Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 14162–14167, December 1997
Plant Biology

The roles of specific xanthophylls in photoprotection

KRISHNA K. NIYOGI*, OLLE BJÖRKMAN, AND ARTHUR R. GROSSMAN

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

Contributed by Olle Björkman, October 3, 1997

ABSTRACT **Xanthophyll pigments have critical structural and functional roles in the photosynthetic light-harvesting complexes of algae and vascular plants. Genetic dissection of xanthophyll metabolism in the green alga *Chlamydomonas reinhardtii* revealed functions for specific xanthophylls in the nonradiative dissipation of excess absorbed light energy, measured as nonphotochemical quenching of chlorophyll fluorescence. Mutants with a defect in either the α- or β-branch of carotenoid biosynthesis exhibited less nonphotochemical quenching but were still able to tolerate high light. In contrast, a double mutant that was defective in the synthesis of lutein, loroxanthin (α-carotene branch), zeaxanthin, and antheraxanthin (β-carotene branch) had almost no nonphotochemical quenching and was extremely sensitive to high light. These results strongly suggest that in addition to the xanthophyll cycle pigments (zeaxanthin and antheraxanthin), α-carotene-derived xanthophylls such as lutein, which are structural components of the subunits of the light-harvesting complexes, contribute to the dissipation of excess absorbed light energy and the protection of plants from photo-oxidative damage.**

The xanthophylls comprise a diverse group of oxygenated carotenoids with varied structures and multiple functions (1). In almost all photosynthetic eukaryotes, the majority of xanthophylls are bound with chlorophyll (Chl) molecules to proteins of integral membrane, light-harvesting complexes (LHCs) (2–5). The LHCs absorb and transfer excitation energy to the photosynthetic reaction centers to drive electron transport; these reactions convert light energy into chemical energy that is used to fix atmospheric CO_2 into sugars. Xanthophylls can function as accessory light-harvesting pigments, as structural entities within the LHC, and as molecules required for the protection of photosynthetic organisms from the potentially toxic effects of light.

The importance of carotenoids in photoprotection is evident from the phenotypes of organisms that cannot synthesize carotenoids, either as a consequence of mutations or treatment with herbicides (e.g., norflurazon) that block carotenoid biosynthesis (6–9). There are several mechanisms by which carotenoids function to protect plants against photodamage. Specific xanthophylls are involved in the de-excitation of singlet Chl (^{1}Chl) that accumulates in the LHC under conditions of excessive illumination (10–14). This de-excitation, measured as nonphotochemical quenching of Chl fluorescence (NPQ), depends on a large transthylakoid proton gradient that becomes established in excessive light. The development of NPQ correlates with the synthesis of zeaxanthin (Z) and antheraxanthin (A) from violaxanthin (V) via the xanthophyll cycle, which is depicted in Fig. 1. Estimates of excited state energy levels suggest that the lowest singlet state (2^1A_g or S_1) of Z and A can accept excitation energy directly from ^{1}Chl (15–17); the excited xanthophylls return to ground state by nonradiative heat dissipation. ^{1}Chl can also enter the triplet state (^{3}Chl) by intersystem crossing, and ^{3}Chl can facilitate the formation of the highly toxic singlet oxygen molecule ($^1O_2$). The triplet states of xanthophylls can de-excite both ^{3}Chl and $^1O_2$ (18). The arrangement of the xanthophyll and Chl molecules in the LHC (19) allows for singlet and triplet energy transfer between these pigments either by a coulomb or a Dexter electron exchange mechanism (20). Finally, xanthophylls may be involved in inhibiting lipid peroxidation (21).

To elucidate the roles of specific xanthophylls in photoprotection, we used a video imaging system to isolate mutants of the unicellular green alga *Chlamydomonas reinhardtii* that were impaired in NPQ and xanthophyll metabolism (22). Analysis of the *npq1* mutant, which is unable to convert V to A and Z, demonstrated that operation of the xanthophyll cycle is not required for all pH-dependent NPQ in *C. reinhardtii* (22). In this report, we provide genetic evidence of a role in NPQ for xanthophylls derived from α-carotene, in addition to the xanthophyll cycle pigments, which are derived from β-carotene (Fig. 1). Characterization of single and double mutants has enabled us to define the specific xanthophylls that are required for protection of the photosynthetic apparatus from photo-oxidative damage in excessive light.

MATERIALS AND METHODS

Strains and Growth Conditions. The *npq1* mutant was isolated as described (22). The *lor1* mutant (originally designated *pg-101*) (23, 24) was obtained from the *Chlamydomonas* Genetics Center (Duke University). Although it was first described as lacking only loroxanthin, *lor1* also is unable to make α-carotene and lutein and is therefore likely defective in ε-cyclase activity. Genetic crosses were performed according to established methods (25). Mutants were backcrossed three times as *mt*− parents to the standard wild-type strain CC-125 (*mt*+) to eliminate other mutations that might be present in the different strain backgrounds, including the chloroplast *sr-u-2–60* mutation that was in the background of the original *npq1* isolate (22).

Cells were grown photoautotrophically in 100 ml minimal (high-salt) medium (25) with shaking in air in sterile beakers at 25°C with a 15 hr light/9 hr dark cycle (22). Viable cell number was determined by using a hemacytometer as described (26). Strain stocks were maintained in very low light (10 μmol photons $m^{-2}{\cdot}s^{-1}$) at 27°C on agar medium containing acetate (Tris–acetate phosphate) (25).

