxanthin (data not shown).

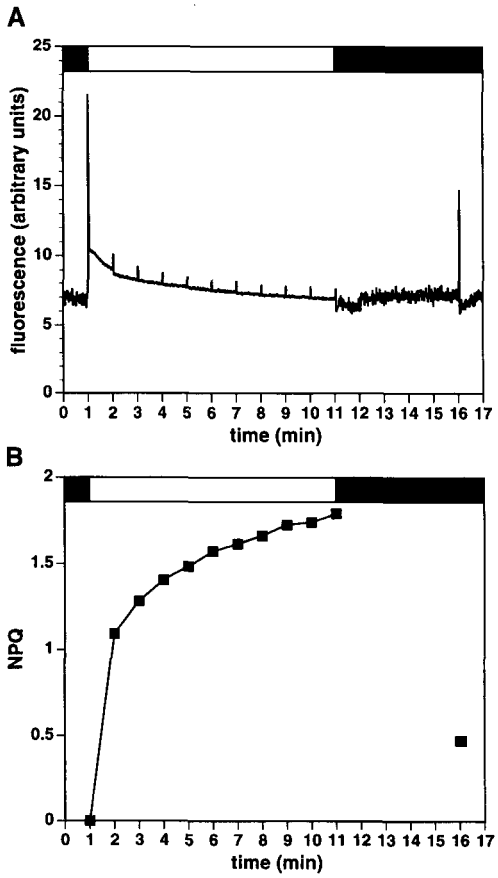

Figure 2. Induction of NPQ in Wild-Type Chlamydomonas.

Black bars above each graph indicate periods of illumination with weak far-red background light; white bars indicate periods of actinic illumination with high light (800 μmol photons m^{-2} sec^{-1}).
(A) Fluorescence recording during induction of NPQ. Chlamydomonas strain CC-425 was exposed to high light as described in Figure 1C. Fluorescence was measured with a PAM fluorometer, as described in Methods. For determination of F_m and F_m', saturating pulses of light were given just before the start of illumination with high light, at 1-min intervals during illumination, and at 5 min after illumination.
(B) NPQ was calculated from the fluorescence data as $(F_m - F_m')/F_m'$.

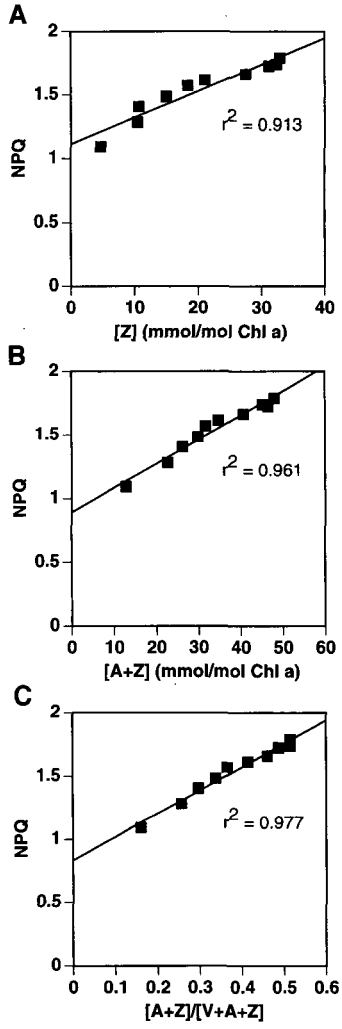

Figure 3. Correlations of NPQ with the Levels of Xanthophyll Cycle Pigments.

Data from Figures 1C and 2 were replotted, and linear regressions (r^2) were calculated using DeltaGraph (DeltaPoint, Inc., Monterey, CA). Chl, chlorophyll; A, antheraxanthin; V, violaxanthin; Z, zeaxanthin.
(A) Plot of NPQ versus the level of zeaxanthin.
(B) Plot of NPQ versus the level of zeaxanthin plus antheraxanthin.
(C) Plot of NPQ versus zeaxanthin plus antheraxanthin, expressed relative to the total xanthophyll cycle pool size.

