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Arabidopsis Mutants Define a Central Role for the Xanthophyll Cycle in the Regulation of Photosynthetic Energy Conversion

Krishna K. Niyogi,[1] Arthur R. Grossman, and Olle Björkman

Carnegie Institution of Washington, Department of Plant Biology, 260 Panama Street, Stanford, California 94305

A conserved regulatory mechanism protects plants against the potentially damaging effects of excessive light. Nearly all photosynthetic eukaryotes are able to dissipate excess absorbed light energy in a process that involves xanthophyll pigments. To dissect the role of xanthophylls in photoprotective energy dissipation in vivo, we isolated Arabidopsis xanthophyll cycle mutants by screening for altered nonphotochemical quenching of chlorophyll fluorescence. The *npq1* mutants are unable to convert violaxanthin to zeaxanthin in excessive light, whereas the *npq2* mutants accumulate zeaxanthin constitutively. The *npq2* mutants are new alleles of *aba1*, the zeaxanthin epoxidase gene. The high levels of zeaxanthin in *npq2* affected the kinetics of induction and relaxation but not the extent of nonphotochemical quenching. Genetic mapping, DNA sequencing, and complementation of *npq1* demonstrated that this mutation affects the structural gene encoding violaxanthin deepoxidase. The *npq1* mutant exhibited greatly reduced nonphotochemical quenching, demonstrating that violaxanthin deepoxidation is required for the bulk of rapidly reversible nonphotochemical quenching in Arabidopsis. Altered regulation of photosynthetic energy conversion in *npq1* was associated with increased sensitivity to photoinhibition. These results, in conjunction with the analysis of *npq* mutants of Chlamydomonas, suggest that the role of the xanthophyll cycle in nonphotochemical quenching has been conserved, although different photosynthetic eukaryotes rely on the xanthophyll cycle to different extents for the dissipation of excess absorbed light energy.

INTRODUCTION

Plants in nature experience variations in incident light quantity over several orders of magnitude on a daily basis. Although the reactions that convert solar energy into chemical energy are remarkably efficient, the capacity of these reactions is limited; therefore, plants often absorb more light energy than they are able to use for photosynthesis. In addition, many environmental stresses, including drought, extremes of temperature, or nutrient deprivation (Demmig-Adams and Adams, 1992), can further limit the ability of a plant to utilize light energy. Absorption of excessive light energy can lead to sustained depressions in photosynthetic efficiency (photoinhibition), often due to oxidative damage to the photosynthetic apparatus.

Photosynthetic organisms have evolved multiple mechanisms to cope with the absorption of excessive light and its consequences. Interception of incident light can be decreased by reorientation and/or movement of chloroplasts within cells (Brugnoli and Björkman, 1992) and, in some plants, by leaf movements (Björkman and Demmig-Adams, 1994). Carotenoids, including the xanthophylls, have essential photoprotective roles as quenchers of triplet chlorophyll (^{3}Chl) and singlet oxygen ($^1O_2$) and as inhibitors of lipid peroxidation (Cogdell and Frank, 1987; Frank and Cogdell, 1993; Demmig-Adams et al., 1996). Another important lipid-soluble antioxidant is α-tocopherol (vitamin E), which can quench reactive oxygen species in the thylakoid membrane and prevent fatty acid peroxidation (Fryer, 1992). Abundant soluble antioxidants in the chloroplast include ascorbate (vitamin C) and glutathione (Foyer et al., 1994). In addition, some reactive oxygen species can be scavenged by enzymes such as superoxide dismutase (Bowler et al., 1994) and ascorbate peroxidase (Asada, 1994). Photorespiratory oxygen metabolism can maintain linear electron transport and utilization of absorbed light energy, especially under conditions of CO_2 limitation (Osmond, 1981; Heber et al., 1996; Kozaki and Takeba, 1996; Park et al., 1996; Osmond et al., 1997). Nevertheless, damage to critical protein subunits and pigment cofactors of photosystem II (PSII), such as the D1 protein in particular, appears to occur as a consequence of normal reaction center function, resulting in inactivation of entire reaction centers and necessitating protein turnover and subsequent reaction center repair (Aro et al., 1993).

The dissipation of excess absorbed light energy is believed to play a key role in regulating light harvesting and

[1]To whom correspondence should be addressed. Current address: Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720-3102. E-mail niyogi@nature.berkeley.edu; fax 510-642-4995.

electron transport and appears to be critical for the prevention of photooxidative damage to the photosynthetic apparatus. An increase in the proton gradient (ΔpH) across the thylakoid membrane in excessive light triggers the harmless dissipation of excess energy as heat in the light-harvesting complexes (LHCs) associated with PSII (reviewed in Demmig-Adams and Adams, 1992; Björkman and Demmig-Adams, 1994; Horton et al., 1994, 1996; Gilmore, 1997). This process, which is measured (and referred to) as nonphotochemical quenching (NPQ) of chlorophyll fluorescence, occurs in almost all photosynthetic eukaryotes. Although pH-dependent energy dissipation (also called qE) is the major component of NPQ under most conditions, decreases in PSII fluorescence due to state transitions (qT) and photoinhibition (qI) may also contribute to what is measured as NPQ (Krause and Weis, 1991). The pH-dependent part of NPQ can usually be distinguished based on the kinetics of its relaxation, which occurs rapidly in darkness because of loss of the light-induced ΔpH.

The majority of NPQ is thought to occur in the PSII antenna pigment bed (Demmig-Adams and Adams, 1992; Horton et al., 1994). A key role of the ΔpH may involve protonation of acidic amino acids on the chlorophyll *a*/*b* binding polypeptides of the PSII LHCs (reviewed in Crofts and Yerkes, 1994; Horton and Ruban, 1994; Gilmore, 1997), resulting in a conformational change that is required for NPQ. This hypothesis is supported by experiments showing that dicyclohexylcarbodiimide, an inhibitor of NPQ in vitro, binds to glutamate side chains of specific LHC polypeptides (Jahns and Junge, 1990; Walters et al., 1994, 1996; Pesaresi et al., 1997). In addition, evidence correlating the association of a conformational change with NPQ has come from measurements of a light-induced spectral absorbance change at 535 nm in intact leaves (Bilger et al., 1989; Bilger and Björkman, 1990, 1994; Ruban et al., 1993). Recently, NPQ in isolated thylakoids has been associated with the formation of a complex with a discrete, decreased lifetime of chlorophyll fluorescence (Gilmore et al., 1995, 1996).

Xanthophyll pigments in the LHCs also appear to have a critical role in NPQ. The extent of NPQ in plants is strongly correlated with the levels of zeaxanthin and antheraxanthin that are formed from violaxanthin via the xanthophyll cycle (see Figure 1) (Demmig-Adams, 1990; Pfündel and Bilger, 1994; Gilmore et al., 1995, 1996; Demmig-Adams and Adams, 1996a, 1996b; Eskling et al., 1997). In low or limiting light, the enzyme zeaxanthin epoxidase converts zeaxanthin to violaxanthin via the intermediate antheraxanthin. In excessive light, when the $[H^+]$ in the thylakoid lumen reaches a critical threshold, the enzyme violaxanthin deepoxidase is activated and converts violaxanthin back to antheraxanthin and then zeaxanthin. Experiments using DTT as an inhibitor of violaxanthin deepoxidase (Yamamoto and Kamite, 1972) in isolated thylakoids and detached leaves have shown that most, and in some cases all, NPQ depends on formation of zeaxanthin and antheraxanthin by the xanthophyll cycle (Bilger and Björkman, 1990; Demmig-Adams et al., 1990; Gilmore and Yamamoto, 1993). However, other studies with isolated thylakoids have suggested that zeaxanthin acts to amplify NPQ within a certain ΔpH range but that the ΔpH is all that is absolutely required for NPQ (Noctor et al., 1991).