Measurements of Fluorescence and Oxygen Evolution. Cells (30 μg Chl) were deposited on a filter disc, and fluorescence parameters were measured in air (without CO_2 enrichment) by using a modified pulse-amplitude modulation fluorometer system as described (22), except that the modulated measuring beam was provided by an LED with a peak output at 450 nm

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

Abbreviations: A, antheraxanthin; Chl, chlorophyll; LHC, light-harvesting complex; NPQ, nonphotochemical quenching of Chl fluorescence; PFD, photon flux density; V, violaxanthin; Z, zeaxanthin.

*To whom reprint requests should be addressed. e-mail: niyogi@andrew.stanford.edu.

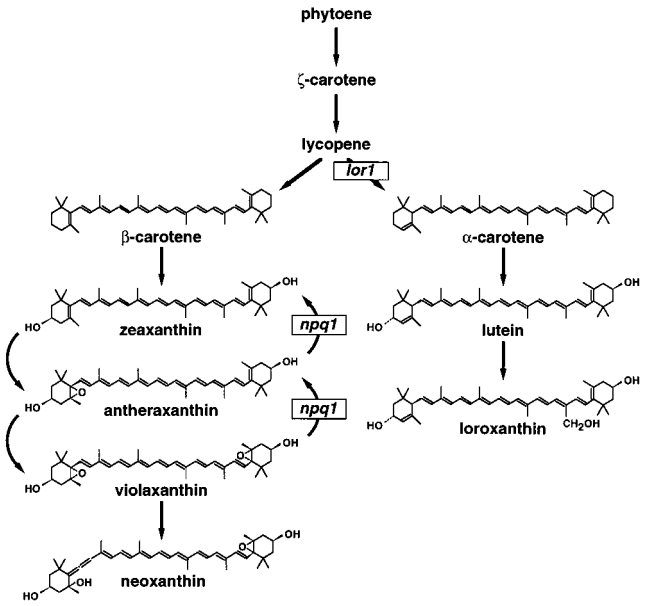

FIG. 1. Carotenoid biosynthetic pathway in *C. reinhardtii*. The defects in xanthophyll metabolism in the *npq1* and *lor1* mutants are indicated.

instead of 655 nm, and the duration of saturating light pulses (for determination of F_m or F'_m) was 250 ms instead of 1 s. Conventional fluorescence nomenclature was used (27). NPQ was calculated as $(F_m - F'_m)/F'_m$.

The light- and CO_2-saturated rate of O_2 evolution was measured by using a DW2/2 oxygen electrode chamber (Hansatech, Norfolk, U.K.) with 1.5 ml of cells (7.5 μg Chl/ml) and 4 mM $NaHCO_3$ at 25°C. Actinic illumination (1090 μmol photons $m^{-2}\cdot s^{-1}$) was provided by a 20 W halogen lamp via a fiber optic.

Pigment Determination. Cells were frozen immediately in liquid nitrogen, and pigments were extracted with 90% (vol/vol) acetone in H_2O. HPLC was performed by using a Spherisorb ODS-1 column as described (22). In addition, duplicate samples were analyzed on a Microsorb-MV (Rainin, Woburn, MA) column (28) to determine the proportion of neoxanthin and loroxanthin, which cochromatographed on the ODS-1 column. Pigments were eluted from the Microsorb-MV column at a flow rate of 1 ml/min with a 7-min linear gradient from 62% to 75% acetone, then an 8-min linear gradient from 75% to 80% acetone, followed by 7 min of 95% acetone, and finally a 3-min linear gradient from 95% to 100% acetone.

RESULTS AND DISCUSSION

A Role in NPQ for Xanthophylls Derived from α-Carotene. The *npq1* mutant is defective in the xanthophyll cycle and could not convert V to A and Z on exposure to excessive light (Figs. 1 and 2) (22). As a consequence, this mutant had a reduced ability to de-excite ^{1}Chl (shown as reduced NPQ in Fig. 3). Nevertheless, excessive light did not impair the growth of *npq1* (Fig. 4 and Table 1) under our growth conditions, probably because substantial NPQ still developed in this strain (22). These results with *npq1* suggested that processes in addition to the xanthophyll cycle are involved in the de-excitation of ^{1}Chl.

The major α-carotene-derived xanthophylls of the *C. reinhardtii* LHCs are lutein and loroxanthin. These xanthophylls are predicted to be energetically similar to A (15–17), suggesting that they might function in the direct de-excitation of ^{1}Chl. This possibility is supported by the finding that the *lor1* mutant of *C. reinhardtii* (23, 24), which cannot synthesize