To determine whether the mutations in *npq1* and *npq2* were caused by insertion of transforming *ARG7* DNA, we examined progeny of crosses to *arg7-8 mt*− strains for cosegregation of the Arg$^+$ and Npq$^-$ phenotypes. In the case of *npq1*, the Arg$^+$ and Npq$^-$ phenotypes segregated independently (data not shown), showing that the *npq1* mutation was not caused by insertion of an expressed copy of the *ARG7* gene. DNA gel blot analyses using hybridization

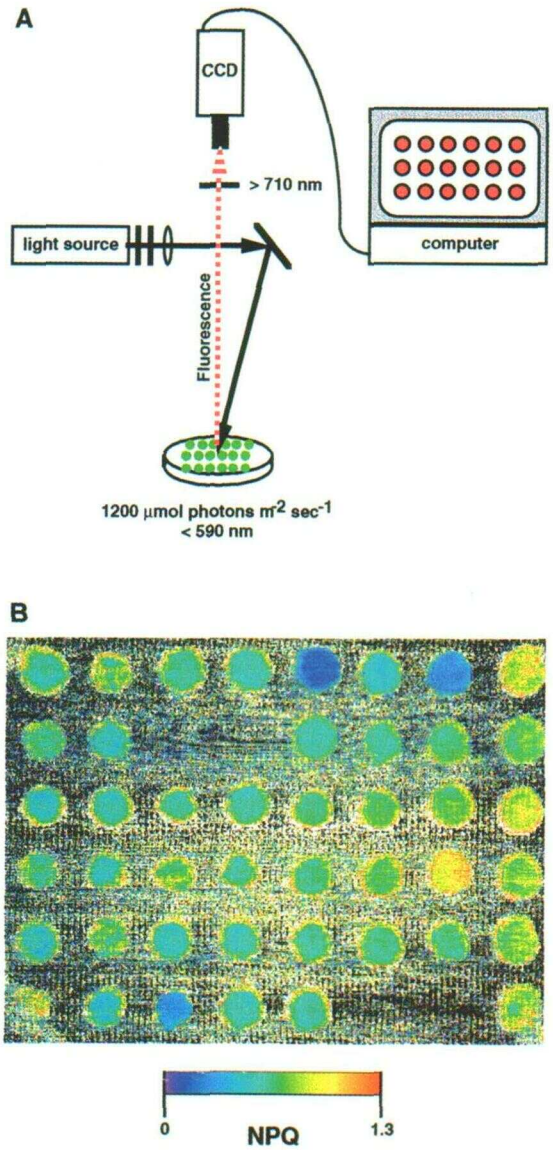

Figure 4. Screening for *npq* Mutants by Digital Video Imaging of Chlorophyll Fluorescence.

(A) Schematic diagram of the video-imaging system. CCD, charge-coupled device.

(B) Example of an NPQ image from the screen for mutants. NPQ occurring between 0 and 5 min of illumination with high light was calculated from digitized images of chlorophyll fluorescence as described in Methods. Colonies with a wild-type level of NPQ appear green, whereas mutants with less NPQ appear blue.

probes derived from the pJD67 plasmid used for transformation failed to reveal any DNA fragments that cosegregated with the Npq^- phenotype (data not shown). The Npq^- phenotype of *npq2* did cosegregate with the Arg^+ phenotype in tetrads (Figures 7C and 7D), suggesting that the *npq2* mutation is closely linked to and most likely caused by a single insertion locus of the *ARG7* plasmid.

Growth of Xanthophyll Cycle Mutants in High Light

To begin to examine the consequences of defects in the xanthophyll cycle for survival of Chlamydomonas, we tested the ability of the *npq1* and *npq2* mutants to grow under various light conditions. Figure 8 shows that photoautotrophic growth of *npq1* and *npq2* was not impaired relative to the wild type in continuous low light (100 μmol photons m^{-2} sec^{-1}) or high light (500 μmol photons m^{-2} sec^{-1}), demonstrating that the xanthophyll cycle is not absolutely required for survival of Chlamydomonas under the conditions of excessive light used here. The different color of all strains grown in high light reflected a decrease in chlorophyll per cell as well as an increased carotenoid:chlorophyll ratio (Table 1). Overall, these results suggest that although zeaxanthin and antheraxanthin have a role in NPQ, processes in the cell unrelated to the xanthophyll cycle are also involved in the dissipation of excess absorbed light energy and survival in high light.