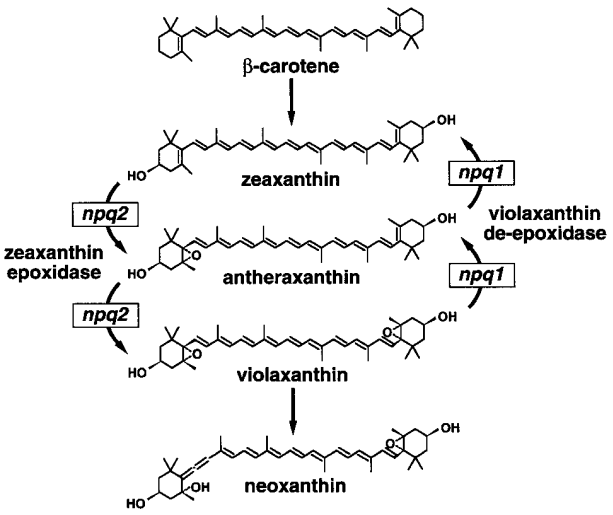

Figure 1. The Xanthophyll Cycle in the β-Carotene Branch of the Carotenoid Biosynthetic Pathway in Plants.

The reactions impaired in the *npq1* and *npq2* mutants are indicated.

The isolation and characterization of mutants of the unicellular green alga Chlamydomonas that are blocked in the synthesis of specific xanthophylls have confirmed a role for the xanthophyll cycle in NPQ and also suggested the involvement of other xanthophylls (Niyogi et al., 1997a, 1997b). The Chlamydomonas *npq1* mutant, which is unable to deepoxidate violaxanthin to zeaxanthin (see Figure 1), is only partially defective in NPQ, suggesting that not all NPQ in Chlamydomonas depends on operation of the xanthophyll cycle. Characterization of the *lor1* mutant (Eichenberger et al., 1986; Chunaev et al., 1991), which lacks xanthophylls derived from α-carotene, revealed a possible role for lutein, which is the most abundant xanthophyll in the thylakoid, in NPQ (Niyogi et al., 1997b). Furthermore, an *npq1 lor1* double mutant lacked almost all NPQ and was very susceptible to photooxidative bleaching in high light (Niyogi et al., 1997b), providing in vivo evidence for the importance of NPQ for photoprotection. This conclusion, however, was complicated by the roles of xanthophylls in several photoprotective processes, including quenching of ^{3}Chl and $^1O_2$ and inhibition of lipid peroxidation.

To what extent are the conclusions drawn from the studies of Chlamydomonas xanthophyll cycle mutants applicable to vascular plants? To address this question, we isolated Arabidopsis mutants that are defective in the xanthophyll cycle. Detailed physiological and molecular genetic analyses demonstrated that although the general features of NPQ are

similar in green algae and plants, the xanthophyll cycle plays a more dominant role in NPQ in Arabidopsis.

RESULTS

Isolation of Arabidopsis Xanthophyll Cycle Mutants

Arabidopsis mutants that exhibited aberrant NPQ when exposed to excessive light were identified using a chlorophyll fluorescence video-imaging system (see Methods), which was used to isolate similar mutants of the unicellular green alga Chlamydomonas (Niyogi et al., 1997a). Approximately 30,000 Arabidopsis M_2 seeds were sown on agar medium, grown photoautotrophically for 12 days at a photon flux density (PFD) of 100 μmol photons m^{-2} sec^{-1}, and screened for an alteration in the extent of rapidly reversible NPQ during exposure to 500 μmol photons m^{-2} sec^{-1} actinic light. Images of maximum chlorophyll fluorescence were captured and digitized after 4 min in high light and then again after a subsequent recovery period of 1 min in the dark. False-color images depicting reversible NPQ were generated from the fluorescence data, allowing identification of *npq* mutants as shown in Figure 2A. Thirteen independent *npq* mutants with defects in the dissipation of excess absorbed light energy were isolated for further characterization.

Pigment analysis of the *npq* mutants before and after treatment with high light uncovered four mutants with blocks in the xanthophyll cycle. Table 1 shows the results of the pigment analysis for two of the mutants, which were selected for further physiological characterization. The *npq1-1* (not shown) and *npq1-2* (Table 1) mutants did not convert violaxanthin to antheraxanthin and zeaxanthin during exposure to high light, suggesting a defect in violaxanthin deepoxidase activity. In contrast, the *npq2-1* (Table 1) and *npq2-2* (data not shown) mutants accumulated high levels of zeaxanthin and contained no detectable antheraxanthin, violaxanthin, or neoxanthin; this phenotype is consistent with a block in the zeaxanthin epoxidase reaction. The previously identified zeaxanthin epoxidase *aba1* mutants of Arabidopsis (Koornneef et al., 1982) exhibit the same xanthophyll profile as *npq2* (Duckham et al., 1991; Rock and Zeevaart, 1991), suggesting that the *npq2* mutants represent new alleles of *aba1.* Furthermore, in the video-imaging assay, the available *aba1* mutants exhibited the same phenotype as the *npq2* mutants (data not shown). The metabolic defects in the *npq1* and *npq2* mutants are indicated in Figure 1.

Absorbance and Fluorescence Characteristics of Xanthophyll Cycle Mutants

The xanthophyll cycle defects in *npq1* and *npq2* mutants were corroborated by measurements of leaf absorbance before, during, and after exposure to high light (1900 μmol

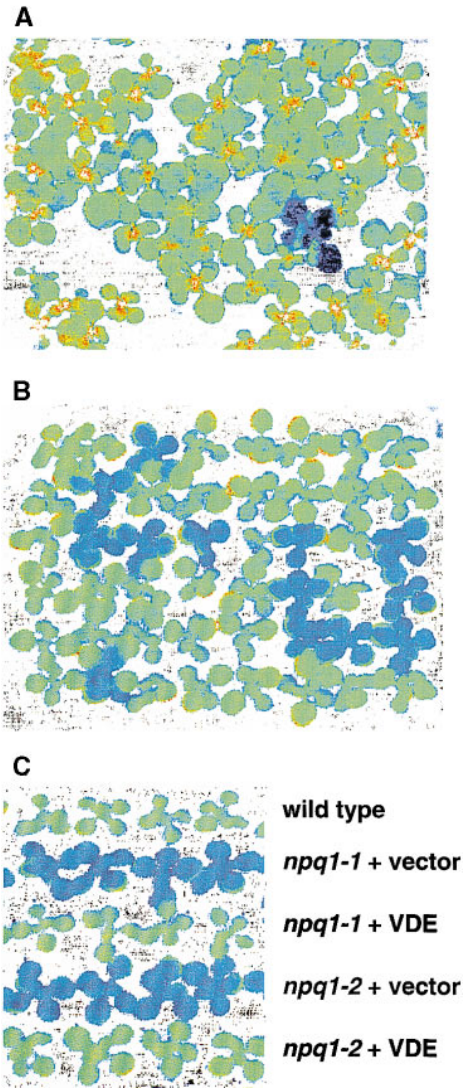

Figure 2. Video Imaging of Chlorophyll Fluorescence Quenching in *npq* Mutants.

(A) Screening for *npq* mutants defective in rapidly reversible NPQ. M_2 plants on agar medium were exposed to 500 μmol photons m^{-2} sec^{-1} for 4 min followed by 1 min in darkness. In this false-color image of reversible NPQ, an *npq* mutant appears dark blue, whereas other (nonmutant) M_2 plants appear green.

