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Zeaxanthin Accumulation in the Absence of a Functional Xanthophyll Cycle Protects *Chlamydomonas reinhardtii* from Photooxidative Stress

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Xanthophylls participate in light harvesting and are essential in protecting the chloroplast from photooxidative damage. To investigate the roles of xanthophylls in photoprotection, we isolated and characterized extragenic suppressors of the *npq1 lor1* double mutant of *Chlamydomonas reinhardtii*, which lacks zeaxanthin and lutein and undergoes irreversible photooxidative bleaching and cell death at moderate to high light intensities. Here, we describe three suppressor strains that carry point mutations in the coding sequence of the zeaxanthin epoxidase gene, resulting in the constitutive accumulation of zeaxanthin in a range of concentrations. The presence of zeaxanthin in these strains was sufficient to prevent photooxidative damage in the *npq1 lor1* background. The size of the light-harvesting antenna in the suppressors decreased in high light in a manner that was proportional to the relative content of zeaxanthin, with the strain having the most zeaxanthin showing a severe reduction in levels of the major light-harvesting complex II proteins in high light. We show that the effect of constitutive zeaxanthin on light harvesting is not the main cause of increased photoprotection, because in the absence of zeaxanthin, a strain with a smaller light-harvesting antenna showed only minor protection against photobleaching in high light. Furthermore, the zeaxanthin-accumulating suppressors were able to tolerate higher levels of exogenous reactive oxygen than their parental strain under conditions that did not affect light harvesting. Our results are consistent with an antioxidant role of zeaxanthin in the quenching of singlet oxygen and/or free radicals in the thylakoid membrane in vivo.

INTRODUCTION

Maintaining a balance between the capture and the use of light energy is essential for the survival of all oxygenic photosynthetic organisms. When absorption of light energy exceeds the capacity for photosynthesis, plants are at risk of photooxidative damage to the chloroplast (Foyer et al., 1994). Excess absorbed light energy can lead to the overreduction of components of the photosynthetic electron transport chain, and this can result in sequential one-electron transfers to ground-state oxygen, producing the superoxide anion, hydrogen peroxide, and the hydroxyl radical. In the light-harvesting antenna of photosystem II (PSII), unused light energy increases the lifetime of the singlet excited state of chlorophyll (^{1}Chl*), which can be converted to the triplet excited state (^{3}Chl*) through the photophysical process of intersystem crossing. ^{3}Chl* itself is not harmful, but it is long-lived and can transfer energy to ground-state oxygen to generate highly reactive singlet oxygen ($^1O_2$*). Under extreme light stress, when the quenching capacity of the chloroplast presumably has been overwhelmed, net formation of $^1O_2$* has been reported (Hideg et al., 1998). $^1O_2$*, superoxide, and other reactive forms of oxygen irreversibly oxidize lipids, proteins, and pigments in their immediate vicinity. Thylakoid lipids are especially susceptible to oxidative damage because of the abundance of unsaturated fatty acid side chains. Reactive oxygen attack of these lipids initiates peroxyl-radical chain reactions, which eventually can destroy the thylakoid membrane (Knox and Dodge, 1985). In the PSII reaction center, the formation of $^1O_2$* under excess light is thought to be the cause of direct damage to structural protein components, which require de novo protein synthesis to be repaired (Melis, 1999).

To cope with imbalances between the absorption of excitation energy and its use, algae and plants regulate photosynthetic light harvesting and make use of a series of antioxidant molecules and enzymes to detoxify reactive oxygen species and free radicals once they have been formed (reviewed by Niyogi, 1999). Some of the xanthophylls (oxygenated carotenoids) present in the thylakoid membrane participate in both of these protective functions. In particular, zeaxanthin and lutein are necessary under high-light stress for the efficient transition of the light-harvesting complexes (LHCs) of PSII from a conformation that favors light harvesting to one that allows for thermal dissipation of part of the excess excitation energy (Niyogi et al., 1997b, 2001; Pogson et al., 1998). This thermal dissipation of excess light energy, which might involve a direct quenching of excess ^{1}Chl*, is measured and often referred to as nonphotochemical quenching (NPQ) of chlorophyll fluorescence (Horton et al., 1996; Müller et al., 2001).

Because carotenoids are distributed in the thylakoid pigment-protein complexes in close proximity to the chlorophylls, and thus to the potential sites of $^1O_2$* formation (Yamamoto and

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Bassi, 1996), they are the most important quenchers of electronically excited states in the thylakoid membrane. ^{3}Chl* or $^1O_2$* transfers its excitation energy to a nearby carotenoid molecule to form a carotenoid triplet that decays harmlessly to the ground state by thermal dissipation. Carotenoids also can protect against lipid peroxidation by reacting with free radicals directly (Palozza and Krinsky, 1992), forming a carotenoid radical that could be regenerated by interaction with tocopherols and ascorbate in the lipid phase of the membrane (Edge et al., 1997). The importance of carotenoids in the protection of plants from photooxidative stress is evident from the fact that mutants that lack carotenoids, or plants in which carotenoid synthesis has been inhibited, are completely unable to sustain growth in the light in the presence of oxygen (Oelmüller, 1989).

In plant and algal mutants with altered xanthophyll composition (Pogson et al., 1996, 1998; Niyogi et al., 1997b) and in experiments with purified recombinant LHCs in vitro (Bassi and Caffarri, 2000), it has been shown that some of the carotenoid binding sites in the LHC proteins are interchangeable. Despite this flexibility in the xanthophyll composition of photosynthetic organisms, it is becoming increasingly clear that the different xanthophylls vary in their ability to quench reactive intermediates and provide photoprotection (Niyogi et al., 1997b; Pogson et al., 1998). Many factors contribute to these differences, including the number of conjugated double bonds, which influences the excited-state energies of carotenoids. In addition, the polarity and stereochemistry of a carotenoid influence its position in the thylakoid membrane or in the LHC, and this in turn influences the chlorophyll-carotenoid distances that play a key role in energy transfer reactions. For this reason, the roles of the different xanthophylls in photoprotection cannot be extrapolated directly from their measured characteristics in organic solvents. In the past few years, the isolation of several mutants with specific defects in xanthophyll biosynthesis has helped clarify those individual roles (reviewed by Baroli and Niyogi, 2000).

Some of those studies have shown that lutein, the most abundant xanthophyll in the thylakoid membrane, is not essential for light harvesting and photoprotection when zeaxanthin is present. Lutein can be replaced in the LHC by other xanthophylls, such that the size of the xanthophyll pool with respect to chlorophyll remains unchanged, and lutein-deficient mutants show no apparent defect in light harvesting under laboratory conditions (Pogson et al., 1996). However, lutein deficiency has been shown to alter the molecular organization of the LHC in Arabidopsis (Lokstein et al., 2002), *Chlamydomonas reinhardtii* (Chunaev et al., 1991), and *Scenedesmus obliquus* (Bishop, 1996). Analysis of the Chlamydomonas *lor1* mutant demonstrated that lutein contributes substantially to the development of NPQ (Niyogi et al., 1997b), although it is not clear whether the pigment plays a direct role in ^{1}Chl* quenching (Niyogi et al., 1997b) or whether the aberrant conformation of the LHC caused by the lack of lutein prevents the development of NPQ (as discussed by Lokstein et al., 2002). Lutein content increases when Chlamydomonas cells are exposed to light stress, but lutein deficiency did not cause enhanced susceptibility to light stress, at least under the relatively moderate high-light conditions tested (Niyogi et al., 1997b).

Chlamydomonas and Arabidopsis mutants that contain only the xanthophylls violaxanthin and neoxanthin are subject to photobleaching under light stress, suggesting that these xanthophylls do not perform a sufficient protective function in the chloroplast (Niyogi et al., 1997b, 2001). Among all the xanthophylls, zeaxanthin is the only one that accumulates exclusively under excess light, by deepoxidation of existing violaxanthin in the so-called xanthophyll cycle (Eskling et al., 1997) (Figure 1). It is widely thought that the main function of zeaxanthin is as a quencher of the ^{1}Chl* state through the process of NPQ (Müller et al., 2001). However, it has been suggested that zeaxanthin may protect from light stress by directly quenching $^1O_2$* and free radicals (Havaux and Niyogi, 1999) and by making the thylakoid membrane less permeable to oxygen (Gruszecki, 1999).

We are using Chlamydomonas mutants with specific defects in xanthophyll composition (Figure 1) to investigate further the functions of each xanthophyll in photoprotection (Niyogi, 1999). The *npq1* mutant has a complete inhibition of the deepoxidation of violaxanthin that occurs normally in the wild type in response to excess light (Niyogi et al., 1997a). The *lor1* mutation blocks the accumulation of lutein and its derivative loroxanthin (Chunaev et al., 1991), but the *lor1* strain has a normal xanthophyll cycle (Niyogi et al., 1997b). Neither single mutation has a lethal effect on growth at moderate- to high-light conditions (Niyogi et al., 1997b). The double mutant *npq1 lor1*, which accumulates violaxanthin and neoxanthin as the only oxygenated carotenoids irrespective of light conditions, does not develop NPQ when transferred to high light and undergoes irreversible bleaching at irradiances of >500 $\mu mol \cdot m^{-2} \cdot s^{-1}$, conditions in which the wild type and the single mutants grow normally (Niyogi et al., 1997b). This bleaching phenotype is not attributable solely to the lack of NPQ in *npq1 lor1*, because a similar

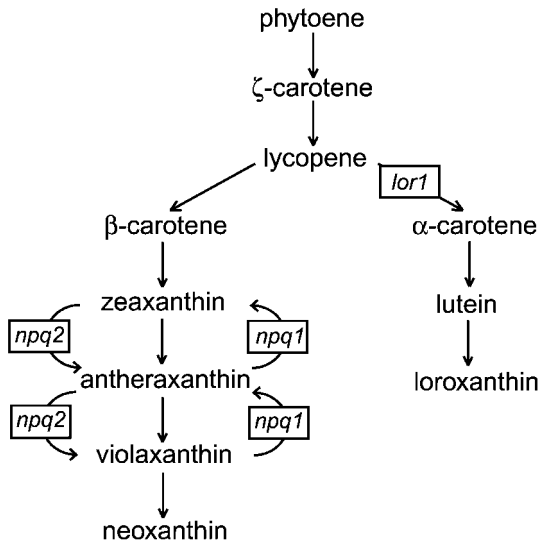

Figure 1. The Xanthophyll Biosynthetic Pathway in Chlamydomonas.