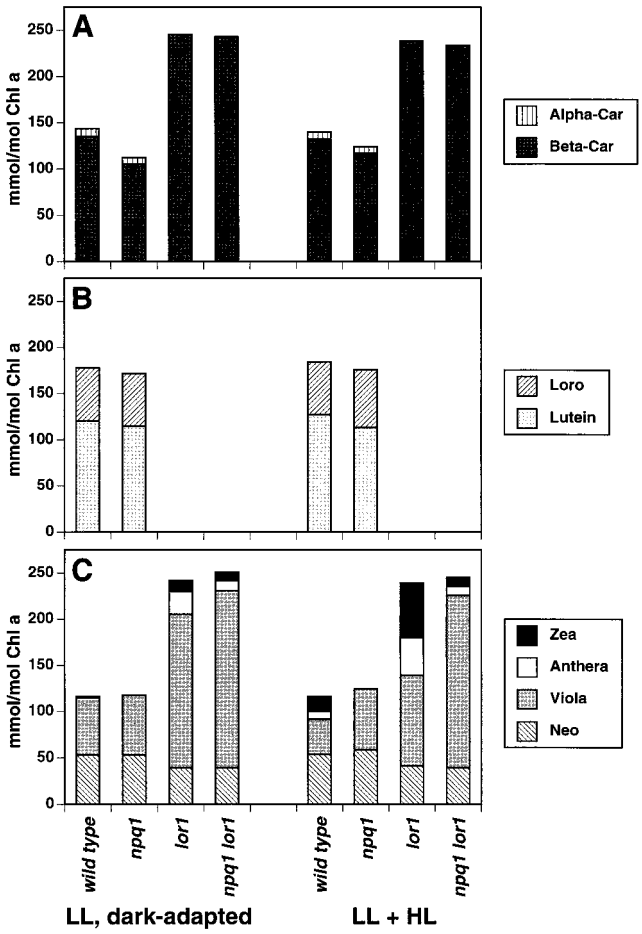

FIG. 2. Carotenoid composition of *C. reinhardtii* strains. Cells were grown at an incident photon flux density (PFD) of 70 μmol photons $m^{-2}\cdot s^{-1}$ (low light, LL) and then dark-adapted overnight. Carotenes (*A*), α-carotene-derived xanthophylls (*B*), and β-carotene-derived xanthophylls (*C*) were determined by HPLC analysis of cell extracts before (LL, dark-adapted) and after exposure to 1,160 μmol photons $m^{-2}\cdot s^{-1}$ for 15 min [LL + high light (HL)] by using the same conditions as for measurements of fluorescence. Values are the means of two independent experiments. Loro, loroxanthin; Alpha-Car, α-carotene; Zea, zeaxanthin; Anthera, antheraxanthin; Viola, violaxanthin; Neo, neoxanthin; Beta-Car, β-carotene.

α-carotene, lutein, and loroxanthin (Figs. 1 and 2), had reduced NPQ (Fig. 3). Reduced NPQ in *lor1* is unlikely to be caused by a lack of α-carotene, because wild-type cells (or *npq1*) accumulated very little α-carotene (Fig. 2), and a mutant that contained elevated levels of α-carotene did not exhibit increased NPQ (unpublished results). Hence, lutein, loroxanthin, or both must contribute to the de-excitation of ^{1}Chl. The pH-dependent, rapidly reversible NPQ that remains in the *lor1* strain can be attributed to the formation of Z and A via the xanthophyll cycle; these xanthophylls accumulated in *lor1* to a greater extent than in wild-type cells (Fig. 2 and Table 2). Like the *npq1* mutant, *lor1* did not appear to be impaired significantly in photoautotrophic growth in high light (Fig. 4 and Table 1).

The *C. reinhardtii lor1* mutant exhibited an elevated Chl *a*/Chl *b* ratio (Table 2) and decreased amount of neoxanthin (Fig. 2), which reflects a partial defect in assembly or stability of the peripheral LHCII (23). Similar pigment changes were reported for an analogous mutant of the green alga *Scenedesmus obliquus* (29). However, it is unlikely that the NPQ defect in *lor1* is solely the consequence of altered assembly or stability of the peripheral LHCII; several observations suggest that lutein has a more direct role in NPQ. Recent data indicate that

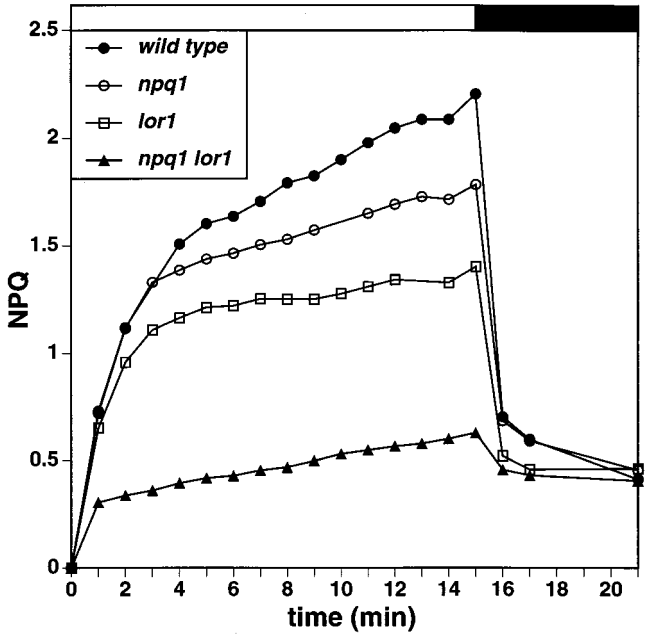

FIG. 3. Induction of NPQ in *C. reinhardtii* strains. Cells were grown at an incident PFD of 70 μmol photons $m^{-2}\cdot s^{-1}$ and dark-adapted overnight. Fluorescence was measured before, during, and after exposure to actinic light (1,160 μmol photons $m^{-2}\cdot s^{-1}$) in air, and NPQ was calculated as $(F_m - F'_m)/F'_m$. The white bar above the graph indicates the period of illumination with high light (HL); the black bar indicates illumination with weak far red background light.