(B) NPQ image of *npq1-2* F_2 progeny (*npq1-2*/*npq1-2* × *NPQ1*/*NPQ1*). Gross NPQ occurring after 4 min in 500 μmol photons m^{-2} sec^{-1} is depicted.

(C) Complementation of *npq1* with the violaxanthin deepoxidase gene. Gross NPQ occurring after 4 min in 500 μmol photons m^{-2} sec^{-1} is shown for wild-type and transgenic *npq1* mutant plants transformed with either the pBIN19 vector only (+ vector) or the pVC1 plasmid containing the wild-type violaxanthin deepoxidase gene (+ VDE).

Table 1. HPLC Analysis of Pigment Composition of the Wild Type and Xanthophyll Cycle Mutants before and after Exposure to High Light[a]

Pigment[b] (mmol/mol Chl *a*)	Wild Type		*npq1-2*		*npq2-1*	
	Before HL[c]	After HL[c]	Before HL	After HL	Before HL	After HL
Neoxanthin	46.6	49.3	50.5	44.2	0.3	0.3
Violaxanthin	46.0	17.4	70.3	62.2	1.2	0.3
Antheraxanthin	1.5	4.0	1.0	1.3	1.0	0.0
Zeaxanthin	1.9	27.2	0.8	0.8	130.7	145.6
β-Carotene	110.5	105.6	131.3	116.9	123.2	129.6
Lutein	246.3	266.0	241.8	224.5	197.7	221.5
Chl *b*	373.7	376.2	370.6	360.8	360.7	320.3
V + A + Z	49.4	48.6	72.2	64.3	133.0	145.8
(A + Z)/(V + A + Z)	0.068	0.642	0.025	0.033	0.991	0.998
Chl *a* (mmol m^{-2})	257.6	226.8	242.1	273.5	248.7	263.0

[a] Six leaves of each genotype were detached, floated on water, and exposed to light from a xenon lamp.
[b] Pigments are expressed relative to chlorophyll *a* (mmol/mol Chl *a*), except for chlorophyll *a*, which is based on leaf area. Values are from a single representative experiment. A, antheraxanthin; V, violaxanthin; Z, zeaxanthin.
[c] Pigment composition of leaves was determined either before or after exposure to high light (HL, 2000 μmol photons m^{-2} sec^{-1}) for 30 min.

photons m^{-2} sec^{-1}). A light-induced absorbance change at 505 nm is associated with xanthophyll cycle activity and is due to differences in absorbance between violaxanthin and zeaxanthin (Yamamoto and Kamite, 1972). Figure 3 shows that both *npq1* and *npq2* mutants failed to exhibit a significant ΔA_{505} in difference spectra (high light minus dark), whereas the wild-type strain exhibited a very prominent ΔA_{505} band. The absorbance change at 535 nm, which is attributed to a conformational change within the thylakoid membrane that occurs upon protonation of the LHCs in the presence of zeaxanthin (Bilger et al., 1989; Bilger and Björkman, 1990, 1994; Ruban et al., 1993), was observed in wild-type and *npq2* leaves (Figure 3). However, the ΔA_{535} was undetectable in the *npq1* mutant, which lacks zeaxanthin, confirming that zeaxanthin is necessary for this absorbance change.

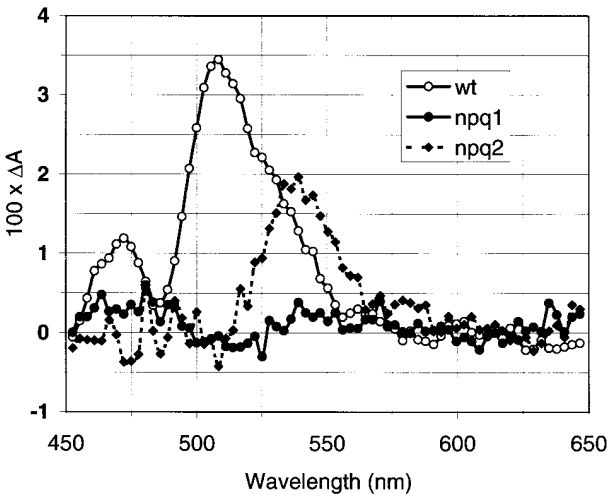

Figure 3. Light-Induced Spectral Absorbance Changes in Leaves of the Wild Type, *npq1*, and *npq2*.

Curves show the changes caused by a 3-min exposure to a PFD of 1900 μmol photons m^{-2} sec^{-1}. Each point represents the mean of two or three determinations. wt, wild type.

The induction kinetics and extent of NPQ in the xanthophyll cycle mutants were examined by measurements of modulated fluorescence that were performed simultaneously with the absorbance change measurements. As shown in Figure 4, induction of NPQ in wild-type Arabidopsis leaves occurred during exposure to high light, reaching a maximum extent within 3 min. The ΔA_{505} band due to zeaxanthin formation appeared at the same time as the NPQ induction in the wild type (data not shown). Upon subsequent darkening, the NPQ in wild-type leaves relaxed rapidly because of the loss of the light-induced ΔpH across the thylakoid membrane. In contrast, the *npq1* mutant exhibited very little NPQ in high light. Most of the NPQ in *npq1* developed within the first minute of illumination, and this NPQ was only partially reversible within 5 min in the dark. Induction of NPQ in the *npq2* mutant occurred more rapidly than it did in the wild type. A similar, rapid induction of NPQ was observed for the Arabidopsis *aba1* mutant (Tardy and Havaux, 1996; Hurry et al., 1997). In addition, the NPQ of *npq2* was more slowly reversible after darkening of the leaf. The slower recovery in *npq2* explains why these mutants were recovered in the video-imaging screen, which targeted mutants impaired in rapidly reversible NPQ.

To examine more closely the early induction of NPQ in wild-type, *npq1*, and *npq2* leaves, saturating pulses were applied at shorter time intervals after a transition from darkness to moderately high light (1087 μmol photons m^{-2} sec^{-1}). Figure 5 shows that the NPQ induction in wild-type leaves was biphasic at this PFD, with a rapid initial increase

in NPQ followed by a slight lag and then a secondary increase. The *npq1* mutant retained the rapid initial increase in NPQ (during the first 10 sec of illumination), but further increases occurred more slowly. These results suggest that induction of NPQ in the wild type involves a rapid phase that is independent of xanthophyll cycle operation followed by a xanthophyll cycle–dependent phase. The induction kinetics of NPQ in the *npq2* mutant showed only a single, rapid phase, resulting overall in much faster NPQ induction than was observed in the wild type. In the presence of constitutively high levels of zeaxanthin, NPQ in the *npq2* mutant is presumably driven solely by induction of the ΔpH.