The reactions impaired in the *npq1*, *npq2*, and *lor1* mutants are indicated.

NPQ-defective mutant with normal xanthophyll composition is not particularly prone to chlorophyll bleaching in high light (Niyogi et al., 1997b). The turnover rate of the D1 protein of PSII in the *npq1 lor1* mutant is not affected (I. Baroli and K.K. Niyogi, unpublished results), suggesting that the bleaching phenotype is caused not by increased rates of photoinhibitory damage to the core of PSII but rather by increased chlorophyll-sensitized formation of reactive oxygen species in the thylakoid membranes caused by the lack of protective xanthophylls. Thus, in Chlamydomonas, although both lutein and zeaxanthin are known to be involved in the quenching of ^{1}Chl* (Niyogi et al., 1997b), they also may play a role in photoprotection by inactivating other intermediate excited states produced during photosynthesis, especially $^1O_2$*.

A fruitful strategy to identify interacting components of a complex biological system for which mutants are available is to isolate extragenic mutations that modify (by either enhancing or suppressing) a mutant phenotype (Prelich, 1999). The *npq1 lor1* double mutant of Chlamydomonas is particularly suitable for extragenic suppressor analysis. The individual mutations are very stable and likely null alleles, and the bleaching phenotype imposes a strong selective pressure for suppressors that survive in high light. At least three types of suppressor mutations can be envisioned. Mutations that affect xanthophyll metabolism may lead to the accumulation of precursors not found normally in the chloroplast that are able to quench reactive oxygen more efficiently than the epoxidized xanthophylls violaxanthin and neoxanthin. Another type of suppressor mutation could cause the increased accumulation of nonxanthophyll antioxidants, which may fulfill the antioxidant role normally performed by lutein or zeaxanthin. A third kind of suppressor mutation could interfere with LHC assembly, decreasing the antenna size and preventing the formation of excess ^{1}Chl* under light stress (Hippler et al., 2000).

Here, we describe suppressors that affect xanthophylls and present evidence that constitutive accumulation of zeaxanthin, independent of the function of the xanthophyll cycle and in the absence of lutein, is sufficient to prevent photobleaching under light or other oxidative stress conditions in Chlamydomonas.

RESULTS

Isolation of Suppressors of *npq1 lor1*

Our physiological experiments were performed on Chlamydomonas cells grown photoautotrophically in minimal medium under two light conditions, 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (low light [LL]) and 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (high light [HL]). It is known that in HL, the wild type shows no signs of light stress; rather, it exhibits an acclimation response characterized by an increased number of photosynthetic units, an increased PSII:PSI stoichiometry (Neale and Melis, 1986), and a greater degree of xanthophyll deepoxidation (Niyogi et al., 1997b) relative to LL conditions. Figure 2 shows the growth phenotypes of the wild type and the zeaxanthin- and lutein-deficient *npq1 lor1* double mutant in minimal medium under the two light conditions. Both strains grew in LL, but the double mutant showed no growth in HL, in contrast to the wild type. The HL sensitivity of *npq1 lor1* was rescued partially by growth under a lower oxygen tension (data not shown). Growth in acetate-containing medium, however, did not prevent cell death, although bleaching was retarded (data not shown). These results suggest that excess reactive oxygen species, such as $^1O_2$*, that are generated in the *npq1 lor1* double mutant in HL affect essential cellular processes besides photosynthesis.

To investigate the contribution of the different xanthophylls to the process of photoprotection, we isolated and characterized genetic suppressors of the HL sensitivity of the *npq1 lor1* mutant. We observed that when the *npq1 lor1* double mutant was exposed to HL, stable suppressors arose spontaneously as green-colored colonies in a bleached background, with a frequency of $\sim 10^{-4}$. Among a collection of $\sim$200 suppressed strains able to grow in HL, 40% showed a change in pigment composition relative to the parental strain, and the vast majority in this class showed an increased and constitutive accumulation of zeaxanthin or of both zeaxanthin and antheraxanthin. Under LL growth conditions, the fraction of β-carotene–derived xanthophylls that was accumulated as zeaxanthin varied in these suppressor strains, from those with a complete block in

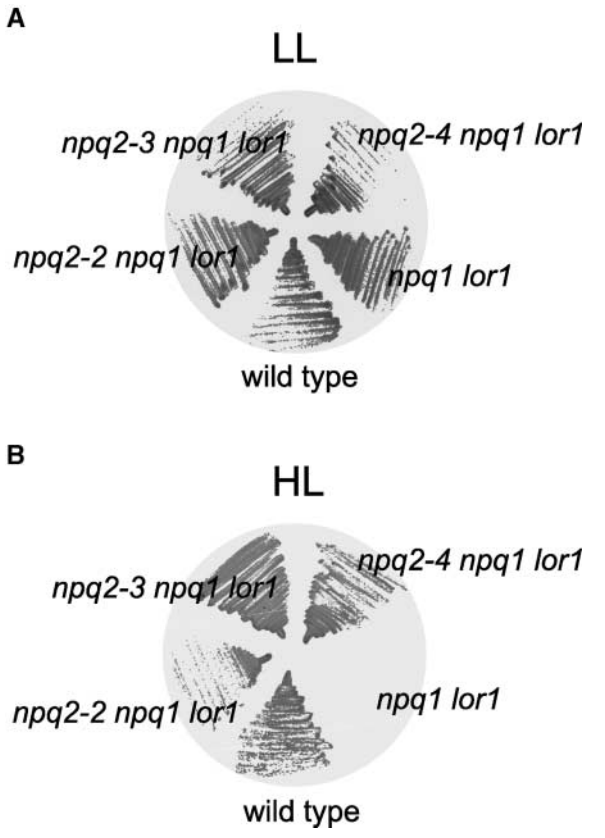

Figure 2. Growth of Chlamydomonas Strains in Minimal Medium under Continuous Light.

(A) Cells grown in LL (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 7 days.
(B) Cells grown in HL (500 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 7 days.

zeaxanthin epoxidation (accumulation of only zeaxanthin) to those with a molar ratio of zeaxanthin:antheraxanthin:violaxanthin of 0.6:0.4:1.

Three independently isolated suppressors, which exemplify varying degrees of constitutive accumulation of zeaxanthin, were selected for further genetic and physiological characterization. As shown below, these suppressed strains were found to contain mutated alleles of the *NPQ2* gene; therefore, they are called *npq2-2 npq1 lor1*, *npq2-3 npq1 lor1*, and *npq2-4 npq1 lor1*, in decreasing order of relative zeaxanthin accumulation. As shown in Figure 2, all three suppressed strains grew at approximately wild-type rates in both LL and HL under photoautotrophic conditions.

Pigment Content and Photosynthetic Characteristics of Suppressors

Figure 3 compares the relative content of β-carotene–derived xanthophylls in wild-type and mutant strains grown under either LL or HL conditions. When the wild-type strain was grown in HL, the content of deepoxidized xanthophylls increased significantly compared with that in LL, such that the xanthophyll cycle deepoxidation state ([zeaxanthin + antheraxanthin]/[violaxanthin + antheraxanthin + zeaxanthin], [Z+A]/[V+A+Z]) increased from 0.11 ± 0.01 in LL to 0.62 ± 0.10 in HL (Figures 3A and 3B). Also in the HL-grown wild type, there was a doubling of the total xanthophyll cycle pool relative to chlorophyll *a* (Chl *a*) (Figure 3A). The double mutant *npq1 lor1* showed a deepoxidation state of 0.20 ± 0.02 under continuous LL, which is greater than in the LL-grown wild type (Figure 3B). This increased deepoxidation state in *npq1 lor1* is not attributable to the light-induced activity of violaxanthin deepoxidase, which is blocked by the *npq1* mutation, but is attributable to competition between LHC proteins and zeaxanthin epoxidase for the binding of zeaxanthin and antheraxanthin (I. Baroli and K.K. Niyogi, unpublished results). The greater xanthophyll cycle pool size in *npq1 lor1* (Figure 3A) is the result of increased metabolic flux into the β-carotene branch caused by the *lor1* mutation (Niyogi et al., 1997b).

When grown in LL and compared with the wild type, the suppressor strains showed increased ratios of xanthophyll cycle pigments to Chl *a*, similar to their *npq1 lor1* parent. Under continuous HL, the xanthophyll cycle pigment content was increased highly in these suppressors, especially in *npq2-2 npq1 lor1*, in which the xanthophyll cycle pigment/Chl *a* ratio was approximately three times higher than in the HL-grown wild type. The *npq2-3 npq1 lor1* and *npq2-4 npq1 lor1* strains also showed highly increased xanthophyll cycle pigment contents in HL, although the xanthophyll cycle pigment/Chl *a* ratio was significantly smaller than in *npq2-2 npq1 lor1*. The β-carotene content also was increased greatly in *npq2-2 npq1 lor1* compared with the wild type in LL and especially in HL. The *npq2-3 npq1 lor1* and *npq2-4 npq1 lor1* showed increased accumulation of β-carotene only in HL (Figure 3C).