the inner rather than the peripheral LHC is the site of xanthophyll-related NPQ (30–35), and there is no evidence of a significant alteration of the inner LHC in the *lor1* mutant. The F_v/F_m value of the *lor1* mutant (and the *lor1 npq1* double mutant) grown in low light was not decreased compared with that of wild-type cells (Table 1), suggesting that the *lor1* lesion does not perturb the efficient transfer of absorbed light energy to the photosystem II reaction centers. As shown in Table 1, the F_v/F_m values of low light grown *lor1* and *npq1 lor1* were significantly greater than that of wild-type *C. reinhardtii*. Finally, the *lut2* mutant of *Arabidopsis thaliana*, which is analogous to the *lor1* mutant, has a normal Chl *a*/Chl *b* ratio (36), but is still impaired in NPQ (O.B., B. Pogson, D. DellaPenna, A.R.G., and K.K.N., unpublished data).

Specific Xanthophylls Required for Photoprotection. Analysis of the *npq1 lor1* double mutant demonstrated that blocking the synthesis of specific xanthophylls derived from both α- and β-carotene resulted in a cumulative reduction in NPQ. This double mutant was unable to synthesize α-carotene, lutein, and loroxanthin or to convert V to A and Z (Figs. 1 and 2). On exposure to high light, this strain displayed almost no reversible NPQ (Fig. 3), suggesting that it is severely impaired in the de-excitation of ^{1}Chl. The reversible NPQ remaining in *npq1 lor1* (Fig. 3) probably reflects the low levels of A and Z (Fig. 2 and Table 2) that accumulate as intermediates in the synthesis of V (Fig. 1).

The α-carotene-derived lutein and loroxanthin and the β-carotene-derived Z and A function redundantly in protecting *C. reinhardtii* from photo-oxidative damage. The *npq1* and *lor1* single mutants grew relatively normally in high light (Fig. 4 and Table 1), although the photosystem II efficiency (F_v/F_m) for *lor1* appeared to be lower than that of wild-type cells (Table 1). In contrast, the *npq1 lor1* double mutant, which attained a similar rate of growth in low light to wild-type cells, grew much more slowly than wild-type cells in high light (Table 1) and rapidly bleached (Fig. 4).

Increases in Z, A, and lutein during growth at elevated light intensities are consistent with a role of these xanthophylls in photoprotection. Wild-type *C. reinhardtii* grown at 350 μmol photons $m^{-2}\cdot s^{-1}$ had greatly increased levels of Z and A, an increased xanthophyll cycle pool (V + A + Z), and elevated lutein relative to Chl *a* (Table 2). Increases in the xanthophyll cycle pool and lutein in high light occurred even in *npq1*, despite the inability of this strain to convert V to A and Z. Vascular plants and many algae grown in high light exhibit similar increases in xanthophyll cycle pigments (37–39). The greater accumulation of lutein in wild type and *npq1* in high light was accompanied by a lower level of loroxanthin (Table 2). Light intensity-dependent changes in loroxanthin levels have been observed previously for the green alga *Scenedesmus obliquus* (40). The xanthophyll cycle pool in the *lor1* strains was considerably elevated relative to that of wild-type or *npq1* cells, even when *lor1* was grown in low light (Table 2). This finding suggests that in the *lor1* mutant the intermediates normally metabolized by the α-carotene biosynthetic pathway are diverted to the β-carotene branch; in agreement with this interpretation, the sum total of the carotenes and xanthophylls in all of the strains was remarkably similar for each specific growth condition. Together, these results suggest that Z, A, and lutein but not loroxanthin have critical functions in

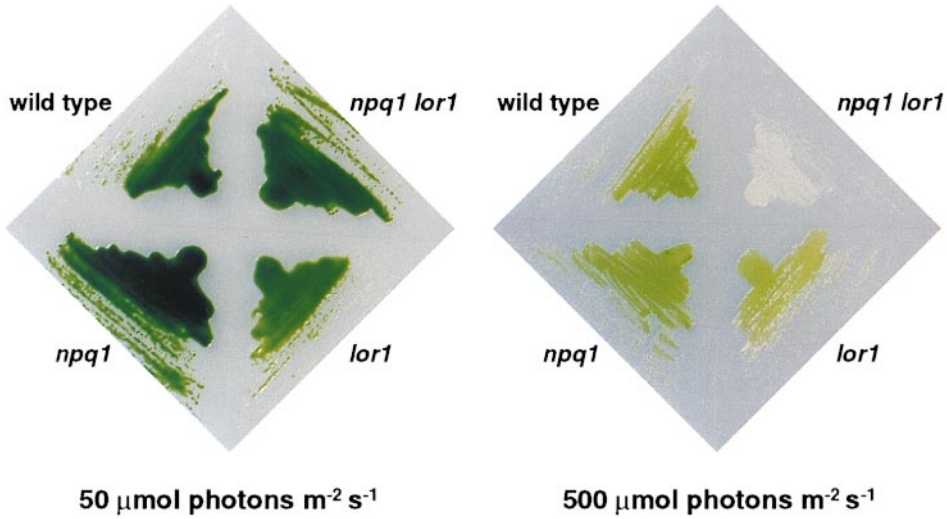

FIG. 4. Growth of *C. reinhardtii* strains. Cells were streaked on minimal agar medium, incubated overnight at 50 μmol photons $m^{-2}\cdot s^{-1}$, and grown photoautotrophically for 6 days at the indicated PFD.