Light–response curves for chlorophyll fluorescence parameters revealed altered regulation of photosynthesis in xanthophyll cycle mutants. Figure 6 shows steady state values of gross NPQ, the estimated reduction state of the first stable PSII electron acceptor Q_A, the efficiency of PSII photochemistry, and the relative rate of PSII photochemistry for an increasing series of PFDs. At elevated PFDs, the *npq1* mutant exhibited lower steady state NPQ than did the wild type (Figure 6A). Although the final extent of NPQ at the highest PFD did reach a value of 1.1 in the *npq1* mutant, approximately half of this NPQ was irreversible within 5 min (data not shown), indicating that it was not due to pH-dependent energy dissipation in the LHCs. Consistent with an impaired ability to dissipate excess absorbed light energy, the *npq1* mutant exhibited a higher reduction state of Q_A at each PFD (Figure 6B). In contrast, the *npq2* mutant had slightly elevated levels of NPQ (Figure 6A) and a lower reduction state of Q_A (Figure 6B), especially at moderate PFDs. However, despite the observed effects on Q_A reduction state, the efficiency of PSII photochemistry (Figure 6C) at each PFD and thus the relative rates of PSII photochemistry (Figure 6D) were only slightly affected in *npq1* compared with the wild type and *npq2.*

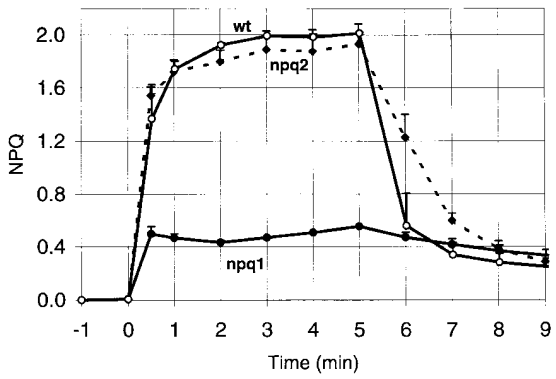

Figure 4. Time Courses for Induction and Relaxation of NPQ in Leaves of the Wild Type, *npq1*, and *npq2.*

Measurements of gross NPQ were made simultaneously with the absorbance changes shown in Figure 3. Actinic light (1900 μmol photons m^{-2} sec^{-1}) was switched on at time zero, and then leaves were left in the dark after 5 min. Each point represents the mean of two or three determinations. For points lacking error bars, the error was smaller than the symbol size. wt, wild type.

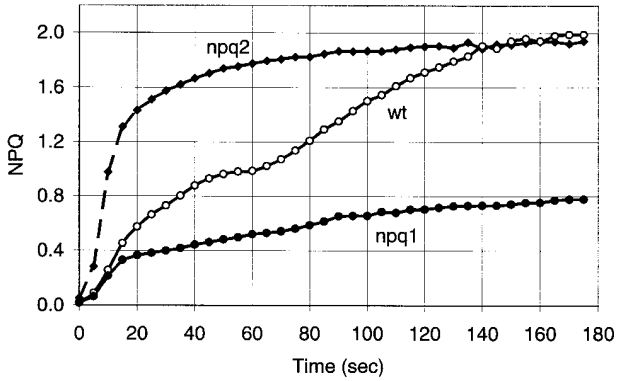

Figure 5. Rapid Time Course of NPQ Induction in Leaves of the Wild Type, *npq1*, and *npq2.*

Leaves were exposed to a PFD of 1083 μmol photons m^{-2} sec^{-1} at time zero, and gross NPQ was measured at 5-sec intervals. Each point represents the mean of two or three determinations. wt, wild type.

Sensitivity of *npq1* to Excessive Light

To begin to assess the effects of impaired violaxanthin deepoxidation on growth of Arabidopsis in excessive light, we maintained wild-type and *npq1* plants together in natural sunlight after germination under controlled conditions (see Methods for details). No obvious differences in the coloration, size, or fertility of well-watered wild-type and *npq1* plants were observed (data not shown).

However, leaves of the *npq1* mutant experienced greater photoinhibition than did wild-type leaves when exposed to full sunlight under conditions that would limit photosynthetic gas exchange. Mature plants acclimated to 450 μmol photons m^{-2} sec^{-1} in a growth chamber were moved outdoors and gradually exposed to natural sunlight during a 2-day period. Compared with plants grown at 230 μmol photons m^{-2} sec^{-1} (Table 1), leaves of these wild-type and *npq1* plants had a xanthophyll cycle pool size that was approximately two times higher (data not shown). Detached leaves were floated on water and exposed to full sunlight (2000 μmol photons m^{-2} sec^{-1}) for 70 min. The xanthophyll cycle pool was mostly deepoxidated (84% zeaxanthin plus antheraxanthin) in wild-type leaves, whereas only 3% of the xanthophyll cycle pool in *npq1* leaves was present as zeaxanthin plus antheraxanthin. Table 2 shows that after exposure to full sunlight, the PSII efficiency at this PFD was 37% lower in *npq1* leaves than in wild-type leaves. Furthermore, during recovery at very low PFD, *npq1* leaves exhibited sustained depressions in PSII efficiency and maximum fluorescence compared with the wild type.

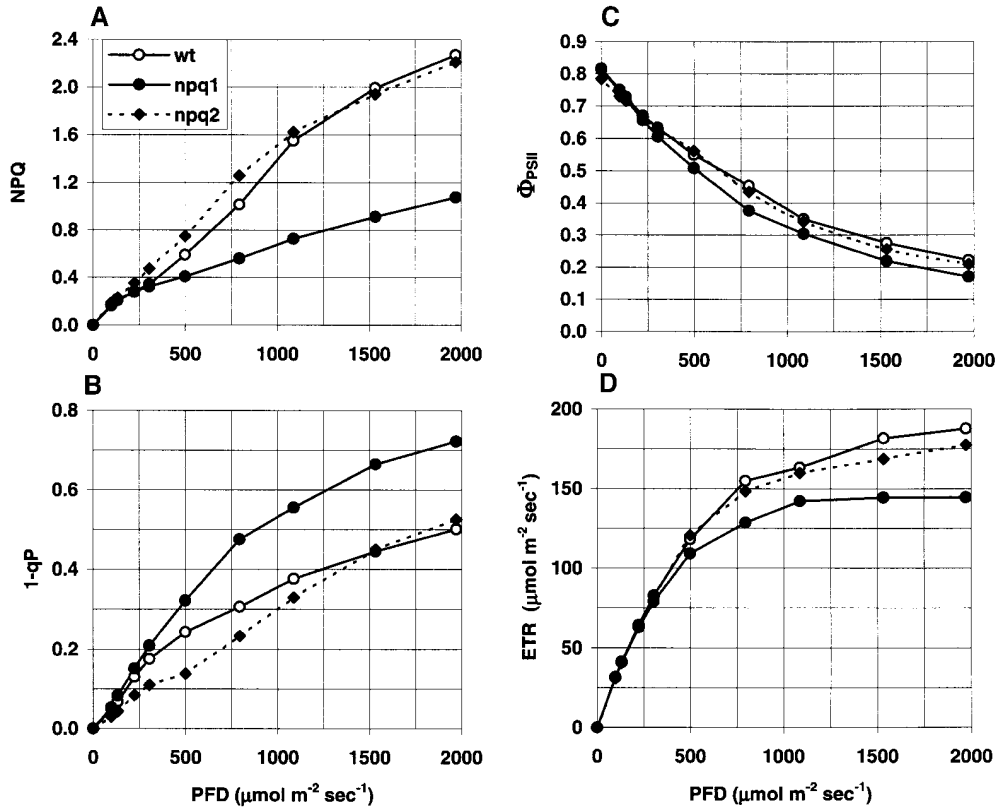

Figure 6. Light Dependence of Steady State Chlorophyll Fluorescence Parameters in Leaves of the Wild Type, *npq1*, and *npq2.*

Each point represents the mean of two or three determinations. wt, wild type.

(A) Gross NPQ. The standard error for each point is <10% of the mean.

(B) Estimated reduction state of Q_A ($1 - qP$). The fraction of Q_A in the reduced state was estimated as $(F_s - F_o')/(F_m' - F_o')$. The standard error for each point is <15% of the mean.

(C) Efficiency of PSII photochemistry (Φ_{PSII}). PSII efficiency was calculated as $(F_m - F_o)/F_m$ in the absence of actinic light and as $(F_m' - F_s)/F_m'$ in the light. The standard error for each point is <10% of the mean.