The xanthophyll composition of the suppressors suggested that these strains are partially impaired, to different degrees, in the activity of zeaxanthin epoxidase (Figure 1). The *npq2-2 npq1 lor1* strain had undetectable levels of neoxanthin and violaxanthin, and the deepoxidation state was 1.0 irrespective of growth conditions (Figure 3B), indicating a severe block in zeaxanthin epoxidation. The block was not complete in this strain, though, because low levels of antheraxanthin accumulated in both light conditions (Figure 3A). The other two suppressor strains, *npq2-3 npq1 lor1* and *npq2-4 npq1 lor1*, showed intermediate levels of xanthophyll epoxidation with respect to the parental strain and the more extreme case of *npq2-2 npq1 lor1*. They showed some change in deepoxidation state when grown in HL, but they maintained high levels of zeaxanthin and antheraxanthin under both light conditions (Figure 3B).

Table 1 compares the photosynthetic performances of the wild type and the strains with xanthophyll deficiencies. The

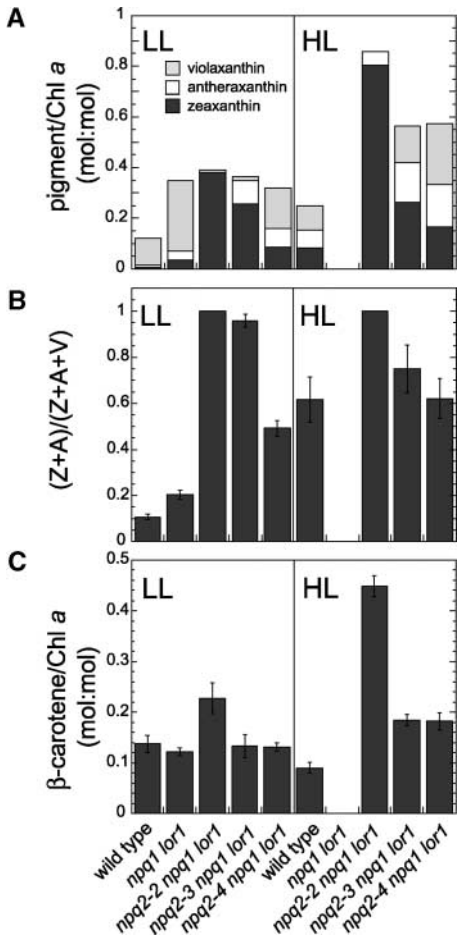

Figure 3. Pigment Characteristics of Chlamydomonas Strains.

(A) Relative content of xanthophyll-cycle pigments.
(B) Deepoxidation state, (Z+A)/(V+A+Z).
(C) Relative content of β-carotene.
Cells were grown on plates in minimal medium under either continuous LL or HL. Values shown are means of three to five independent experiments. In **(A)**, the standard deviation was ≤15% of the means. In **(B)** and **(C)**, the error bars represent standard deviations.

Table 1. Photosynthetic Characteristics of Chlamydomonas Strains

Strain	Growth Irradiance ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Total Cellular Chlorophyll Content (pg)	Chl *a*/Chl *b* Ratio (mol:mol)	F_v/F_m	Maximum Rate of Oxygen Evolution (nmol oxygen·$min^{-1} \cdot 10^{-6}$ cells)	Relative Photosynthetic Efficiency (arbitrary units)
Wild type	50	2.66 ± 0.71 (10)	2.58 ± 0.08 (9)	0.764 ± 0.006 (3)	8.18 ± 1.38 (7; 2)	0.027
npq1 lor1	50	1.93 ± 0.58 (10)[a]	3.45 ± 0.21 (10)[a]	0.777 ± 0.009 (3)	11.30 ± 1.12 (5; 2)	0.030
npq2-2 npq1 lor1	50	1.56 ± 0.70 (6)[a]	2.91 ± 0.33 (5)[a]	0.715 ± 0.005 (3)[a]	9.01 ± 1.78 (5; 2)	0.023
npq2-3 npq1 lor1	50	1.94 ± 0.38 (5)[a]	2.88 ± 0.09 (4)[a]	0.701 ± 0.005 (3)[a]	12.12 ± 1.05 (2; 2)	0.025
npq2-4 npq1 lor1	50	2.49 ± 0.81 (5)	3.05 ± 0.27 (5)[a]	0.764 ± 0.008 (3)	7.43 ± 1.06 (5; 2)	0.018
Wild type	500	1.38 ± 0.29 (6)	2.93 ± 0.18 (7)	n. d.	7.19 ± 1.49 (5; 3)	0.021
npq1 lor1	500	No growth	No growth	No growth	No growth	No growth
npq2-2 npq1 lor1	500	0.82 ± 0.43 (8)[a]	5.93 ± 1.20 (9)[a]	n. d.	11.23 ± 0.68 (2; 1)	0.012
npq2-3 npq1 lor1	500	0.88 ± 0.30 (4)[a]	5.45 ± 0.76 (4)[a]	n. d.	5.66 ± 0.98 (4; 2)	0.015
npq2-4npq1 lor1	500	1.04 ± 0.32 (6)	5.13 ± 0.74 (6)[a]	n. d.	6.73 ± 1.09 (5; 3)	0.017

Values are given as means ± SD (or, for $n = 2$, means ± difference from the mean), with the number of independent cultures analyzed in parentheses. In the case of the maximum rate of oxygen evolution, both the number of measurements and the number of independent cultures are given as: (measurements; cultures). n.d., not determined.

[a] Significantly different from wild-type values under the same conditions using a two-tailed *t* test ($P < 0.05$).

wild-type strain showed the expected response to light (Neale and Melis, 1986), with a slightly increased Chl *a*/Chl *b* ratio, twofold lower cellular chlorophyll content, and decreased photosynthetic efficiency in HL compared with LL. All of these changes are thought to reflect an acclimation of the photosynthetic apparatus to the ambient irradiance (MacIntyre et al., 2002). Compared with the wild type under each light condition, the suppressors showed alterations in cellular chlorophyll content and in Chl *a*/Chl *b* ratios. The degree of change was approximately proportional to the xanthophyll deepoxidation index. The *npq2-2 npq1 lor1* and *npq2-3 npq1 lor1* suppressors showed significantly decreased chlorophyll content in both LL and HL. In *npq2-4 npq1 lor1*, the strain that retained higher levels of violaxanthin, the chlorophyll content was similar to that of the wild type in both light conditions. All three suppressor strains had an increased Chl *a*/Chl *b* ratio, and this was more evident in HL, with an almost twofold increase in the ratio between LL and HL conditions. Because the ratio of the two chlorophylls did not change significantly in the HL-grown *lor1* and *npq1* single mutants (Niyogi et al., 1997b), we conclude that the extreme Chl *a*/Chl *b* values observed in HL in the triple mutants are caused by the lack of violaxanthin and/or neoxanthin, which may make the LHC polypeptides more unstable. The Chlamydomonas *npq2 lor1* mutant has been shown to have a smaller PSII light-harvesting antenna than the wild type (Polle et al., 2001), and we observed the same effect in the suppressors (see below).

Overall photosynthesis, measured as the saturated rate of oxygen evolution, showed no significant trend with respect to xanthophyll epoxidation, but there was a high degree of variance in the oxygen evolution data. However, the relative photosynthetic efficiency, measured from the slope of photosynthesis-irradiance response curves, showed an inverse relationship with zeaxanthin content when the strains were grown in HL (Table 1). These results, together with the changes in Chl *a*/Chl *b* ratios, indicate that the suppressors with higher zeaxanthin content had a decreased efficiency of light harvesting compared with the wild type.

When grown in LL, the suppressors with high degrees of zeaxanthin accumulation (*npq2-2 npq1 lor1* and *npq2-3 npq1 lor1*) showed a significant decrease in the efficiency of photochemical conversion at PSII, measured as the chlorophyll fluorescence parameter F_v/F_m (maximum photochemical efficiency of PSII in the dark-adapted state; Table 1). The suppressor *npq2-4 npq1 lor1*, which has a xanthophyll cycle pigment content more similar to that of the wild type, showed wild-type values of F_v/F_m. Induction of NPQ during the exposure of LL-grown cells to 1300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ remained aberrant in the *npq2 npq1 lor1* strains (data not shown), consistent with measurements of NPQ in the zeaxanthin-accumulating *npq2-1*::*ARG7* mutant of Chlamydomonas (Niyogi et al., 1997a).

Genetic Characterization of the Zeaxanthin-Accumulating Suppressors

To determine the genetic basis of the suppression phenotype in the zeaxanthin-accumulating strains, we backcrossed these mutants to the corresponding *npq1 lor1* strain of the opposite mating type (Table 2) and analyzed progeny from dissected tetrads for their resistance to HL and their pigment composition. Figure 4 shows an example of tetrad analysis in a backcross of *npq2-3 npq1 lor1 mt*− and *npq1 lor1 mt*+. In crosses of all of the suppressed strains, the HL-bleaching phenotype segregated with a 2:2 ratio, demonstrating that the suppression of HL sensitivity results from a single nuclear mutation. HPLC analysis of the meiotic progeny showed perfect cosegregation of the HL resistance phenotype and the zeaxanthin accumulation phenotype (Table 2).

Analysis of vegetative diploid strains homozygous for *npq1* and *lor1* and heterozygous for the suppressor showed that the three *npq2* suppressor mutations were recessive in terms of both pigment accumulation and bleaching in HL. As shown in Table 3, the presence of one wild-type copy of the suppressor gene in the three heterozygous diploid strains abolished the accumulation of zeaxanthin to higher levels and did not allow wild-type rates of growth in HL. We observed that all of the diploid strains were able to sustain some growth in HL, even though the zeaxanthin content was low, and this was true for the *npq1/npq1 lor1/lor1* homozygous diploid strain as well (Table 3).