DISCUSSION

We have isolated Chlamydomonas mutants defective in NPQ by high-resolution digital video imaging that sensitively

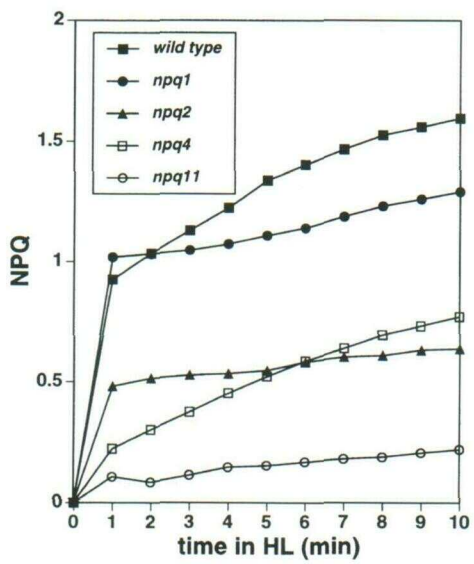

Figure 5. Induction of NPQ in Representative *npq* Mutants.

Strains were grown as given in Figure 1C and analyzed as given in Figure 2. Wild type refers to the wt strain described in Methods. HL, high light.

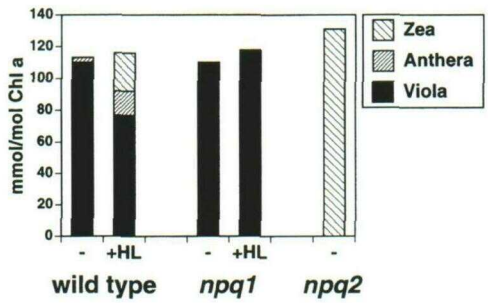

Figure 6. HPLC Analysis of Xanthophyll Cycle Pigments in *npq* Mutants.

Cells of the wild type, *npq1*, and *npq2* were grown photoautotrophically on agar medium in low light (−, 50 μmol photons m^{-2} sec^{-1}) and then exposed to high light for 30 min (+HL, 500 μmol photons m^{-2} sec^{-1}). Chl, chlorophyll; Zea, zeaxanthin; Anthera, antheraxanthin; Viola, violaxanthin.

detects differences in chlorophyll fluorescence quenching during illumination of algal colonies with saturating light. From 15,000 mutagenized colonies, *npq* mutants were recovered at a frequency of ~1%. This high frequency was likely due to the dependence of NPQ on the ΔpH that is generated by photosynthetic electron transport. Most of the mutants that were isolated were partial loss-of-function mutations that affected electron transport (data not shown) and thus indirectly altered NPQ. By using HPLC analysis of pigments as a secondary screen, we identified two mutants that were defective in the xanthophyll cycle. The *npq1* mutant was unable to de-epoxidize violaxanthin, whereas the *npq2* mutant was unable to epoxidize zeaxanthin (Figure 1). In addition, we identified a single mutant, *npq3*, that lacked chlorophyll *b* (data not shown) and appeared to be a new allele of the previously characterized *CBN1* gene (Chunaev et al., 1987). The isolation of single alleles of *npq1*, *npq2*, and *npq3* suggests that our screen for *npq* mutants was probably not saturating. The majority of transformants generated by the insertional mutagenesis procedure had a single insertion locus of the plasmid DNA containing *ARG7*. Assuming random insertion, a Chlamydomonas genome size of 100 Mb, and an average gene size of 10 kb, we would have to screen 30,000 transformants to attain a 95% probability of obtaining an insertion in a particular gene.

Chlorophyll fluorescence has been used for decades to screen for mutants of Chlamydomonas (Bennoun and Levine, 1967) and vascular plants (Miles and Daniel, 1974; Meurer et al., 1996) that are defective in photosynthesis. Most of these previously described mutants were isolated as high chlorophyll fluorescence (*hcf*) strains that exhibited high steady state fluorescence upon excitation with relatively low intensity actinic light; these mutants are defective in photochemical quenching of chlorophyll fluorescence. In contrast, screening with the video-imaging system allowed us to isolate mutants with altered kinetics and/or extent of NPQ during illumination with saturating actinic light. The calculation of NPQ as $(F - F')/F'$ from digitized images of chlorophyll fluorescence normalizes for differences in fluorescence that arise from variations in chlorophyll content or in PFD across the field of illumination. Video imaging of NPQ has been used previously to visualize the topography of photosynthetic activity in leaves (Daley et al., 1989), to diagnose viral infections in