(D) Relative PSII electron transport rate (ETR). The rate of electron transport through PSII was calculated as $\Phi_{PSII} \times PFD \times \alpha \times 0.5$, where α is the fraction of incident light absorbed by the leaf. In all leaves, α was in the range 0.84 to 0.86. The standard error for each point is <10% of the mean.

Genetic Characterization of *npq1* and *npq2*

The *npq1* and *npq2* mutants were backcrossed to the wild type (ecotype Columbia [Col-0]) to determine the genetic basis for the mutant phenotypes. The data in Table 3 show that in the F_1 generation, all progeny exhibited wild-type levels of NPQ (Npq^+). Furthermore, the *npq1*/*NPQ1* heterozygous plants were able to deepoxidate violaxanthin to zeaxanthin, and the *npq2*/*NPQ2* heterozygotes had normal levels of all xanthophylls (data not shown). In the subsequent F_2 generation, the Npq^- phenotype appeared in ~25% of the progeny (Table 3 and Figure 2B), demonstrating that both *npq1* and *npq2* are single, recessive nuclear mutations.

Crosses between *npq1-1* and *npq1-2* yielded only Npq^- progeny (Table 4). The lack of complementation suggests that *npq1-1* and *npq1-2* are independent mutations in the same gene. Similarly, a complementation test between *npq2-1* and *aba1-3* confirmed that *npq2-1* is a new allele of the gene that was defined previously by the *aba1* mutations (Koornneef et al., 1982; Table 4).

The *npq1* Mutants Are Defective in the Gene Encoding Violaxanthin Deepoxidase

The genetic map position of *npq1* was determined by testing the segregation of simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994) among F_2 progeny of a cross between *npq1-1* and a polymorphic wild-type Arabidopsis strain (ecotype Landsberg *erecta* [L*er*-0]). Tight linkage was observed between *npq1* and the nga63 marker on chromosome 1 (Figure 7A). Also located

on chromosome 1 is the violaxanthin deepoxidase gene (Bugos and Yamamoto, 1996), which is immediately adjacent to the *KNOLLE* gene (Lukowitz et al., 1996) (GenBank accession number U39452). The available physical mapping data for chromosome 1 (http://cbil.humgen.upenn.edu/~atgc/physical-mapping/physmaps.html) revealed that nga63 and the violaxanthin deepoxidase gene are closely linked (they are found on a single yeast artificial chromosome clone). Therefore, these results placed the *npq1-1* mutation close to the Arabidopsis violaxanthin deepoxidase gene and suggested that *npq1-1* may contain a mutation in the violaxanthin deepoxidase gene.

Genomic DNA containing the violaxanthin deepoxidase gene was amplified from the wild type and *npq1-1* by polymerase chain reaction (PCR). Direct sequencing of the PCR products from independent reactions revealed a single base pair difference between the wild type and *npq1-1.* Figure 7B shows that the G-to-A transition mutation is predicted to change a conserved cysteine to a tyrosine in the violaxanthin deepoxidase protein sequence. Repeated attempts to amplify the violaxanthin deepoxidase gene from *npq1-2* were unsuccessful (data not shown), suggesting that the fast neutron–induced *npq1-2* allele may involve a deletion or rearrangement of the violaxanthin deepoxidase gene.

The *npq1-1* and *npq1-2* mutations were complemented by a wild-type copy of the violaxanthin deepoxidase gene (Figure 2C). Wild-type genomic DNA containing the violaxanthin deepoxidase gene was subcloned between T-DNA borders and introduced into *npq1-1* and *npq1-2* plants by Agrobacterium-mediated transformation. Whereas control transgenic plants containing only the pBIN19 vector still showed the Npq$^-$ mutant phenotype, *npq1* plants that were transformed with the wild-type violaxanthin deepoxidase gene exhibited the wild-type, Npq$^+$ phenotype (Figure 2C).

DISCUSSION

Mutations in the Arabidopsis Genes Encoding Xanthophyll Cycle Enzymes

To isolate plants defective in NPQ and the xanthophyll cycle, we used a digital video-imaging technique that was used previously to isolate Chlamydomonas mutants (Niyogi et al., 1997a). By screening mutagenized plants growing photoautotrophically in Petri plates, ~100 Arabidopsis plants could be analyzed simultaneously, and *npq* mutants were recovered at a frequency of approximately one in 2000. This frequency is considerably lower than that obtained from the screening of Chlamydomonas mutants, suggesting that the Arabidopsis growth conditions may have been less permissive for partial loss-of-function mutations affecting photosynthetic electron transport. Perhaps as a result of this increased stringency, a relatively large fraction (four of 13) of the Arabidopsis *npq* mutants exhibited defects in the xanthophyll cycle.

The Arabidopsis *npq1* and *npq2* mutations affect the structural genes encoding the enzymes of the xanthophyll cycle. Several lines of evidence demonstrated that the *npq1* mutants are defective in the Arabidopsis violaxanthin deepoxidase gene (Bugos and Yamamoto, 1996). The *npq1-1*

Table 2. Sensitivity of *npq1* and Wild-Type Leaves to Photoinhibition[a]

		Φ_{PSII}[b]			F_m'		
Time	No.	*npq1-2*	Wild Type	*npq1-2*/ Wild Type	*npq1-2*	Wild Type	*npq1-2*/ Wild Type
Morning	6	0.812 ± 0.003	0.801 ± 0.002	1.01	831 ± 8	802 ± 1	1.04
0 min	9	0.123 ± 0.002	0.194 ± 0.008	0.63	243 ± 8	162 ± 9	1.50
14 min	6	0.517 ± 0.019	0.639 ± 0.014	0.81	323 ± 8	366 ± 15	0.88
34 min	6	0.641 ± 0.015	0.752 ± 0.006	0.85	482 ± 15	593 ± 6	0.81
57 min	6	0.657 ± 0.012	0.754 ± 0.007	0.87	532 ± 14	633 ± 10	0.84
68 min	6	0.672 ± 0.014	0.757 ± 0.007	0.89	551 ± 14	663 ± 9	0.83
85 min	6	0.680 ± 0.013	0.765 ± 0.006	0.89	570 ± 16	675 ± 5	0.84
115 min	6	0.702 ± 0.009	0.764 ± 0.005	0.92	589 ± 10	678 ± 12	0.87
174 min	6	0.724 ± 0.006	0.771 ± 0.005	0.94	626 ± 13	696 ± 19	0.90
237 min	6	0.730 ± 0.006	0.772 ± 0.005	0.95	639 ± 13	714 ± 19	0.90
297 min	6	0.737 ± 0.007	0.776 ± 0.005	0.95	654 ± 16	717 ± 7	0.91
Next morning[c]	6	0.798 ± 0.005	0.824 ± 0.001	0.97	759 ± 6	759 ± 10	1.00

[a] Leaves were detached, floated on water, and exposed to natural sunlight (2000 μmol photons m^{-2} sec^{-1}) for 70 min until time zero. The recovery of treated leaves was then followed for 5 hr at a PFD of 1 μmol photons m^{-2} sec^{-1}. Fluorescence parameters were measured as described in Methods and expressed as means ±SE.

[b] The efficiency of PSII photochemistry (Φ_{PSII}) was calculated as $(F_m' - F_s)/F_m'$. All measurements of Φ_{PSII} were made at 1 μmol photons m^{-2} sec^{-1} except for the time zero measurement, which was made at 2000 μmol photons m^{-2} sec^{-1}.

[c] Measurements were also made the following morning after 20 hr of recovery.