The Suppressors Are New Alleles of *npq2*

The pigment composition of the suppressed strains suggested that, in addition to the original *npq1* and *lor1* mutations, the strains contain a mutation that results in the impairment of zeaxanthin epoxidation. The Chlamydomonas *npq2-1*::*ARG7* mutation, which causes the constitutive accumulation of zeaxanthin in the chloroplast independently of light conditions, has been described previously (Niyogi et al., 1997a). To examine whether the suppressor mutations were alleles of *npq2*, both linkage and complementation tests were performed. We tested the *npq2-2* suppressor for allelism with *npq2-1*::*ARG7* by crossing *npq2-2 npq1 lor1* to an *npq2-1*::*ARG7 lor1* strain of the opposite mating type (the presence of the *lor1* mutation in the *npq2-1*::*ARG7* strain facilitated the pigment analysis). No recombinant progeny with wild-type zeaxanthin epoxidation were observed by HPLC analysis among the meiotic progeny from seven tetrads, strongly suggesting that this zeaxanthin-accumulating suppressor strain carried a mutation in the *NPQ2* gene. The segregation of the mating-type locus alleles occurred at the expected 2:2 ratio among the same progeny.

For the other two suppressors, we tested allelism by complementation analysis with the *npq2-2* allele. Stable vegetative diploid strains were recovered as Arg prototrophs after crosses between *npq2-2 npq1 lor1 arg7-1 mt+* and either *npq2-3 npq1 lor1 arg7-8 mt−* or *npq2-4 npq1 lor1 arg7-8 mt−*. As shown in Table 3, HPLC analysis of the pigment content of the diploid strains showed that the characteristic accumulation of violaxanthin of the initial *npq1 lor1* strain was not recovered, demonstrating that there was no complementation of the zeaxanthin epoxidation defect in the diploid strains. Therefore, we conclude that the three suppressor strains carry mutated alleles of the *NPQ2* gene that confer different degrees of zeaxanthin epoxidation.

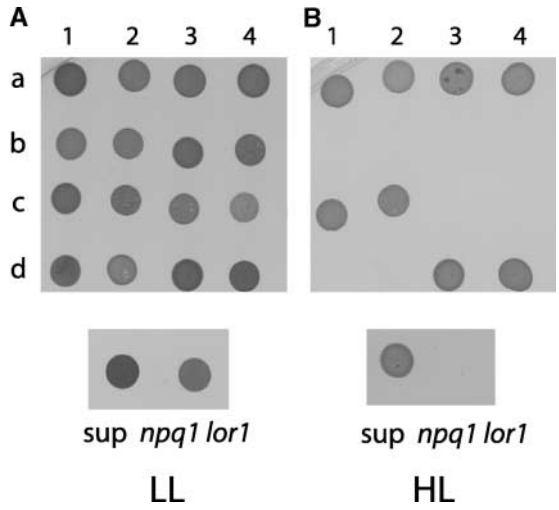

Figure 4. Genetic Analysis of a Suppressor of the HL Sensitivity of *npq1 lor1*.

An example of tetrad analysis from a backcross between the parental strain *npq1 lor1 arg7-1 mt+* and *npq2-3 npq1 lor1 arg7-8 mt−* (sup). Progeny from four tetrads were grown under continuous LL (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or HL (500 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in minimal medium for 7 days. Each vertical column represents a tetrad (labeled a–d). Both parental strains grown under the same conditions are shown as controls.

The Molecular Nature of the *npq2* Mutations

The four mutant alleles of *npq2* (*npq2-1*::*ARG7* and the three *npq2* suppressors of *npq1 lor1*) showed a defect in the epoxidation of zeaxanthin to antheraxanthin and violaxanthin, so we sought to determine whether the *NPQ2* gene corresponds to the structural gene that encodes the chloroplast-localized enzyme zeaxanthin epoxidase. Chlamydomonas ESTs with similarity to known zeaxanthin epoxidase genes from plants (Marín et al., 1996) were found in the GenBank database, and full-length cDNA sequences were obtained for genes from Chla-

Table 2. Genetic Analysis of the Zeaxanthin-Accumulating Suppressor Strains: Test for Cosegregation of Resistance to HL and Zeaxanthin Accumulation Phenotypes

Cross	Number of Independent Progeny Analyzed (Number of Complete Tetrads)	HL Resistant:HL Sensitive	Parental:Mutant Pigment Composition	Recombinant Progeny
npq2-2 npq1 lor1 mt+ × *NPQ2 npq1 lor1 mt−*	41 (3)	21:20	21:20	0
NPQ2 npq1 lor1 mt+ × *npq2-3 npq1 lor1 mt−*	18 (0)	10:8	10:8	0
NPQ2 npq1 lor1 mt+ × *npq2-4 npq1 lor1 mt−*	37 (4)	18:19	18:19	0

mydomonas and from the marine Chlamydomonas strain W80 (Miyasaka et al., 2000). The deduced amino acid sequence of the open reading frame in the Chlamydomonas cDNA clone consisted of 763 amino acid residues (Figure 5) with a predicted molecular mass of 81 kD. The cDNA from strain W80 exhibited 66.7% identity to the Chlamydomonas sequence at the DNA level, and it contained an open reading frame of 727 amino acids (Figure 5).

A multiple sequence alignment of the deduced Chlamydomonas zeaxanthin epoxidase proteins, both from Chlamydomonas and strain W80, with several known plant zeaxanthin epoxidases revealed a high degree of identity (Figure 5). Both predicted proteins contain less highly conserved, N-terminal extensions that likely function as chloroplast transit peptides. A Basic Local Alignment Search Tool (BLAST) comparison of the protein precursor sequences showed that amino acid identity of the Chlamydomonas zeaxanthin epoxidase is 59% with the *Nicotiana plumbaginifolia* and Arabidopsis enzymes, 58% with *Prunus armeniaca*, and 54% with rice. The zeaxanthin epoxidase precursor proteins from the two Chlamydomonas species are 62% identical and contain all of the motifs found in the plant ZEP proteins analyzed to date (Figure 5). Two of the three short motifs typical of the lipocalin family of proteins (Hieber et al., 2000) overlap with the central, 200-residue flavoprotein monooxygenase domain that contains the catalytic site of the enzyme. The phosphopeptide binding domain (Forkhead-associated or FHA domain), which is thought to be involved in protein–protein interactions (Durocher and Jackson, 2002), also is present. The Chlamydomonas protein also contains an Ala-rich domain in the C-terminal region that is not present in the higher plant proteins (Figure 5).

Genomic DNAs from the wild type and the *npq2* mutants were analyzed for restriction fragment length polymorphisms (RFLPs) that affect the zeaxanthin epoxidase gene by DNA gel blot hybridization. The *npq2-1*::*ARG7* mutation was isolated after insertional mutagenesis with plasmid DNA containing the argininosuccinate lyase (*ARG7*) gene and was shown to be caused by the insertion of the *ARG7* plasmid at a single locus (Niyogi et al., 1997a). Figure 6 shows that *npq2-1*::*ARG7* exhibited RFLPs with two different restriction enzymes, suggesting that it does contain a DNA insertion in the zeaxanthin epoxidase gene. By contrast, the other three *npq2* alleles present in the zeaxanthin-accumulating suppressors showed no RFLPs with any of the enzymes tested compared with wild-type DNA (Figure 6). These results suggest that in the suppressors, the spontaneously arising mutations in the zeaxanthin epoxidase gene probably are not caused by transposon insertions or by large deletions or rearrangements in the coding region of the gene and that the mutant phenotypes may be attributable to point mutations.

To determine the precise sites of the mutations in the suppressor *npq2* alleles, we compared the genomic sequences of the zeaxanthin epoxidase genes from the wild type and the suppressor strains. Based on the cDNA sequence, primers were designed to amplify and sequence overlapping genomic DNA fragments of the zeaxanthin epoxidase gene, and a 6369-bp contig of genomic sequence was assembled for the wild-type strain that contained the entire gene. Comparison of the cDNA and genomic DNA sequences revealed that the zeaxanthin epoxidase gene in Chlamydomonas consists of 10 exons (varying in length from 84 to 710 bp) and 9 introns (varying in length from 157 to 644 bp), as shown in Figure 7A. Sequencing of the gene from the *npq2* suppressor strains showed that all three suppressors contain single point mutations in the zeaxanthin epoxidase coding region (Figures 5 and 7B). The strain that carries the *npq2-2* allele, which shows the strongest zeaxanthin epoxidase mutant phenotype, has an A-to-G mutation in the predicted translation start codon. The point mutations in the other two *npq2* alleles, *npq2-3* and *npq2-4*, which show partial loss of function of zeaxanthin epoxidase, both appear in the conserved monooxygenase domain, and they are only 16 bp apart (Figure 6B). The mutation in *npq2-3* changes the coding sequence so that a Gly at position 304 is replaced by Asp, introducing a negative charge into an otherwise neutral to positively charged region of the protein. The mutation in *npq2-4*, the mildest of the three alleles, causes the replacement of the bulkier Phe at position 309 by Leu.