Table 1. Growth and photosynthesis characteristics of *C. reinhardtii* strains

Strain	Growth PFD, μmol photons $m^{-2}\cdot s^{-1}$	Chl doubling time, hr (n)	F_v/F_m (n)	O_2 evolution, μmol O_2 $cell^{-1}\cdot hr^{-1}$ (n)
Wild type	70	20 ± 0 (4)	0.763 ± 0.011 (6)	1.21 ± 0.06 (2)
npq1	70	19 ± 2 (4)	0.771 ± 0.009 (6)	0.99 ± 0.07 (2)
lor1	70	23 ± 1 (4)	0.793 ± 0.010 (6)	0.96 ± 0.01 (2)
npq1 lor1	70	21 ± 1 (4)	0.802 ± 0.004 (6)	1.14 ± 0.16 (2)
Wild type	350	28 ± 2 (4)	0.769 ± 0.009 (3)	0.90 ± 0.23 (2)
npq1	350	29 ± 3 (4)	0.761 ± 0.004 (3)	0.92 ± 0.06 (2)
lor1	350	34 ± 4 (4)	0.718 ± 0.066 (3)	0.36 ± 0.17 (2)
npq1 lor1	350	67 ± 16 (4)	0.477 ± 0.050 (3)	0.09 ± 0.00 (2)

Each value is the mean ± SD (or for $n = 2$, mean ± difference from the mean). The maximum rate of O_2 evolution was determined at light and CO_2 saturation as described.

photoprotection and that the NPQ defect in the *lor1* mutant is probably caused by the absence of lutein.

Multiple Levels of Xanthophyll-Dependent Photoprotection. Z, A, and lutein have 10 or more conjugated double bonds and at least one cyclohexenyl ring with a single oxygen substituent (see Fig. 1). Xanthophylls with these characteristics could alter the fluidity of the photosynthetic membranes (41, 42) and/or the aggregation state of the LHCs within the membranes (43). The lack of an epoxide on at least one cyclohexenyl ring of Z, A, and lutein may facilitate a direct photochemical reaction with $^1O_2$ (44), whereas having additional conjugated double bonds might make these xanthophylls more effective in preventing lipid peroxidation (21). Furthermore, the energies of the lowest singlet and triplet excited states decrease with increasing conjugation length. It is more energetically feasible for a direct transfer of excitation energy to occur from ^{1}Chl to the singlet states of Z, A, and lutein than to V or neoxanthin (15, 16), which have fewer than 10 conjugated double bonds. Direct interaction between Chl and xanthophylls is consistent with the results of our genetic analysis, which revealed roles for Z, A, and lutein in the de-excitation of ^{1}Chl *in vivo*. Alternatively, unique structural features of Z, A, and lutein may be required for protonation-induced conformation changes of specific inner LHC proteins to which these xanthophylls are bound; these changes could result in de-excitation of ^{1}Chl that does not involve direct transfer of excitation energy from Chl to xanthophyll (14, 45).

The absence of xanthophyll-dependent de-excitation of ^{1}Chl in the LHCs of the *npq1 lor1* mutant would lead to elevated formation of ^{3}Chl and $^1O_2$. The latter species could cause irreversible photo-oxidative damage unless it were de-excited by interactions with carotenoids or scavenged by antioxidants such as α-tocopherol. β-Carotene, V, and neoxanthin (plus very low levels of A and Z) are essentially the only carotenoids in the *npq1 lor1* mutant. The predominant carotenoids associated with the LHCs of this strain are likely to be V and neoxanthin, because these two xanthophylls, but not β-carotene, can substitute for lutein in LHC reconstitution assays (46). Both V and neoxanthin are probably able to function in photoprotection as they can de-excite ^{3}Chl, thereby minimizing $^1O_2$ production (18). However, V and neoxanthin have fewer than 10 conjugated double bonds, making these pigments less effective in the de-excitation of any $^1O_2$ that does form in the *npq1 lor1* strain (47). In contrast, Z, A, and lutein would be efficient scavengers of both ^{3}Chl and $^1O_2$; this is supported by the finding that *C. reinhardtii* mutants that are unaffected in their xanthophyll composition, but that cannot quench ^{1}Chl (they exhibit essentially no NPQ) (22), can still survive high intensity illumination (unpublished results). Hence, the accumulation of V and neoxanthin in the *npq1 lor1* strain is not sufficient for photoprotection in high light (Fig. 4); photoprotection in this strain is impaired at several levels because of a lack of Z, A, and lutein.