Table 3. Results of Crosses between Xanthophyll Cycle Mutants and the Wild Type[a]

Cross	Type	Total	Npq$^+$	Npq$^-$	χ^2
NPQ1/*NPQ1* × *npq1-1*/*npq1-1*	F_1	2	2	0	
	F_2	121	92	29	0.069[b]
npq1-2/*npq1-2* × *NPQ1*/*NPQ1*	F_1	11	11	0	
	F_2	48	33	13	0.33[b]
npq2-1/*npq2-1* × *NPQ2*/*NPQ2*	F_1	28	28	0	
	F_2	48	33	15	1.0[c]

[a] Pollen from the male parent (listed first) was crossed onto stigmas of the female parent to generate F_1 seeds. The heterozygous F_1 plants were allowed to self-pollinate to generate F_2 seeds. The phenotypes of F_1 and F_2 plants were scored by fluorescence video imaging after 12 days of growth on agar medium.

[b] The 3:1 segregation of the Npq phenotype in the F_2 generation is consistent with the hypothesis that *npq1-1* and *npq1-2* are single recessive nuclear mutations ($P > 0.5$).

[c] The 3:1 segregation of the Npq phenotype in the F_2 generation is consistent with the hypothesis that *npq2-1* is a single recessive nuclear mutation ($P > 0.1$).

mutation mapped to the same chromosomal location as the cloned violaxanthin deepoxidase gene, and DNA sequencing revealed a single base pair substitution in the gene from *npq1-1* plants (Figure 7). In addition, transformation of *npq1-1* and *npq1-2* with the violaxanthin deepoxidase gene resulted in rescue of the mutant phenotypes (Figure 2C). The *npq2-1* mutation failed to complement the *aba1-3* mutation (Table 4), which defines the zeaxanthin epoxidase gene of Arabidopsis (Marin et al., 1996), suggesting that *npq2-1* and *npq2*-2 are new mutations affecting the zeaxanthin epoxidase gene. The *aba1* mutants were originally identified as abscisic acid–deficient mutants (Koornneef et al., 1982), and consistent with this finding is the observation that the *npq2-2* mutant exhibited a dramatic "wilty" phenotype (data not shown). However, the *npq2-1* mutant that we used for physiological and genetic analyses was not particularly susceptible to wilting, presumably because it synthesizes sufficient abscisic acid. It is likely that *npq2-1* is not a complete loss-of-function mutation affecting zeaxanthin epoxidase activity, as evidenced by the very low but detectable levels of antheraxanthin, violaxanthin, and neoxanthin in *npq2-1* (Table 1).

Synthesis of Zeaxanthin by the Xanthophyll Cycle Is Required for the Bulk of pH-Dependent NPQ in Arabidopsis

Induction of NPQ in excessive light was severely impaired in the *npq1* mutant (Figures 4 and 5), demonstrating that deepoxidation of violaxanthin to antheraxanthin and zeaxanthin is necessary for most of the rapidly reversible NPQ in Arabidopsis. This finding is consistent with the results of experiments in which DTT was effective as an inhibitor of NPQ in detached leaves of many but not all plants (Bilger and Björkman, 1990; Demmig-Adams et al., 1990; Johnson et al., 1994). In Arabidopsis, providing 2 mM DTT to wild-type leaves via the petiole resulted in an exact phenocopy of the *npq1* mutation, but treatment with DTT had no effect on *npq1* leaves (data not shown). These results confirm that DTT affects NPQ primarily by inhibition of violaxanthin deepoxidase activity (Bilger and Björkman, 1990).

However, the *npq1* mutant retained a small but significant amount of reversible NPQ that was induced very rapidly after the transition from darkness to excessive light (Figures 4 and 5), suggesting that a component of reversible NPQ in Arabidopsis is independent of the xanthophyll cycle. The residual NPQ could be due to the very low but persistent levels of antheraxanthin and zeaxanthin (Table 1) that accumulate in the *npq1* mutant as intermediates in the biosynthesis of violaxanthin (see Figure 1). Recent modeling of pH- and xanthophyll-dependent NPQ based on chlorophyll fluorescence lifetime measurements has suggested that even small amounts of antheraxanthin and zeaxanthin can lead to significant NPQ (Gilmore, 1997). Alternatively, the reversible NPQ in the *npq1* mutant may involve the xanthophyll lutein, which is derived from α-carotene. A role for lutein in NPQ was suggested by characterization of Chlamydomonas xanthophyll mutants (Niyogi et al., 1997b). The issue of whether lutein or antheraxanthin is responsible for the residual NPQ in *npq1* can be addressed by using existing lutein-deficient Arabidopsis mutants (Pogson et al., 1996). A third possibility is that the remaining NPQ in *npq1* mutants is pH dependent but xanthophyll independent (Noctor et al., 1993; Gilmore et al., 1995, 1996).

The *npq1* mutants of Arabidopsis and Chlamydomonas have provided unequivocal molecular genetic evidence that violaxanthin deepoxidase activity is involved in NPQ, but it is apparent that the extent to which different organisms rely on the xanthophyll cycle can be very different (Johnson et al., 1994; Casper-Lindley and Björkman, 1998). When transferred from darkness to high light, reversible NPQ was impaired in the Chlamydomonas *npq1* mutant by only 25% (Niyogi et al., 1997b). In contrast, 85% of the reversible NPQ was inhibited in the Arabidopsis *npq1* mutant (Figure 4), suggesting that there is a greater relative contribution of xanthophyll cycle–dependent NPQ in Arabidopsis compared with Chlamydomonas. It is possible that the ΔpH has a

Table 4. Results of Complementation Tests[a]

Cross	Type	Total	Npq$^+$	Npq$^-$
npq1-2/*npq1-2* × *npq1-1*/*npq1-1*	F_1	8	0	8
aba1-3/*aba1-3* × *npq2-1*/*npq2-1*	F_1	21	0	21

[a] Pollen from the male parent (listed first) was crossed onto stigmas of the female parent to generate F_1 seeds. The phenotypes of F_1 plants were scored by fluorescence video imaging after 12 days of growth on agar medium.

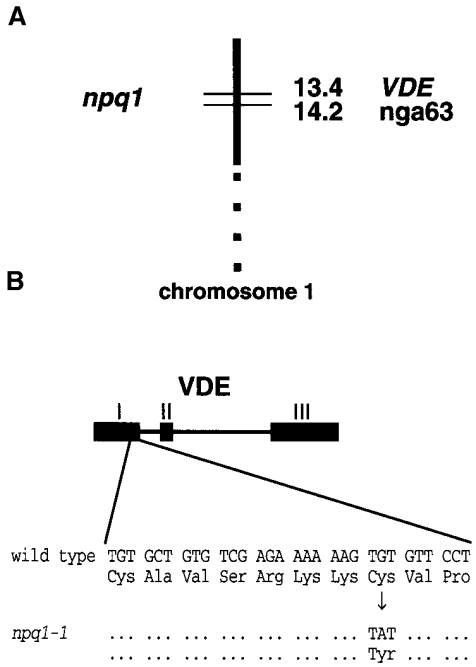

Figure 7. The *npq1* Mutations Are in the Violaxanthin Deepoxidase Gene.