Effect of Light Stress on Lipid Peroxidation in *npq1 lor1* and Suppressors

To determine whether the constitutive accumulation of zeaxanthin prevented the photooxidation of membrane lipids, we fol-

Table 3. Results of Dominance and Complementation Tests

Type of Test	Genotype[a]	Pigment Phenotype in LL ([A+Z]/[V+A+Z])[b]	HL Growth[c]
Control	Wild-type diploid strain	0.36 ± 0.03 (3)[d]	++++
Control	*npq1/npq1*; *lor1/lor1*	0.14 ± 0.01 (2)	+
Dominance	*npq2-2/NPQ2; npq1/npq1*; *lor1/lor1*	0.12 ± 0.02 (2)	+
Dominance	*npq2-3/NPQ2; npq1/npq1*; *lor1/lor1*	0.16 ± 0.02 (2)	+
Dominance	*npq2-4/NPQ2; npq1/npq1*; *lor1/lor1*	0.24 ± 0.03 (2)	+
Complementation	*npq2-3/npq2-2; npq1/npq1*; *lor1/lor1*	0.99 ± 0.01 (6)	n.d.
Complementation	*npq2-4/npq2-2; npq1/npq1*; *lor1/lor1*	0.73 ± 0.04 (6)	n.d.

[a] Only the relevant genotype is shown. Diploid strains were generated by complementation of the *arg7-1* and *arg7-8* mutations.
[b] Values shown are mean ± SD (or, for $n = 2$, means ± difference from the mean).
[c] ++++, wild-type rates of growth on agar plates in HL; +, very slow growth with bleaching on agar plates in HL; n.d., not determined.
[d] The wild-type diploid strain repeatedly exhibited an increased deepoxidation state in LL compared with the wild-type haploid strain (Figure 3B).

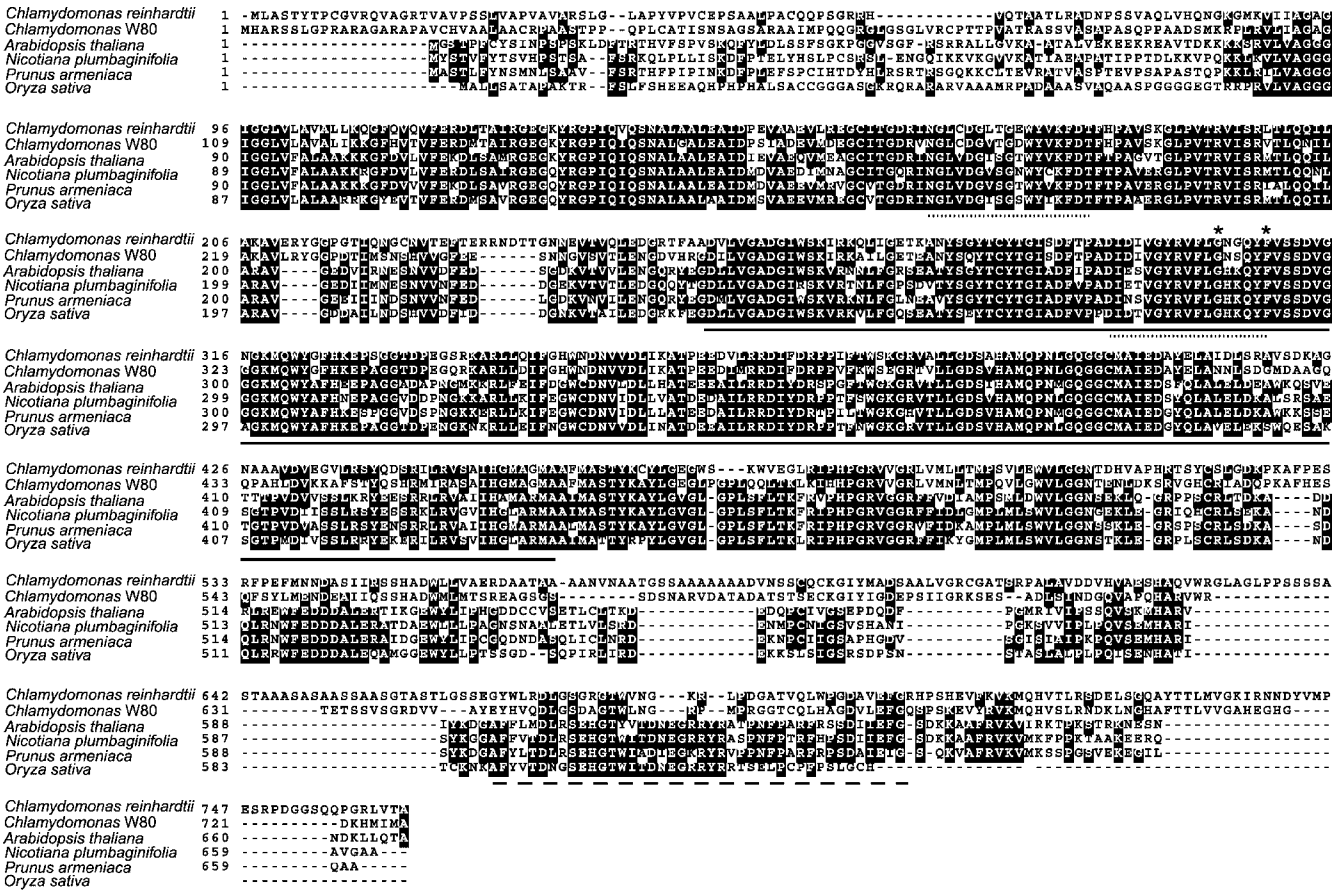

Figure 5. Alignment of the Deduced Amino Acid Sequences of Zeaxanthin Epoxidase Precursor Proteins from Chlamydomonas and Selected Plant Species.

Residues that are identical in at least three of the sequences are shaded in black. The long monooxygenase domain, which contains the putative ADP and flavin adenine dinucleotide binding sites, is shown with a solid underline, the two lipocalin conserved motifs are shown with a dotted underline, and the FHA (Forkhead-associated) domain is shown with a dashed underline. The mutation sites in the *npq2-3* and *npq2-4* alleles are indicated with asterisks. The mutation in *npq2-2* causes a change in the translation initiation codon.

lowed the accumulation of lipid peroxides in liquid cultures of the double mutant *npq1 lor1* and the suppressor strains after a transfer from continuous LL to continuous HL. The initial content of lipid peroxides, measured as thiobarbituric acid–reactive substances, in LL-grown cells was similar in all strains, in the range of 10 to 20 pmol/10^6 cells. Figure 8 shows the relative increase in lipid peroxides when cells were transferred from LL to HL. Wild-type cultures did not accumulate significant amounts of peroxidized lipids in HL, whereas in the double mutant *npq1 lor1*, the lipid peroxide content increased sixfold in the course of 48 h. The suppressor *npq2-2 npq1 lor1*, which accumulates zeaxanthin and minor amounts of antheraxanthin only, showed wild-type levels of lipid peroxidation in HL. The other two suppressor strains, with intermediate xanthophyll epoxidation levels, showed a level of lipid peroxidation during the treatment that appeared to be inversely proportional to the relative amount of xanthophyll accumulated as zeaxanthin. The measurements of lipid peroxidation as thiobarbituric acid–reactive substances were corroborated by measurements based on thermoluminescence (M. Havaux, I. Baroli, and K.K. Niyogi, unpublished results). These results show that zeaxanthin accumulation prevents lipid peroxidation induced by reactive oxygen species in HL.

Effect of Zeaxanthin Accumulation on the Organization of the Light-Harvesting Antenna

Chlamydomonas regulates the size of the PSII LHC in response to light conditions (Neale and Melis, 1986). Also, mutations that alter the chloroplast xanthophyll content are known to cause abnormalities in the composition of the LHC in algae (Chunaev et al., 1991; Bishop, 1996). Thus, we expected that zeaxanthin and antheraxanthin accumulation at the expense of violaxanthin and neoxanthin would markedly affect the LHC composi-

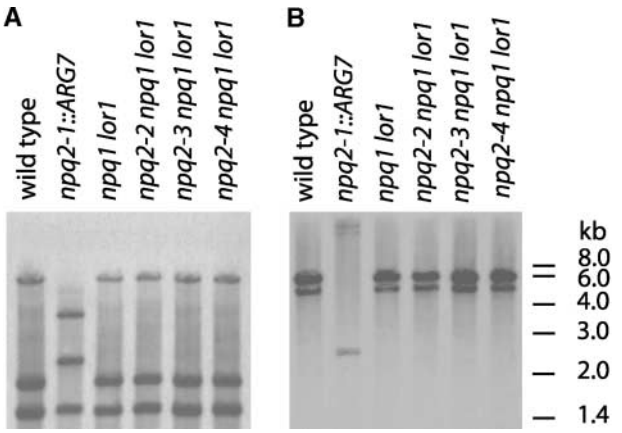

Figure 6. DNA Gel Blot Analysis of Wild-Type Chlamydomonas, the *npq1 lor1* Mutant, and Strains Carrying Mutations in the *NPQ2* Gene.

Genomic DNA was digested with restriction enzymes and probed with a 1.8-kb fragment corresponding to the 5′ portion of the zeaxanthin epoxidase cDNA.

(A) DNA digested with PstI.

(B) DNA digested with BamHI and SpeI.

tion in the suppressors. Indeed, in the double mutant *npq2 lor1*, the presence of zeaxanthin as the only xanthophyll has been shown to reduce the size of the PSII antenna complex in cells grown in LL (Polle et al., 2001). This decrease was not the result of the decreased expression of a particular LHC polypeptide, because qualitatively, all of the wild-type antenna polypeptides were detected in the mutant.

Immunoblot analysis of the *npq2 npq1 lor1* suppressor strains showed that the amounts of LHCII polypeptides were affected in LL in a manner that correlated with the relative content of zeaxanthin (Figure 9). That is, higher deepoxidation states of the xanthophyll cycle pool were associated with lower contents of LHC polypeptides. This effect was more extreme when the cells were grown under continuous HL, such that the content of the major LHC polypeptides was reduced to <20% of wild-type levels in the *npq2-2 npq1 lor1* strain. These decreased levels of LHC polypeptides were consistent with the increased Chl *a*/Chl *b* ratios observed in the suppressors (Table 1).