A Model for NPQ with Roles for Xanthophylls Derived from Both α- and β-Carotene. The finding that lutein plays an important role in NPQ emphasizes how the generation and characterization of mutants can be used to dissect photoprotection in plants. The results presented here have enabled us to develop a model for NPQ that incorporates functions for both the α- and β-carotene-derived xanthophylls. Because LHCs can assemble in mutants lacking lutein [although there is some difference in the extent of assembly of LHCs in algal and plant mutants deficient in lutein (24, 29, 36, 48)], other xanthophylls must be able to fulfill the structural role of lutein in LHC assembly. In the case of the *npq1 lor1* double mutant (and the *lor1* single mutant grown in low light), V and neoxanthin (rather than lutein) must be the xanthophylls that form the "cross brace" in each LHC monomer (19). Because V can replace lutein in the LHCs, we propose that the xanthophylls derived from α- and β-carotene bind to the same sites in the LHCs of wild type. Based on the substoichiometric levels of β-carotene-derived xanthophylls per LHC monomer, it was suggested that these xanthophylls bind to sites on the LHC distinct from those to which lutein binds (19, 49). As an alternate interpretation, the substoichiometric amounts of some of the xanthophylls may simply reflect the heterogeneous

Table 2. Pigment characteristics of *C. reinhardtii* strains

Strain	Growth PFD, μmol photons $m^{-2}\cdot s^{-1}$	Chl *a*/Chl *b* (n)	(A+Z)/(V+A+Z) (n)	(V+A+Z)/Chl *a*, mmol/mol (n)	Lutein/Chl *a*, mmol/mol (n)	Loroxanthin/Chl *a*, mmol/mol (n)
Wild type	70	2.64 ± 0.04 (6)	0.066 ± 0.020 (2)	72.1 ± 5.4 (2)	115.2 ± 6.0 (2)	58.9 ± 3.1 (2)
npq1	70	2.64 ± 0.03 (6)	0.000 ± 0.000 (2)	73.6 ± 5.1 (2)	113.1 ± 6.3 (2)	57.7 ± 2.8 (2)
lor1	70	4.02 ± 0.10 (6)	0.213 ± 0.004 (2)	221.5 ± 12.1 (2)	0.0 ± 0.0 (2)	0.0 ± 0.0 (2)
npq1 lor1	70	4.04 ± 0.07 (6)	0.108 ± 0.001 (2)	219.1 ± 15.1 (2)	0.0 ± 0.0 (2)	0.0 ± 0.0 (2)
Wild type	350	2.87 ± 0.05 (3)	0.598 ± 0.035 (2)	114.3 ± 1.9 (2)	209.8 ± 1.2 (2)	20.2 ± 1.7 (2)
npq1	350	2.90 ± 0.02 (3)	0.001 ± 0.001 (2)	125.3 ± 12.1 (2)	215.3 ± 20.4 (2)	21.3 ± 0.1 (2)
lor1	350	4.17 ± 0.11 (3)	0.696 ± 0.075 (2)	320.8 ± 22.3 (2)	0.0 ± 0.0 (2)	0.0 ± 0.0 (2)
npq1 lor1	350	4.70 ± 0.29 (3)	0.195 ± 0.001 (2)	440.0 ± 46.8 (2)	0.0 ± 0.0 (2)	0.0 ± 0.0 (2)

Pigment determination by HPLC was performed on samples of exponentially growing cells that were taken during the middle of the light period. Each value is the mean ± SD (or for $n = 2$, mean ± difference from the mean).

xanthophyll population that can assemble with the LHC monomers (e.g., two luteins, one lutein and one V, or one lutein and one Z) (30). Because V is de-epoxidated efficiently in the *lor1* mutant (Fig. 2), the V that assembles with the LHC must be accessible, either directly while still bound to the LHC polypeptides, or on exchange with a pool of free pigment, to the de-epoxidase that catalyzes the conversion of V to Z. This binding site would position Z (or lutein) in close proximity to the Chl *a* molecules that assemble with the LHC polypeptides and allow for the direct transfer of excitation energy from ^{1}Chl to Z (or lutein).

Xanthophyll-dependent de-excitation of ^{1}Chl also depends on acidification of the thylakoid lumen and/or localized thylakoid membrane domains (10–12, 14). Protonation of the LHC, probably specific polypeptides of the inner LHC (29–34), may promote a conformation change (14, 50) that favors the transfer of excitation energy from ^{1}Chl to the xanthophylls that are bound to these particular proteins. Therefore, only a subset of the Z, A, and lutein pools are directly involved in the de-excitation mechanism. Because the lowest singlet energy state of lutein is likely to be higher than that of Z (and therefore closer to the lowest energy state of ^{1}Chl), lutein may be a weaker quencher at pH values not saturating for NPQ. A greater proton gradient may be required to facilitate energy transfer from ^{1}Chl to lutein either by causing a more substantial conformation change that brings the lutein closer to the Chl molecules or by changing the local electric field, which could provide sufficient spectral overlap between the lutein and proximal Chl molecules or alter the dipole strength of the lutein S_1 to S_0 transition (15).

This model accommodates roles in NPQ for both α- and β-carotene-derived xanthophylls and explains both *in vivo* and *in vitro* results, some of which have suggested that all NPQ depends on Z and A. From experiments with isolated thylakoid membranes, Gilmore and Yamamoto (51) derived the linear equation $NPQ = \chi_1[H^+][Z + A] + \chi_2[H^+] + c$. This equation includes the term $\chi_2[H^+]$ that relates NPQ to the proton concentration and is independent of [Z] and [A]. This term may reflect the contribution of the invariant α-carotene-derived xanthophylls such as lutein. Therefore, we suggest that the equation be modified to $NPQ = \chi_1[H^+][Z + A] + \chi_3[H^+][L] + c$, where [L] is the lutein concentration and $\chi_3[L] = \chi_2$. Assuming that χ_3 is relatively small compared with χ_1, which is consistent with lutein being a weaker quencher than Z, lutein-dependent NPQ would be most apparent at a high $[H^+]$, which occurs during the induction of photosynthesis on sudden illumination of dark-adapted cells with high light (the way in which the experiments in this study were performed). This interpretation is also consistent with the observation that only the rapid phase of NPQ is observed on illumination of the *npq1* mutant (Fig. 2) (22). Others have noted xanthophyll cycle-independent NPQ under conditions likely to generate a high lumenal $[H^+]$, such as during the illumination of leaves in an atmosphere of 1% O_2 and 0% CO_2 (52). The contribution of the lutein concentration to NPQ would be less when the lumenal $[H^+]$ is only moderately high, conditions that occur during steady-state photosynthesis in high light or when the ATP synthase operates in reverse in isolated thylakoids in the dark. These latter conditions were used in many experiments that led to the conclusion that essentially all NPQ was dependent on Z and A (53–55).