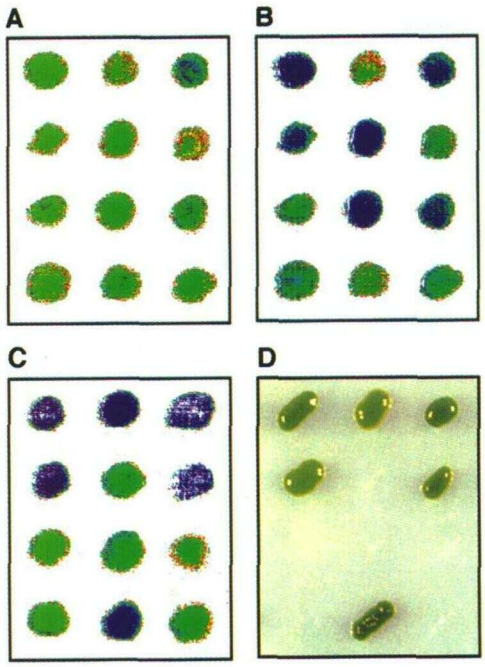

Figure 7. Tetrad Analysis of *npq* Mutants.

Each vertical column of four colonies is a tetrad from a cross between a mutant and the wild type. The background color in **(A)**, **(B)**, and **(C)** was changed to white.

(A) NPQ image of tetrads from *NPQ1 mt+* × *npq1 mt−* cross. NPQ occurring between 0 and 1 min of illumination with high light was calculated for colonies grown on HS medium. Note the similar (wild-type) phenotype of all progeny (green).

(B) NPQ image of tetrads from *NPQ1 mt+* × *npq1 mt−* cross. NPQ occurring between 1 and 10 min of illumination with high light was calculated for colonies grown on HS medium. Note the 2:2 segregation ratio of the Npq^+ (green) and Npq^- (blue) phenotypes. The *npq1* mutation affects the slower second phase of NPQ that occurs between 1 and 10 min but not the initial component of NPQ that occurs between 0 and 1 min.

(C) NPQ image of tetrads from *NPQ2 arg7-8 mt+* × *npq2::ARG7 arg7-8 mt−* cross. NPQ occurring between 0 and 5 min of illumination with high light was calculated for colonies grown on HS plus 50 μg/mL L-arginine.

(D) Arg phenotype of tetrads from *NPQ2 arg7-8 mt+* × *npq2::ARG7 arg7-8 mt−* cross. Tetrad progeny were patched on TAP medium without L-arginine and grown for 7 days.

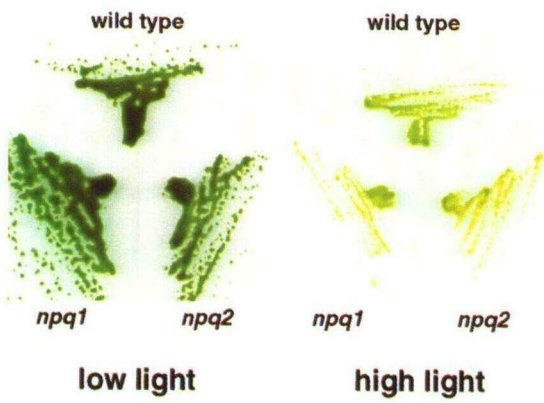

Figure 8. Growth of *npq* Mutants.

Chlamydomonas strains were streaked on HS agar medium and grown photoautotrophically for 8 days at 50 μmol photons m^{-2} sec^{-1} (low light) or 500 μmol photons m^{-2} sec^{-1} (high light).

tobacco plants (Balachandran et al., 1994), and to study stomatal movements (Cardon et al., 1994).

Characterizations of the *npq* mutants confirmed a role for the xanthophyll cycle in NPQ. The *npq1* and *npq2* strains were impaired in the slower second phase of NPQ (Figure 5), suggesting that this feature is related to the accumulation of additional zeaxanthin and antheraxanthin. The *npq1* mutant was unable to convert violaxanthin to antheraxanthin and zeaxanthin, whereas the *npq2* mutant accumulated zeaxanthin under all light conditions and did not form any additional zeaxanthin during short-term illumination with excessive light. The assignment of the second phase of NPQ as zeaxanthin and antheraxanthin dependent is consistent with the results in wild-type Chlamydomonas in which the accumulation of zeaxanthin plus antheraxanthin best correlates with the slowly developing component of NPQ (Figure 3).