Effect of a Small Light-Harvesting Antenna on the High-Light Sensitivity of *npq1 lor1*

To determine if the protective effect of zeaxanthin and antheraxanthin accumulation against photooxidation could be explained by an indirect effect on the size of the light-harvesting antenna, we examined the effect of a mutation that greatly reduced the abundance of LHC polypeptides without affecting xanthophyll content, in the background of the *npq1 lor1* double mutant. The *cbn1* mutation leads to a lack of Chl *b* (Chunaev et al., 1991), and this defect causes a severe reduction in LHC size (Allen and Staehelin, 1994). We constructed a *cbn1 npq1 lor1* triple mutant and measured the xanthophyll and LHC composition of this strain. The triple mutant maintained the xanthophyll composition of the parental *npq1 lor1* strain, with a deepoxidation state of 0.11 ± 0.004 and a xanthophyll cycle pool size ([V+A+Z]/Chl *a*) of 0.634 ± 0.174 in LL. The LHCII proteins were undetectable in this mutant, even when grown in LL (Figure 9). The smaller light-harvesting antenna had a slight protective effect in HL (Figure 10), but the strain showed clear signs of photobleaching and did not grow nearly as well as the wild type or the zeaxanthin-accumulating *npq2* suppressors (Figure 2). The smaller LHC conferred by the *cbn1* mutation had no protective effect in an *npq1 lor1* background at higher light intensities. At 700 and 1100 $\mu mol \cdot m^{-2}\ s^{-1}$, the zeaxanthin-accumulating suppressors still were able to grow at wild-type rates, but the *cbn1 npq1 lor1* triple mutant did not survive (data not shown). Together, these results demonstrate that a smaller PSII LHC is not sufficient to explain the ability of the *npq2 npq1 lor1* suppressor strains to survive in HL.

Effect of Zeaxanthin Accumulation on Oxidative Stress Induced in Low Light

An alternative explanation for how zeaxanthin accumulation prevents bleaching and lipid peroxidation in HL involves a direct antioxidant function of zeaxanthin. Therefore, we tested the *npq2 npq1 lor1* suppressor strains for their ability to quench

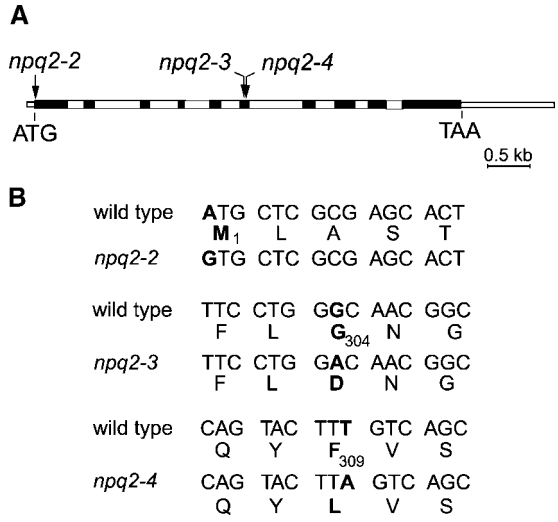

Figure 7. Structure of the Chlamydomonas Zeaxanthin Epoxidase Gene and Positions of Point Mutations.

(A) Scheme of the zeaxanthin epoxidase locus in Chlamydomonas. The gene has 10 exons (indicated in black) and 9 introns (indicated in white). The 5′ and 3′ untranslated regions are indicated by the thinner boxes. The positions of the three point mutations in the *npq2* suppressors are indicated by arrows.

(B) Sequence of the zeaxanthin epoxidase gene in the regions surrounding the point mutations showing the amino acid changes.

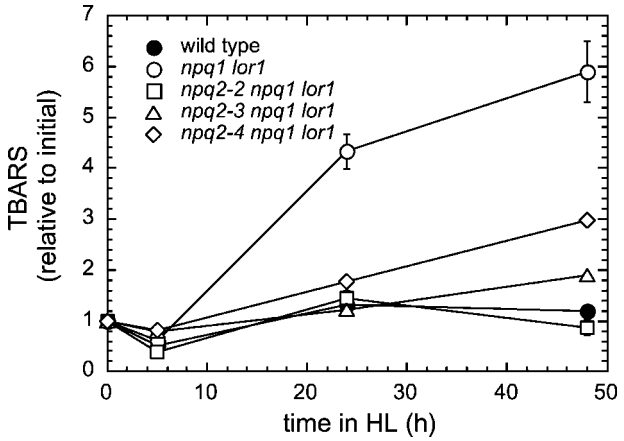

Figure 8. Lipid Peroxidation in Suppressors of *npq1 lor1* after Transfer to High Light.

Exponentially growing liquid cultures were transferred from LL to HL conditions at time 0. Lipid peroxides were measured as thiobarbituric acid–reactive substances (TBARS). The results are shown relative to the initial culture content of TBARS, which was similar in all strains tested. Data are from three independent cultures. Error bars represent standard deviations and are not shown if smaller than the symbols.

$^1O_2$* in vivo under LL conditions in which the effect of the *npq2* mutations on the LHC was minimal. Figure 11 shows that the *npq1 lor1* parental strains were more sensitive than the wild type to the presence of the $^1O_2$* photosensitizer, rose bengal, in the growth medium. Survival in the presence of exogenously generated $^1O_2$* was restored in the *npq2* suppressor strains. The degree of protection against $^1O_2$* was approximately proportional to the amount of zeaxanthin and antheraxanthin that accumulated in the mutants, because the suppressor strains showed the following order of survival: *npq2-2 npq1 lor1* $\simeq$ *npq2-3 npq1 lor1* $>$ *npq2-4 npq1 lor1* (Figure 11). These phenotypes were observed in LL, but not in the dark, consistent with the well-known light-dependent generation of $^1O_2$* by rose bengal. These results suggest that zeaxanthin and antheraxanthin are much more effective $^1O_2$* quenchers than the epoxidized xanthophylls, violaxanthin and neoxanthin, and that zeaxanthin can replace lutein as a $^1O_2$* quencher. These data also demonstrate that chloroplast-localized xanthophylls are able to protect cells from the lethal effects of $^1O_2$*.

DISCUSSION

npq2 Mutations Affect the Chlamydomonas Zeaxanthin Epoxidase Gene

The *npq2* suppressors of *npq1 lor1* led to an accumulation of zeaxanthin in the absence of lutein that was sufficient to prevent photooxidative bleaching in Chlamydomonas. We recovered a range of suppressors that showed different degrees of impairment of zeaxanthin epoxidation, from a deepoxidation state ([Z+A]/[V+A+Z]) of 1.0 to 0.5. The *npq2-2 npq1 lor1* strain, with β-carotene and zeaxanthin as the only carotenoids, showed wild-type rates of growth in both LL and HL conditions and behaved normally in crosses, indicating that the severe alteration of xanthophyll composition was not an impediment to the growth and reproduction of Chlamydomonas.

In contrast to Chlamydomonas, the *lut2 aba1* mutant of Arabidopsis, which has a pigment composition analogous to that of the Chlamydomonas *npq2-2 npq1 lor1* mutant described here, showed reduced seedling viability, poor growth, and a virescent phenotype (Pogson et al., 1998). In a developing plant seedling, the lack of specific xanthophylls may affect the assembly of the light-harvesting antenna during the greening process, thereby altering the further development of the plant. Chlamydomonas cells are capable of chlorophyll synthesis in the dark and do not undergo greening when grown in continuous illumination, so the effect of altered xanthophyll composition might not be as apparent. Also, because violaxanthin and neoxanthin can be precursors of abscisic acid (Schwartz et al.,

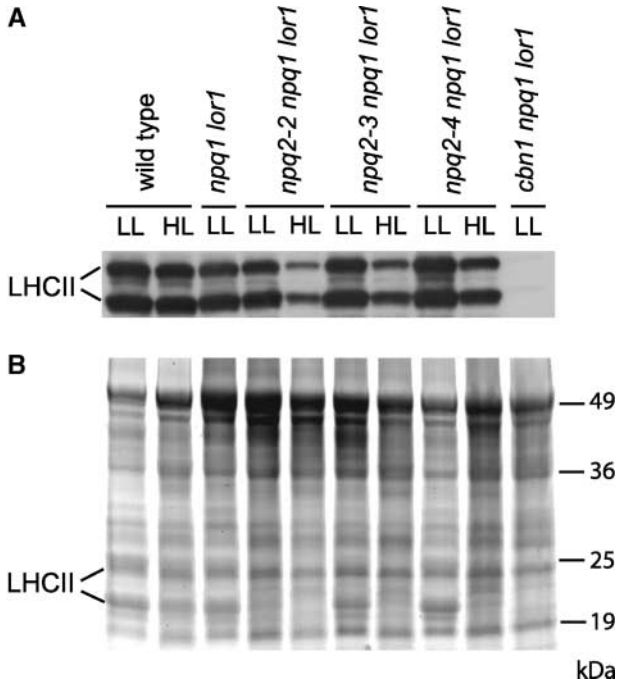

Figure 9. Protein Analysis of Chlamydomonas Whole Cell Extracts.

Cells were grown for several generations under continuous LL or HL conditions, except for mutants *npq1 lor1* and *cbn1 npq1 lor1*, which were grown only in LL. Lanes were loaded with an equal number of cells.

(A) Immunoblot analysis of the LHC of PSII. The major LHC polypeptides of PSII (LHCII) were visualized with the anti-P17 antibody (Bassi and Wollman, 1991).

(B) Coomassie blue staining of a protein gel identical to the one used in **(A)** for immunoblot analysis.