Measurements of Chl fluorescence lifetime distributions in isolated thylakoids have distinguished two distinct components that contribute to a ΔpH-dependent decrease in the fluorescence yield (32, 54). A xanthophyll cycle pigment-independent shift in the fluorescence lifetime from 2.0 to 1.6 ns occurs on acidification of isolated thylakoid membranes. This shift, which we propose is a consequence of the de-excitation of ^{1}Chl by lutein, involves the entire LHC population, probably because all of the LHC monomers contain at least one lutein (two luteins, lutein and V, or lutein and neoxanthin). When Z and A are present, a new low pH-dependent fluorescence lifetime component of 0.4 ns appears at the expense of the 1.6-ns component. The fractional intensity of this 0.4-ns component is correlated with the concentration of Z and A. This result is explained by our model, because Z and A would replace lutein, V, or neoxanthin in the LHC and lead to the more efficient de-excitation of ^{1}Chl, thereby decreasing the fluorescence lifetime from 1.6 ns to 0.4 ns.

A similar process may occur when xanthophylls are added to isolated LHC particles and fluorescence quenching is measured on acidification (34, 56). The LHC particles used in these experiments contained mainly lutein and some V. They exhibited a certain level of NPQ that increased when Z was added to the preparations and decreased when V was added. According to our model the addition of Z would enhance quenching by exchanging for lutein and V within the LHC. Addition of V would inhibit quenching because some lutein bound in the LHC would be replaced by V, which is unable to de-excite ^{1}Chl efficiently. Addition of lutein to the preparation would be predicted to have little or no effect.

In summary, the work presented here resolves some important questions concerning the xanthophyll dependence of NPQ and suggests a model for NPQ that accounts for several recent observations and that can now be tested further by using mutants. The analysis of mutants so far has demonstrated that almost all rapidly reversible NPQ depends on specific xanthophylls derived from both α- and β-carotene. Lutein is present constitutively in the LHCs and may function in NPQ mainly at high $[H^+]$. These conditions activate the conversion of V to Z; the latter is a more effective acceptor of excitation energy from ^{1}Chl, thereby allowing dynamic increases in the extent of photoprotection in excessive light. However, the results also emphasize that the xanthophylls can interact with ^{1}Chl, ^{3}Chl, and $^1O_2$, proffering several lines of defense against photooxidative damage.

We thank Connie Shih for excellent technical assistance, Elizabeth Harris (*Chlamydomonas* Genetics Center, Duke University, Durham, NC) for strains, and Catharina Casper-Lindley and Dennis Wykoff for reviewing the manuscript. This work was supported by National Science Foundation Grant IBN 950-6254 (to A.R.G. and O.B.). K.K.N. is a Department of Energy–Energy Biosciences Fellow of the Life Sciences Research Foundation. K.K.N. was also supported by a fellowship from the Department of Energy/National Science Foundation/U.S. Department of Agriculture Training Program in Plant Biology at Stanford University. This is Carnegie Institution of Washington, Department of Plant Biology, publication no. 1346.

1. Britton, G. (1995) *FASEB J.* **9,** 1551–1558.
2. Green, B. R. & Durnford, D. G. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47,** 685–714.
3. Grossman, A. R., Bhaya, D., Apt, K. E. & Kehoe, D. M. (1995) *Annu. Rev. Genet.* **29,** 231–288.
4. Jansson, S. (1994) *Biochim. Biophys. Acta* **1184,** 1–19.
5. Paulsen, H. (1995) *Photochem. Photobiol.* **62,** 367–382.
6. Griffiths, M., Sistrom, W. R., Cohen-Bazire, G. & Stanier, R. Y. (1955) *Nature (London)* **176,** 1211–1215.
7. Sager, R. & Zalokar, M. (1958) *Nature (London)* **182,** 98–100.
8. Anderson, I. C. & Robertson, D. S. (1960) *Plant Physiol.* **35,** 531–534.
9. Böger, P. & Sandmann, G. (1994) *Photosynthetica* **28,** 481–493.
10. Demmig-Adams, B. (1990) *Biochim. Biophys. Acta* **1020,** 1–24.
11. Demmig-Adams, B. & Adams, W. W., III (1992) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43,** 599–626.
12. Demmig-Adams, B., Gilmore, A. M. & Adams, W. W., III (1996) *FASEB J.* **10,** 403–412.
13. Gilmore, A. M. (1997) *Physiol. Plant.* **99,** 197–209.
14. Horton, P., Ruban, A. V. & Walters, R. G. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47,** 655–684.
15. Owens, T. G. (1994) in *Photoinhibition of Photosynthesis: From Molecular Mechanisms to the Field*, eds. Baker, N. R. & Bowyer, J. R. (BIOS, Oxford), pp. 95–109.