However, the characterizations of the *npq* mutants established that the formation of zeaxanthin and antheraxanthin by the xanthophyll cycle is not absolutely required for NPQ. The *npq1* mutant, which cannot convert violaxanthin to zeaxanthin and antheraxanthin, exhibited only 20% less NPQ than did wild-type cells. A small amount of antheraxanthin (<5%) is sometimes present in the *npq1* mutant and in dark-adapted wild-type cells (Figures 1C and 6). This antheraxanthin probably accumulates as an intermediate in the epoxidation of zeaxanthin to violaxanthin. The rapidly developing NPQ, which is observed in wild-type Chlamydomonas and which remains in the *npq1* mutant, is nigericin sensitive (data not shown), suggesting that it is pH dependent and xanthophyll cycle independent.

Xanthophyll cycle–independent NPQ has been observed previously. Equations that best describe the extent of NPQ in lettuce and pea thylakoids (Gilmore and Yamamoto, 1993) include contributions from both pH-dependent, xanthophyll cycle–dependent NPQ and pH-dependent, xanthophyll cycle–independent NPQ. Studies of chlorophyll fluorescence lifetime distributions in isolated photosynthetic membranes have confirmed that an elevated ΔpH, even in the presence of little zeaxanthin, can result in NPQ (Gilmore et al., 1995, 1996). Furthermore, leaves treated with DTT to inhibit the formation of zeaxanthin and antheraxanthin from violaxanthin often exhibit 25 to 40% of the NPQ of control leaves (Adams et al., 1990; Bilger and Björkman, 1990). The DTT-insensitive NPQ was greater in leaves during the induction of photosynthesis (immediately after the transition from darkness to light) and during illumination in 2% O_2 in the absence of CO_2 (Adams et al., 1990)—conditions that could sustain a higher ΔpH. Indeed, in some experiments with isolated thylakoids, the extent of NPQ at high ΔpH was the same in the presence and in the absence of zeaxanthin. This has led to the idea that the ΔpH is all that is absolutely required for NPQ and that zeaxanthin acts to amplify NPQ within the ΔpH range generated by photosynthetic electron transport in vivo (Noctor et al., 1991; Horton et al., 1996).

This notion of zeaxanthin as a modulator of NPQ is supported by experiments with the *npq2* mutant, which exhibited development of NPQ already during the 1-sec saturating pulse of light that was applied for determination of F_m (data not shown). The Arabidopsis *aba* mutant, which like *npq2* is defective in zeaxanthin epoxidase activity (Rock and Zeevaart, 1991), also shows accelerated NPQ formation (Tardy and Havaux, 1996; Hurry et al., 1997), suggesting that the presence of zeaxanthin increases the sensitivity of NPQ to a given ΔpH. Thus, the formation of zeaxanthin and antheraxanthin by the xanthophyll cycle provides a means to increase NPQ above a basal capacity for NPQ that depends only on pH.

The operation of the xanthophyll cycle in Chlamydomonas is similar to that in vascular plants, although the conversion of violaxanthin to antheraxanthin and zeaxanthin occurs more slowly (Figure 1C). Slower de-epoxidation of violaxanthin has been observed in other green algae, including *Ulva rotundata* (Franklin et al., 1992), *Chlorella vulgaris* (Maxwell et al., 1995), and *Dunaliella tertiolecta* (Casper-Lindley and Björkman, 1996). The slow kinetics of de-epoxidation in Chlamydomonas may have enabled us to resolve the xanthophyll cycle–dependent and xanthophyll cycle–independent components of NPQ. Also similar to vascular plants, the total xanthophyll cycle pool size (violaxanthin, antheraxanthin, and zeaxanthin) was higher (on a chlorophyll *a* basis) in Chlamydomonas cells grown in high light compared with cells grown in low light (Table 1). Hence, Chlamydomonas, like vascular plants, acclimates to excess excitation of the photosynthetic apparatus over the long term by increasing the level of the potentially photoprotective xanthophyll cycle pigments.