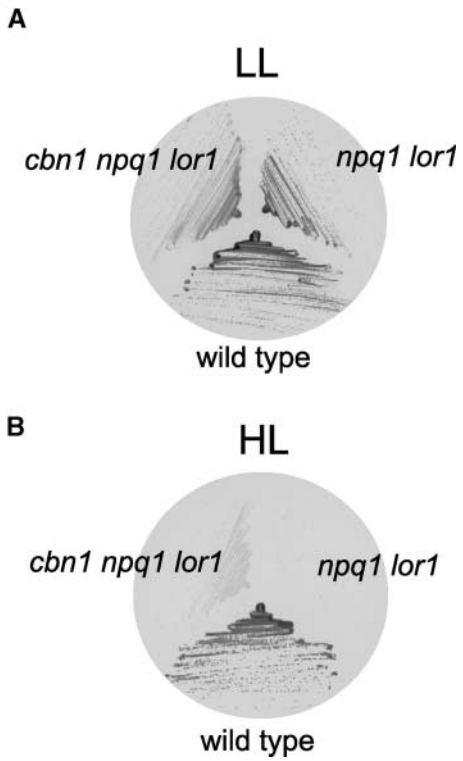

Figure 10. Effect of Light-Harvesting Antenna Size on the HL Sensitivity of *npq1 lor1*.

The *cbn1* mutation, which causes an impairment in Chl *b* biosynthesis, was introduced in the *npq1 lor1* genetic background, and the resulting strain was tested for survival in HL. The wild-type and *npq1 lor1* strains shown here are the same strains shown in Figure 1 and are included for comparison.

(A) Cells grown in minimal medium under continuous LL (50 μmol·m^{-2}·s^{-1}).

(B) Cells grown in minimal medium under continuous HL (500 μmol·m^{-2}·s^{-1}).

1997), the phenotype of the double mutant *lut2 aba1* could be attributable in part to the lack of this hormone rather than to a direct effect of the lack of violaxanthin and neoxanthin, although the delayed greening of the Arabidopsis *lut2 aba1* mutant was not rescued by exogenous abscisic acid (Pogson et al., 1998). As in the Chlamydomonas mutants, Arabidopsis plants that lack the major xanthophylls had increased Chl *a*/Chl *b* ratios and decreased chlorophyll content.

Most of the zeaxanthin-accumulating suppressors also accumulated antheraxanthin, but no strains that accumulated only antheraxanthin were recovered in our screen. These results are consistent with a defect in zeaxanthin epoxidase, which catalyzes the epoxidation of both β-rings of zeaxanthin to produce sequentially antheraxanthin and violaxanthin (Eskling et al., 1997). Zeaxanthin epoxidase is located on the stromal side of the thylakoid membrane, and it is thought that some flexibility must exist in the membrane orientation of the substrates violaxanthin and antheraxanthin for the enzyme to complete the epoxidation of both β-rings. Zeaxanthin epoxidase activity is almost completely absent in the suppressor that carries the *npq2-2* allele. The other two alleles in the suppressors, *npq2-3* and *npq2-4*, allow for incomplete epoxidation of zeaxanthin.

By determining the molecular basis for the defects in four *npq2* alleles, we showed that *NPQ2* is synonymous with the zeaxanthin epoxidase structural gene in Chlamydomonas. Furthermore, the severity of the epoxidation defects in these alleles can be explained by the nature of the mutation in each case. The reference allele, *npq2-1*::*ARG7*, contains an inactivating DNA insertion in the zeaxanthin epoxidase gene (Figure 6) that cosegregates with *ARG7* in a cross (Niyogi et al., 1997a). The *npq2-1*::*ARG7* mutation completely blocks zeaxanthin epoxidation. The *npq2-2* mutation, which allows the synthesis of very low levels of antheraxanthin, affects the predicted initiation codon of the zeaxanthin epoxidase mRNA, changing it from AUG to GUG (Figure 7). In the absence of an alternative in-frame Met codon, it is likely that a very low level of translation initiation

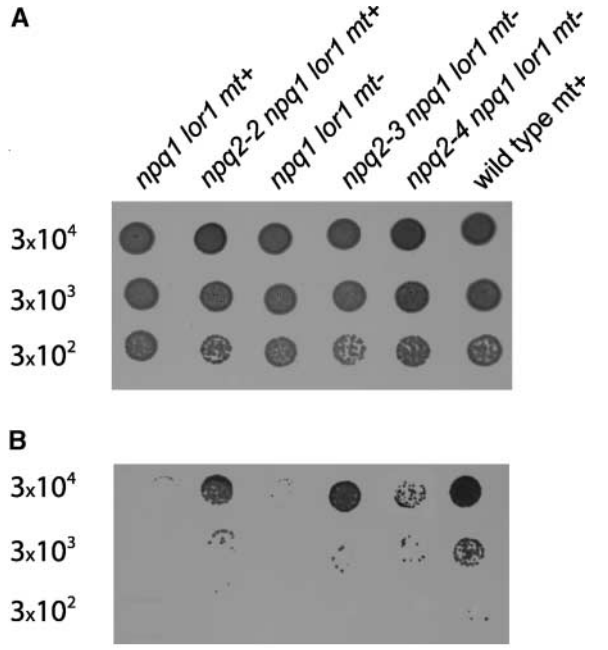

Figure 11. Response of Wild-Type and Mutant Strains of Chlamydomonas to Exogenously Generated Singlet Oxygen.

(A) Cells grown in minimal medium under continuous LL.

(B) Cells grown in minimal medium containing 3.8 μM rose bengal under continuous LL.

Because of strain variability in the response to rose bengal, each of the zeaxanthin-accumulating suppressors is shown with the corresponding parental strain as a control. Strain *npq1 lor1 arg7-8* is the parent of the suppressor carrying the *npq2-2* allele, and strain *npq1 lor1 arg7-1* is the parent of the suppressors carrying the *npq2-3* and *npq2-4* alleles. Each row represents a 10-fold serial dilution of cells at the time of plating. The total number of cells plated is indicated at left.

occurs, resulting in very low levels of zeaxanthin epoxidase enzyme expression. Indeed, site-directed mutagenesis of the start codon has been used to attenuate the expression of essential chloroplast genes in Chlamydomonas (Chen et al., 1995; Majeran et al., 2000). The mutations in *npq2-3* and *npq2-4* affect the conserved monooxygenase domain of zeaxanthin epoxidase (Figure 5), leading to partial losses of function most likely by affecting enzyme kinetics or accumulation. Consistent with the observed deepoxidation states in vivo (Figure 3B), substitution of Asp instead of Gly in *npq2-3*, which introduces a negative charge into an otherwise neutral to positively charged region, might be expected to impair function to a greater extent than the more conservative replacement of a nearby Phe by Leu in *npq2-4*. A more thorough structure-function analysis of zeaxanthin epoxidase could be performed by sequencing additional independent *npq2* alleles recovered in our suppressor screen.

Suppressors with an *npq2* mutant phenotype were recovered in our screen with high frequency ($\sim 4 \times 10^{-5}$). Results of a fluctuation test suggest that most of the suppressor mutations are induced by the HL treatment, rather than arising spontaneously in the LL culture before selection (B. Gutman and K.K. Niyogi, unpublished results). Of nine *npq2* suppressor strains that were tested, none was found to carry insertions of the Chlamydomonas transposons *Tcr1*, *Tcr2*, or *Tcr3* in the zeaxanthin epoxidase gene (I. Baroli and K.K. Niyogi, unpublished results). Thus, higher rates of transposon insertions at the *NPQ2* locus do not appear be the reason for the high frequency of suppressors. However, we cannot exclude the possibility that the *NPQ2* locus may be a "hot spot" for point mutations. Zeaxanthin epoxidase is a large protein with two conserved cofactor binding domains and regions involved in protein–protein interactions, and point mutations that cause even a minor impairment of enzyme function could lead to levels of constitutive zeaxanthin accumulation that are sufficient to allow the growth of *npq1 lor1* in HL.

How Does Zeaxanthin Accumulation Protect Cells from Photooxidation?

Zeaxanthin has multiple functions in the chloroplast that could explain the recovery of *npq2* suppressors of *npq1 lor1*. Zeaxanthin is well known for its role in the quenching of ^{1}Chl* and the thermal dissipation of excess energy (NPQ) (Müller et al., 2001). With its 11 conjugated double bonds, zeaxanthin also is an efficient quencher of ^{3}Chl*, $^1O_2$*, and free radicals, and in the case of mutant strains that lack other xanthophylls, it must fulfill a structural role in the LHC as well.

The double mutant *npq1 lor1* has greatly reduced levels of NPQ, so one conceivable way in which the constitutive accumulation of zeaxanthin could increase photoprotection in the *npq1 lor1* mutant background is by increasing the rate of thermal dissipation. However, it is unlikely that the bleaching phenotype of *npq1 lor1* in HL is caused by the lack of NPQ in the first place (Niyogi et al., 1997b). The *npq5* mutant of Chlamydomonas also shows a severe lack of NPQ but has nearly wild-type xanthophyll composition (Elrad et al., 2002), and it does not exhibit photobleaching under our HL conditions (Niyogi et al., 1997b), suggesting that maintenance of a high NPQ is not necessary for survival in HL. Paradoxically, in the *npq2* suppressors, we observed restoration of NPQ to almost wild-type levels in the strain with the lowest content of zeaxanthin and the highest content of violaxanthin, *npq2-4 npq1 lor1* (data not shown). Consistent with measurements in the *npq2-1*::*ARG7* single mutant (Niyogi et al., 1997a), the extent of NPQ was not increased in *npq2-2 npq1 lor1* with respect to the parental strain *npq1 lor1*, although this suppressor showed normal growth in HL (Figure 2). Together, these data suggest that the restoration of NPQ is not the main mechanism by which zeaxanthin (and antheraxanthin) protect the suppressor strains from photobleaching. This notion is consistent with results from Arabidopsis plants with an increased zeaxanthin content through overexpression of β-carotene hydroxylase, which show enhanced tolerance to light stress under HL, although their NPQ remains unchanged (Davison et al., 2002).