(A) Genetic map positions of *npq1* and the violaxanthin deepoxidase gene (*VDE*) relative to the nga63 SSLP marker on chromosome 1.
(B) Sequence of the *npq1-1* allele of the violaxanthin deepoxidase gene. A schematic diagram of the mature violaxanthin deepoxidase protein (VDE) shows three domains: a cysteine-rich domain (I), a lipocalin signature motif (II), and a highly charged domain (III) (Bugos and Yamamoto, 1996). The *npq1-1* mutant has a single base pair substitution that changes the last cysteine (position 72) of the cysteine-rich domain to a tyrosine. This cysteine is invariant in the deduced wild-type violaxanthin deepoxidase protein sequences from lettuce (GenBank accession number U31462), tobacco (GenBank accession number U34817), and Arabidopsis (GenBank accession number U44133). Dots in the *npql-1* sequence indicate identity to the wild-type sequence.

greater role in regulating NPQ in some photosynthetic eukaryotes such as Chlamydomonas and other green algae, whereas the level of NPQ is controlled to a greater extent by xanthophyll cycling in other organisms such as Arabidopsis and the diatom Phaeodactylum (Casper-Lindley and Björkman, 1998). Nevertheless, the general features of NPQ appear to be similar in Chlamydomonas and Arabidopsis: synthesis of zeaxanthin by the xanthophyll cycle is clearly required for a component of NPQ but not for all NPQ.

Why Have a Xanthophyll Cycle?

Almost all photosynthetic eukaryotes have a xanthophyll cycle (reviewed in Pfündel and Bilger, 1994; Eskling et al., 1997). The cycle involving violaxanthin, antheraxanthin, and zeaxanthin is found in green plants, green algae, and many brown algae, whereas a deepoxidation cycle involving diadinoxanthin and diatoxanthin occurs in all other eukaryotic algae except for rhodophytes and cryptophytes. All organisms that possess a xanthophyll cycle appear to have a chlorophyll-based, LHC type of major peripheral antenna associated with PSII, whereas those that lack a xanthophyll cycle have a PSII antenna composed largely of phycobiliproteins (Grossman et al., 1995; Green and Durnford, 1996). This observation is consistent with the hypothesis that the xanthophyll cycle has an important role in the regulation of photosynthetic energy conversion and the prevention of $^1O_2$ formation. Furthermore, xanthophyll deepoxidation must have evolved relatively early in the history of photosynthetic eukaryotes, at a time before the divergence of the green algae from the other algae. It is also possible that xanthophyll deepoxidation arose independently in different lineages.

Leaves of the Arabidopsis *npq1* mutant showed a greater sensitivity than did the wild type to a short-term photoinhibitory light treatment during which gas exchange was restricted (Table 2), although growth of *npq1* plants in natural sunlight did not appear to be inhibited (data not shown). These initial results suggest that the xanthophyll cycle may be especially important under conditions of high light combined with additional environmental stresses.

Characterization of the *aba1* mutant, which is allelic to the *npq2* mutants, failed to reveal any increased susceptibility to photoinhibition (Hurry et al., 1997), showing that accumulation of zeaxanthin has no deleterious effect in terms of photoprotection. Given that an elevated ΔpH (in addition to zeaxanthin) is required for most of the NPQ in Arabidopsis, what advantage does the xanthophyll cycle provide over constitutive accumulation of zeaxanthin?

One possible disadvantage of having high levels of zeaxanthin present continuously in *npq2* plants is that more energy dissipation occurs at steady state in moderate, subsaturating light (Figure 6A). In addition, the slower reversibility of NPQ in *npq2* (Figure 4) means that NPQ would remain engaged for a longer period of time after a decrease in incident PFD. The presence of high levels of zeaxanthin may amplify the level of NPQ occurring at intermediate ΔpH during relaxation of a high ΔpH (Noctor et al., 1991; Gilmore and Yamamoto, 1993). This enhanced NPQ could decrease the efficiency of photosynthesis in limiting light. Indeed, retention of zeaxanthin has been associated with sustained depressions in photosynthetic efficiency in plants in the laboratory (Jahns and Miehe, 1996; Demmig-Adams et al., 1998) and in the field, especially in overwintering plants (Adams et al., 1994; Adams and Demmig-Adams, 1995; Ottander et al., 1995; Verhoeven et al., 1996). The consequences of zeaxanthin accumulation in *npq2* for growth in different environments, such as fluctuating light, remain to be tested.

In addition to the photoprotective functions of xanthophylls in NPQ and in quenching of ^{3}Chl and $^1O_2$, xanthophylls are essential structural components of the LHCs

(Plumley and Schmidt, 1987). Replacement of violaxanthin and neoxanthin with zeaxanthin in the *aba1* mutant appears to affect the structure of the LHCs, specifically the peripheral, trimeric LHCII (Hurry et al., 1997). Perturbation of LHC structure could affect the efficiency of light harvesting in limiting light.

Although the exact mechanistic role of xanthophylls in NPQ remains unclear (Demmig-Adams and Adams, 1996a; Horton et al., 1996; Young and Frank, 1996; Gilmore, 1997), recent estimates of excited state energy levels have established the feasibility of direct deexcitation of ^{1}Chl by xanthophylls (Chow, 1994; Frank et al., 1994; Owens, 1994). The lowest excited singlet states (S_1) of antheraxanthin and zeaxanthin (and lutein) appear to lie at or below that of ^{1}Chl, suggesting that downhill transfer of excitation energy from Chl to xanthophyll could occur. The excited xanthophyll could then return to ground state by dissipating the excitation energy as heat. In contrast, the S_1 energy level of violaxanthin lies above ^{1}Chl, which is consistent with a function for violaxanthin as an accessory light-harvesting pigment, absorbing light not available to Chl and then transferring the energy to Chl. The xanthophyll cycle would then be involved in interconversion of light-harvesting and energy-dissipating pigments in the LHCs, and this ability may confer a selective advantage in many natural environments. We hope that further characterization of the Arabidopsis xanthophyll cycle mutants will provide additional insights into the various roles of different xanthophylls in plants.

METHODS

Strains, Growth Conditions, and Genetic Crosses

Arabidopsis thaliana M_2 seeds (ecotype Columbia [Col-0]) derived from mutagenesis with ethyl methanesulfonate were generously provided by M. Liscum (University of Missouri, Columbia), and M_2 seeds derived from mutagenesis by fast-neutron bombardment were obtained from Lehle Seeds (Round Rock, TX). The *aba1-3* mutant was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). The *npq1-2* and *npq2-1* alleles were used for all physiological analyses.

For growth in 100 × 25-mm Petri plates, seeds were surface-sterilized and sown on plant nutrient agar medium (Haughn and Somerville, 1986) lacking sucrose. The plates were wrapped with gas-permeable tape (Scotch 394; 3M, St. Paul, MN) and incubated at 22°C under continuous illumination (100 μmol photons m^{-2} sec^{-1}; cool-white fluorescent). Plants were grown routinely in Pro-Mix HP potting mix (Premier, Laguna Beach, CA) in a shaded greenhouse with natural sunlight during the day (maximum of 200 μmol photons m^{-2} sec^{-1}) and supplemental lighting (15 μmol photons m^{-2} sec^{-1}) at night. The greenhouse temperature was 22 ± 4°C during the day and 20 ± 2°C at night. For examination of growth in natural sunlight, plants were maintained in pots in an unshaded location for several weeks during the summer in Stanford, CA.

Plants used for the measurements shown in Figures 3 and 4 were grown in controlled growth chambers at the Carnegie Institution (Stanford, CA), in 7-cm-diameter plastic pots containing potting mix. Leaves used for these experiments had developed at a photon flux density (PFD) of 250 μmol photons m^{-2} sec^{-1} under a photoperiod of 11.5 hr of light at 22°C alternating with a 12.5-hr dark period at 17°C. Light was provided by a bank of fluorescent tubes (cool white; very high output) supplemented with incandescent lamps.