Although the xanthophyll cycle is involved in a component of NPQ, the xanthophyll cycle is not required for survival of Chlamydomonas in high light (Figure 8). The comparable growth of the wild type and the *npq1* mutant in high light

suggests that the pH-dependent, xanthophyll cycle–independent NPQ confers considerable high-light tolerance. Zeaxanthin and antheraxanthin may be necessary for survival (1) at higher PFDs than were used in these analyses, (2) when light levels are rapidly fluctuating, or (3) when high light is combined with additional environmental stresses, such as nutrient limitation. Additional physiological characterizations of these mutants will be described separately.

The xanthophyll cycle phenotypes of the *npq1* and *npq2* mutants are both caused by single nuclear mutations. Although mutagenesis of the starting strain was performed by insertional mutagenesis, the *npq1* mutation was not linked to the single *ARG7* insertion in this strain. The *npq1* mutation must have arisen spontaneously during the mutagenesis procedure. Similarly "untagged" mutants have been recovered in other mutant screens after insertional mutagenesis (Tam and Lefebvre, 1993; Gumpel et al., 1995; Pazour et al., 1995). In contrast to the situation for *npq1*, the *npq2* phenotype cosegregated with the Arg^+ phenotype (Figure 7), suggesting that the insertion of pJD67 was responsible for the *npq2* mutation. pJD67 sequences could be used as hybridization probes to facilitate the isolation of the *NPQ2* gene, which may encode the zeaxanthin epoxidase.

Identification of xanthophyll cycle mutants among the *npq* mutants demonstrates the power and potential of using a genetic approach for analyzing the regulation of photosynthesis. In addition to the *npq1*, *npq2*, and *npq3* mutants, we have identified several other *npq* mutants that have normal pigment composition; these mutants identify other factors and processes that are critical for NPQ. Molecular genetic, biochemical, and physiological characterizations of these mutants will help elucidate the various ways that NPQ is generated and controlled.

METHODS

Strains and Growth Conditions

Chlamydomonas reinhardtii strains were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). The arginine auxotrophic strain CC-425 (*arg7-8 cw15 mt+ sr-u-2-60*) was the parental strain used to generate mutants. Other strains used in the genetic analyses were constructed by standard techniques (Harris, 1989).

Cells were grown photoautotrophically in minimal HS medium (Harris, 1989) or photoheterotrophically in acetate-containing TAP medium (Harris, 1989). Strain stocks were maintained on TAP agar medium (containing 50 μg/mL L-arginine, if necessary) at 27°C and 10 μmol photons m^{-2} sec^{-1} continuous light (cool-white fluorescent; 40 W). Cells on agar plates were also grown routinely at 50 μmol photons m^{-2} sec^{-1} continuous light (cool-white fluorescent; 40 W). For fluorescence measurements, cells were grown in 50 or 100 mL of HS liquid medium with shaking at 150 rpm in a sterile 250-mL polypropylene beaker covered with a polystyrene Petri dish bottom at 25°C and 100 μmol photons m^{-2} sec^{-1} (15-hr-light and 9-hr-dark cycle) (very high output, cool-white fluorescent). "High light" was provided by a 1000-W metal halide lamp.

Mutagenesis and Genetic Crosses

Insertional mutagenesis of the Arg^- auxotroph CC-425 was performed by transformation with pJD67 (linearized with HindIII) plasmid DNA containing the argininosuccinate lyase gene (*ARG7*; Debuchy et al., 1989), as described by Davies et al. (1994). Arg^+ transformants were selected on TAP agar medium lacking arginine. Colonies of transformants were inoculated individually into 200 μL of TAP medium in 96-well microtiter plates, grown at 50 μmol photons m^{-2} sec^{-1} for 5 to 7 days, spotted onto HS agar plates maintained at 50 μmol photons m^{-2} sec^{-1} and TAP agar plates maintained at 10 μmol photons m^{-2} sec^{-1}, and grown for 7 to 10 days before video imaging (see below). Alternatively, transformants were selected on HS agar lacking arginine and screened directly by video imaging. A random transformant clone (designated wt) was used as a control strain in mutant analyses; this Arg^+ reference strain was indistinguishable from the CC-425 parental strain.

Genetic crosses and tetrad analysis were performed according to established methods (Harris, 1989).