16. Frank, H. A., Cua, A., Chynwat, V., Young, A., Gosztola, D. & Wasielewski, M. R. (1994) *Photosynth. Res.* **41,** 389–395.
17. Chow, W. S. (1994) in *Advances in Molecular and Cell Biology*, eds. Bittar, E. E. & Barber, J. (JAI, London), Vol. 10, pp. 151–196.
18. Frank, H. A. & Cogdell, R. J. (1993) in *Carotenoids in Photosynthesis*, eds. Young, A. & Britton, G. (Chapman & Hall, London), pp. 252–326.
19. Kühlbrandt, W., Wang, D. N. & Fujiyoshi, Y. (1994) *Nature (London)* **367,** 614–621.
20. Dexter, D. L. (1953) *J. Chem. Phys.* **21,** 836–850.
21. Frank, H. A. & Cogdell, R. J. (1996) *Photochem. Photobiol.* **63,** 257–264.
22. Niyogi, K. K., Björkman, O. & Grossman, A. R. (1997) *Plant Cell* **9,** 1369–1380.
23. Eichenberger, W., Boschetti, A. & Michel, H. P. (1986) *Physiol. Plant.* **66,** 589–594.
24. Chunaev, A. S., Mirnaya, O. N., Maslov, V. G. & Boschetti, A. (1991) *Photosynthetica* **25,** 291–301.
25. Harris, E. H. (1989) *The Chlamydomonas Sourcebook* (Academic, San Diego).
26. Davies, J. P., Yildiz, F. H. & Grossman, A. (1996) *EMBO J.* **15,** 2150–2159.
27. van Kooten, O. & Snel, J. F. H. (1990) *Photosynth. Res.* **25,** 147–150.
28. Plumley, F. G. & Schmidt, G. W. (1995) *Plant Cell* **7,** 689–704.
29. Bishop, N. I., Urbig, T. & Senger, H. (1995) *FEBS Lett.* **367,** 158–162.
30. Bassi, R., Pineau, B., Dainese, P. & Marquardt, J. (1993) *Eur. J. Biochem.* **212,** 297–303.
31. Härtel, H. & Lokstein, H. (1995) *Biochim. Biophys. Acta* **1228,** 91–94.
32. Gilmore, A. M., Hazlett, T. L., Debrunner, P. G. & Govindjee (1996) *Photosynth. Res.* **48,** 171–187.
33. Jahns, P. (1995) *Plant Physiol.* **108,** 149–156.
34. Ruban, A. V., Young, A. J. & Horton, P. (1996) *Biochemistry* **35,** 674–678.
35. Walters, R. G., Ruban, A. V. & Horton, P. (1994) *Eur. J. Biochem.* **226,** 1063–1069.
36. Pogson, B., McDonald, K. A., Truong, M., Britton, G. & Della-Penna, D. (1996) *Plant Cell* **8,** 1627–1639.
37. Thayer, S. S. & Björkman, O. (1990) *Photosynth. Res.* **23,** 331–343.
38. Demmig-Adams, B. & Adams, W. W., III (1992) *Plant Cell Environ.* **15,** 411–419.
39. Maxwell, D. P., Falk, S. & Huner, N. P. A. (1995) *Plant Physiol.* **107,** 687–694.
40. Senger, H., Wagner, C., Hermsmeier, D., Hohl, N., Urbig, T. & Bishop, N. I. (1993) *J. Photochem. Photobiol. B* **18,** 273–279.
41. Gruszecki, W. I. & Strzalka, K. (1991) *Biochim. Biophys. Acta* **1060,** 310–314.
42. Havaux, M. & Gruszecki, W. I. (1993) *Photochem. Photobiol.* **58,** 607–614.
43. Ruban, A. V., Phillip, D., Young, A. J. & Horton, P. (1997) *Biochemistry* **36,** 7855–7859.
44. Schubert, H., Kroon, B. M. A. & Matthijs, H. C. P. (1994) *J. Biol. Chem.* **269,** 7267–7272.
45. Young, A. J. & Frank, H. A. (1996) *J. Photochem. Photobiol. B* **36,** 3–15.
46. Plumley, F. G. & Schmidt, G. W. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 146–150.
47. Foote, C. S., Chang, Y. C. & Denny, R. W. (1970) *J. Am. Chem. Soc.* **92,** 5216–5218.
48. Bishop, N. I. (1996) *J. Photochem. Photobiol. B* **36,** 279–283.
49. Lee, A. I. & Thornber, J. P. (1995) *Plant Physiol.* **107,** 565–574.
50. Bilger, W. & Björkman, O. (1994) *Planta* **193,** 238–246.
51. Gilmore, A. M. & Yamamoto, H. Y. (1993) *Photosynth. Res.* **35,** 67–78.
52. Adams, W. W., III, Demmig-Adams, B. & Winter, K. (1990) *Plant Physiol.* **92,** 302–309.
53. Gilmore, A. M. & Yamamoto, H. Y. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1899–1903.
54. Gilmore, A. M., Hazlett, T. L. & Govindjee (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2273–2277.
55. Demmig-Adams, B. & Adams, W. W., III (1996) *Trends Plant Sci.* **1,** 21–26.
56. Phillip, D., Ruban, A. V., Horton, P., Asato, A. & Young, A. J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1492–1497.