Plants used for the measurements shown in Table 1 and Figures 5 and 6 were grown in controlled growth chambers at the Research School of Biological Sciences, Australian National University, Canberra. They were grown in 7.5 × 7.5-cm plastic pots in a commercial potting mix obtained from a local nursery. Leaves used for these experiments had developed at a PFD of 230 μmol photons m^{-2} sec^{-1} under a photoperiod of 12 hr of light at 23°C alternating with a 12-hr dark period at 16°C. Light was provided by a bank of fluorescence-coated high-intensity discharge and incandescent lamps. Air humidity during the light was in the range of 60 to 70%.

Plants used for the photoinhibition experiments shown in Table 2 were grown initially under the same conditions as above, except that the PFD during leaf development was 450 μmol photons m^{-2} sec^{-1}. Well-established potted plants were moved outdoors on November 30 (southern summer) and gradually exposed to full daylight. On December 2, plants were kept outdoors in full sunlight until 11:00 AM, when leaves were cut and floated on water. The incident PFD on the cut leaves was 2000 μmol photons m^{-2} sec^{-1} until 12:10 PM, when fluorescence parameters were determined at 1971 μmol photons m^{-2} sec^{-1}. The treated leaves were then moved indoors, and recovery at a PFD of 1.0 to 1.6 μmol photons m^{-2} sec^{-1} was followed over the next 5 hr. Fluorescence parameters were also determined the next morning after recovery overnight.

Plants were hand-watered daily; periodically, a standard nutrient solution was added. All plants, including the *npq2-1* mutant, grew well under these conditions with no signs of wilting. Fully expanded rosette leaves were used for all measurements.

Genetic crosses were performed according to standard procedures (Somerville and Ogren, 1982).

Fluorescence Video Imaging

Screening of mutants by digital video imaging of chlorophyll fluorescence was done as described previously (Niyogi et al., 1997a), with the following modifications. The light source was a 2500 W xenon arc lamp (model XBO 2500 W OFR; Osram, Van Nuys, CA) contained in a lamp housing that provided a collimated, slightly divergent light beam. A wide-band hot mirror (Optical Coating Laboratory, Inc., Santa Rosa, CA) was placed at a 45° angle to the exit beam from the lamp housing, and a cyan dichroic filter (Optical Coating Laboratory, Inc.) was placed normal to the exit beam. A laboratory-built pneumatic shutter system was used to provide 2.5-sec saturating light pulses (2000 μmol photons m^{-2} sec^{-1}) at 1-min intervals during a 4-min illumination with actinic light (500 μmol photons m^{-2} sec^{-1}). A final saturating pulse was applied after a subsequent 1-min dark period. The opening and closing of the shutters were controlled by a laboratory-built digital interface connected to a standard personal computer equipped with the Hewlett-Packard (Palo Alto, CA) Video to Photo frame grabber and software running on the Windows 95 operating system. Color video images of F_m (maximum fluorescence in the dark-adapted state) or F_m' (maximum fluorescence in any light-adapted state) were captured during saturating pulses, and false-color images of nonphotochemical quenching (NPQ) were generated as described previously (Niyogi et al., 1997a).

Pigment Determination

HPLC analysis of carotenoids and chlorophylls was done as described previously (Niyogi et al., 1997a).

Measurements of Fluorescence and Spectral Absorbance Changes

Simultaneous measurements of light-induced changes in absorbance (450 to 650 nm) and fluorescence (Figures 3 and 4) were performed with the apparatus described previously (Brugnoli and Björkman, 1992; Bilger and Björkman, 1994). The fiber-optic arrangement was modified to accommodate the relatively small Arabidopsis leaves. To minimize apparent changes in absorbance caused by chloroplast movements (Brugnoli and Björkman, 1992), predarkened leaves were kept in yellow light ($\sim$8 μmol photons m^{-2} sec^{-1}) for 1 to 2 hr before the measurements. Also, the actinic light used to induce the absorbance and fluorescence changes was passed through a yellow dichroic filter (Optical Coating Laboratory, Inc.) that blocked radiation $<$500 nm.

Determination of conventional fluorescence parameters (Van Kooten and Snel, 1990) was performed with an OS-500 pulse-modulated fluorometer (Optisciences, Tyngsboro, MA). Plants were dark-adapted for 1 to 2 hr before experiments to allow maximal reepoxidation of zeaxanthin and antheraxanthin to violaxanthin and to ensure complete relaxation of the thylakoid pH gradient. An attached, fully expanded rosette leaf was placed in the leaf clip, allowing air to circulate freely on both sides of the leaf. At the start of each experiment, the leaf was exposed to 2 min of far-red illumination (2 to 4 μmol photons m^{-2} sec^{-1}) for determination of F_o (minimum fluorescence in the dark-adapted state). Saturating pulses (1.0 sec) of white light (8000 μmol photons m^{-2} sec^{-1}) were applied to determine F_m or F_m' values. Actinic light provided by a 20-W halogen lamp (EZX/FG, 8°; Philips, Somerset, NJ) was filtered through a wide-band hot mirror (Optical Coating Laboratory, Inc.) and attenuated as necessary with neutral density filters (Melles Griot, Irvine, CA). F_s is the fluorescence yield during actinic illumination. F_o' (minimum fluorescence in the light-adapted state) was measured in the presence of far-red light after switching off the actinic light. Gross NPQ was calculated as $(F_m - F_m')/F_m'$, where F_m is the maximum value taken before exposure to actinic light.

Light curves for fluorescence parameters were determined as follows. The actinic light was increased in steps. At low and moderate actinic intensities, fluorescence measurements at each step were continued until a steady state was reached (usually within 10 min). These F_s and F_m' values (in the light) and the F_o' value recorded immediately after the extinction of the light were used to calculate the fluorescence parameters shown in Figure 6. At actinic light levels $\geqslant$1000 μmol photons m^{-2} sec^{-1}, measurements were limited to 10 min at each step to minimize effects of photodamage.

Genetic Mapping and Molecular Biology

The *npq1-1* mutation was mapped relative to simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994). Genomic DNA was isolated from F_2 plants derived from a cross between *npq1-1*/*npq1-1* (Col-0) and *NPQ1*/*NPQ1* Landsberg *erecta* (L*er*-0) strains, and the segregation of SSLP markers was scored by polymerase chain reaction (PCR) for 50 F_2 plants. For marker nga63, all 14 Npq^- F_2 progeny (*npq1-1*/*npq1-1*) were homozygous for the Col-0 polymorphism, whereas all 36 Npq^+ progeny (*npq1-1*/*NPQ1* or *NPQ1*/*NPQ1*) were either heterozygous or homozygous for the L*er*-0 polymorphism.

Genomic DNA containing the wild-type and *npq1-1* alleles of the violaxanthin deepoxidase gene was amplified by PCR, using oligonucleotide primers KN75 (5′-GGGGAAGATTAGATAGTGTGA-3′) and KN76 (5′-TTACTTTATATGAACCGAACA-3′) and Taq DNA polymerase (Perkin-Elmer, Norwalk, CT). Double-stranded PCR products were purified (Ausubel et al., 1989) and sequenced using a dye terminator cycle sequencing kit and an ABI prism model 310 genetic analyzer (Perkin-Elmer).

For complementation of the *npq1* mutations, a 4-kb EcoRI-BglII genomic DNA fragment from pSE48/B (Lukowitz, 1996) containing the wild-type violaxanthin deepoxidase gene was subcloned into vector pBIN19 digested with EcoRI and BamHI to generate pVC1. Arabidopsis *npq1-1* and *npq1-2* plants were transformed by vacuum infiltration (Bechtold et al., 1993) of *Agrobacterium tumefaciens* GV3101 containing either pVC1 or pBIN19.

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