Fluorescence Measurements

Measurements of fluorescence (*F*) parameters were performed with a modified (Brugnoli and Björkman, 1992) pulse-amplitude modulation fluorometer (model PAM-100; Walz, Effeltrich, Germany). Cultures were collected at the end of an overnight dark period and kept shaking in very low light (<5 μmol photons m^{-2} sec^{-1}). Cells (20 μg of chlorophyll *a*) were deposited on a 25-mm in diameter, 12-μm pore size nitrocellulose filter disc by filtration. The filter disc was then placed in the leaf disc chamber of the PAM system and exposed to 3 min of weak illumination with far-red light (1 μmol photons m^{-2} sec^{-1}) and with the PAM measuring beam at 1.6 kHz for determination of F_o (minimum fluorescence in the dark-adapted state). A saturating pulse (1 sec) of white light (4000 μmol photons m^{-2} sec^{-1}) from a halogen lamp (model DZE/FDS, 150 W; General Electric, Cleveland, OH) was applied for determination of F_m (maximum fluorescence in the dark-adapted state) or F_m' (maximum fluorescence in any light-adapted state). Actinic light (800 μmol photons m^{-2} sec^{-1}) was provided via the fiber optic by a halogen lamp (model ENL, 50 W; General Electric) with a heat filter (wide-band hot mirror; OCLI, Santa Rosa, CA). The measuring beam was switched to 100 kHz during saturating pulses and during actinic illumination. Conventional fluorescence nomenclature is used (Van Kooten and Snel, 1990). NPQ was calculated as $(F_m - F_m')/F_m'$.

Pigment Determination

Determination of chlorophyll concentration was performed spectrophotometrically in 90% (v/v) acetone extracts (Jeffrey and Humphrey, 1975). HPLC analysis of carotenoids and chlorophylls was done as described previously (Thayer and Björkman, 1990), with the following modifications. Pigments were extracted with 90% (v/v) acetone, separated on a Spherisorb ODS-1 column (Alltech Associates, Deerfield,

IL) with solvents A-2 and B (Gilmore and Yamamoto, 1991), and eluted using 100% solvent A-2 for 11.5 min followed by 100% solvent B for 6.5 min.

Digital Video Imaging

Screening of mutants was performed by using digital video imaging of chlorophyll fluorescence. The video-imaging system consisted of a high-intensity light source, a high-resolution color video camera, appropriate lenses and optical filters, and a computer for image detection, storage, and analysis. High light was obtained from a Leitz Pradovit 250 W halogen slide projector (Leica Camera, Northvale, NJ), mounted horizontally, together with an additional collimating lens and a Cyan and Detector Trim filter (OCLI). Nearly all of this light was in the spectral range <590 nm. The light beam was deflected onto the sample (a 100-mm in diameter Petri plate) by a first-surface mirror mounted at a 50° angle so that the beam illuminated the horizontal Petri plate from above at an angle of ~85°. The illuminated area was 48 cm^2 with a maximum photon flux density (PFD) of 1200 μmol photons m^{-2} sec^{-1}. This light intensity was high enough to excite fluorescence (F) approximately equal to F_m at the beginning of the illumination or F_m' during the illumination. The high-resolution charge-coupled device (CCD) color video camera (model 8212-1000; COHU, San Diego, CA) was mounted vertically in a straight line from the Petri plate holder, above the horizontal part of the light beam. The camera was modified during the manufacturing process to make it sensitive to far-red light. An image of a 54 × 72 mm area of the Petri plate was focused on the CCD by a 25-mm, F 1.4 TV lens (Computar, Commack, NY). A far-red transmitting filter (RG9; Schott Optical Glass, Duryea, PA) immediately in front of the lens blocked actinic light. The video camera was connected to a 486-40 MHz personal computer with a SNAPplus desktop video adapter and frame grabber (Cardinal Technologies, Lancaster, PA). Fluorescence images were displayed at 30 frames per second on a 20-inch color monitor, and 24-bit digital images could be saved at 30-sec intervals. For routine screening, images were saved within 1 sec of the beginning of actinic illumination (F) and after 1 and 5 min of illumination (F'). Each image was split into red, green, and blue channels by using image editing software (Picture Publisher; Micrografx, Richardson, TX), and the resulting 8-bit red channel images, which contain most of the fluorescence information, were used for calculation of NPQ as $(F - F')/F'$, using image analysis software (Transform; Spyglass, Inc., Savoy, IL).

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