Zeaxanthin accumulation by the *npq2* suppressors might confer photoprotection indirectly by resulting in a decrease in the size of the light-harvesting antenna that feeds light energy into PSII. In general, a lower content of chlorophyll in the chloroplast might be expected to decrease the levels of ^{3}Chl* and $^1O_2$* formed in HL. Indeed, when grown in HL, the *npq2* suppressors showed lower cellular chlorophyll content and decreased accumulation of LHCII polypeptides; thus, it is possible that part of the observed decreased sensitivity to photodamage in these mutants is caused by a decrease in the photosensitized production of $^1O_2$* and free radicals. By introducing the *cbn1* mutation in the *npq1 lor1* background, we observed that decreased antenna size, in the absence of zeaxanthin and antheraxanthin accumulation, does allow for some degree of protection from photodamage (Figures 9 and 10). However, the *cbn1 npq1 lor1* strain showed partial bleaching at 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and was completely unable to grow at higher light intensities, whereas all of the *npq2 npq1 lor1* strains grew at wild-type rates. We conclude that only partial protection is afforded by a decrease in antenna size in the absence of the protective xanthophylls zeaxanthin and antheraxanthin and that the smaller LHC is not sufficient to explain the recovery of *npq2* suppressors in our screen.

The decreased accumulation of LHCII proteins in the *npq2 npq1 lor1* strains in HL could have multiple underlying causes. It is conceivable that the abnormal xanthophyll composition might interfere with the synthesis, assembly, and/or stability of the LHCs. Experiments performed in vitro have demonstrated that it is possible to reconstitute LHC proteins with chlorophylls and with either violaxanthin or zeaxanthin in place of lutein, but the yield of LHCII-zeaxanthin complexes is substantially lower than that of either LHCII-violaxanthin or LHCII-lutein complexes (Croce et al., 1999; Hobe et al., 2000), consistent with a possible assembly defect in the *npq2* suppressors in vivo. The lack of lutein caused by the *lor1* mutation is known to affect LHCII organization in Chlamydomonas (Chunaev et al., 1991), and we have observed a loss of LHCII proteins in the HL-grown *lor1* single mutant (I. Baroli and K.K. Niyogi, unpublished results) similar to that in the *npq2-2 npq1 lor1* strain (Figure 9). This finding suggests that the absence of lutein, rather than the accumulation of abnormally high levels of zeaxanthin, is responsible for the loss of LHCII proteins in HL. However, despite the

absence of lutein, the *npq2-3 npq1 lor1* and *npq2-4 npq1 lor1* strains in HL exhibited LHCII protein levels that were inversely related to the severity of the block in zeaxanthin epoxidation. In fact, LHCII levels approached wild-type levels in the HL-grown *npq2-4 npq1 lor1* strain (Figure 9). It appears that in the absence of lutein, it is the amount of accumulated antheraxanthin that is correlated with the accumulation of LHCII proteins in HL, consistent with the close structural and conformational similarity between lutein and antheraxanthin. The loss of the LHCs in the *npq2-2 npq1 lor1* strain also might be attributable in part to decreased photoprotection within the LHCs, because the LHCII-zeaxanthin complex reconstituted in vitro was found to be more sensitive to photooxidation than the wild-type complex (Formaggio et al., 2001).

However, the protective effect of zeaxanthin accumulation is enhanced outside of the LHCs, because the *npq2* suppressors were more resistant than the *npq1 lor1* starting strain to $^1O_2$* generated by an exogenous photosensitizer, rose bengal (Figure 11). Rose bengal generates $^1O_2$* throughout the growth medium and within the cell by a type-II photosensitization reaction similar to the interaction between ^{3}Chl* and ground-state O_2. The resistance of the *npq2* suppressors to rose bengal–induced photooxidation was apparent in LL, in which the effect of zeaxanthin accumulation on LHC protein levels was minor (Figures 9 and 11). This result strongly suggests that zeaxanthin could protect Chlamydomonas from photooxidation in HL by quenching $^1O_2$ and/or free radicals directly in the lipid phase of the thylakoid membrane. Alternatively, zeaxanthin might have an unidentified beneficial effect on lipid peroxide metabolism. The high zeaxanthin/chlorophyll ratio and the small antenna in the suppressors, especially the *npq2-2 npq1 lor1* strain grown in HL, suggests that Chlamydomonas chloroplast membranes can maintain a relatively high concentration of free zeaxanthin in the lipid phase. In fact, there is evidence for a role of zeaxanthin as a stabilizer of thylakoid membrane function and structure under stress conditions (Havaux, 1998). A role of zeaxanthin in quenching $^1O_2$ is consistent with the enhanced protection of the PSII reaction center D1 protein from light-induced degradation observed in the *npq2-1*::*ARG7* mutant (Jahns et al., 2000). Our results with Chlamydomonas mutants lacking or accumulating zeaxanthin also are in agreement with recent findings in Arabidopsis that zeaxanthin, besides promoting energy dissipation in the LHC, might protect from photooxidative stress by participating directly in quenching $^1O_2$* and/or free radicals in the thylakoid membrane, thus preventing the accumulation of lipid peroxides (Havaux and Niyogi, 1999; Havaux et al., 2000).

The antioxidant effect of zeaxanthin that we have shown here for Chlamydomonas is not restricted to photosynthetic membranes (Demmig-Adams and Adams, 2002). A role of membrane carotenoids in quenching $^1O_2$* also has been demonstrated in nonphotosynthetic bacteria (Tatsuzawa et al., 2000) and fungi (Schroeder and Johnson, 1995). In animals, zeaxanthin and lutein are thought to have important antioxidant functions (Mares-Perlman et al., 2002). In the primate eye, they are concentrated up to 1000-fold the serum levels in the macula lutea region of the retina, where they may protect polyunsaturated lipid membranes against light-induced damage (Landrum and Bone, 2001). Loss of zeaxanthin and lutein is correlated with the onset of age-related macular degeneration in humans, whereas increased dietary intake of xanthophylls is associated with a decreased risk of macular degeneration (Seddon et al., 1994). Although at much lower concentration than in the macula, zeaxanthin and lutein also are found in the lens epithelium and cortex, and they may help prevent the oxidation of epithelial lipids, an important etiological factor in the development of cataracts (Hammond et al., 2001). Similarities in the fatty acid composition of thylakoid and retinal membranes (an enrichment in polyunsaturated fatty acids) (Giusto et al., 2000) make Chlamydomonas an attractive system in which to study the functions of these antioxidant molecules in vivo.

In summary, we have shown that in the absence of lutein, zeaxanthin accumulation can prevent lipid peroxidation and restore growth in HL to *npq1 lor1*, a light-sensitive, xanthophyll-deficient strain of Chlamydomonas. The mechanism of protection by zeaxanthin most likely involves a direct quenching of $^1O_2$* and/or free radicals in chloroplast membranes. A decrease in the PSII light-harvesting antenna size in zeaxanthin-accumulating suppressors of *npq1 lor1* in HL also might contribute to photoprotection by decreasing the absorption of light and the generation of $^1O_2$* and by liberating additional zeaxanthin into the lipid phase of the membrane, where it could quench $^1O_2$*.

METHODS

Strains and Growth Conditions

The *Chlamydomonas reinhardtii* strains used in this study are described in Table 4. The wild-type strain CC-125 and the chlorophyll (Chl) *b*–less *cbn1-48* mutant CC-1354 were obtained from the Chlamydomonas Genetics Center (Duke University, Durham, NC). For physiological studies, cells were grown photoautotrophically in 100 mL of minimal (HS) medium (Harris, 1989) at 25°C with shaking in air in sterile beakers. Continuous illumination was provided from the top by cool-white fluorescent lights at a PFD of 50 μmol·m^{-2}·s^{-1} (low light [LL]) or 500 μmol·m^{-2}·s^{-1} (high light [HL]). The light path through the cultures was 3.5 cm. For HPLC determination of pigment content and for suppressor screening, cells were exposed to continuous HL or LL on agar plates containing

Table 4. Genotypes of Strains Used in This Study

Genotype	Reference
Wild-type *mt+*	Harris, 1989
npq1 lor1 mt+	Niyogi et al., 1997b
npq2-1::*ARG7 mt+*	Niyogi et al., 1997a
npq2-1::*ARG7 lor1 mt−*	This work
npq1 lor1 arg7-1 mt+	This work
npq1 lor1 arg7-8 mt−	This work
npq2-2 npq1 lor1 arg7-1 mt+	This work
npq2-3 npq1 lor1 arg7-8 mt−	This work
npq2-4 npq1 lor1 arg7-8 mt−	This work
cbn1 npq1 lor1 mt−	This work

minimal medium. Strain stocks were maintained at 10 μmol· m^{-2}·s^{-1} on acetate-containing agar medium (Tris-acetate-phosphate) (Harris, 1989). Cell densities were determined using a hemocytometer. Photon flux densities were measured with a quantum meter (Li-Cor, Lincoln, NE).

For the exposure of cells to singlet oxygen generated by rose bengal, equal numbers of exponentially growing cells were transferred from HS liquid cultures to HS plates that contained 3.8 μM rose bengal. The samples were grown at a light intensity of 60 to 80 μmol·m^{-2}·s^{-1} for 6 days before growth was scored.

Isolation of Suppressors of the *npq1 lor1* High-Light Sensitivity

The double mutant strains *npq1 lor1 arg7-1 mt+* and *npq1 lor1 arg7-8 mt−* were grown in low light in HS medium to the exponential phase. A volume containing 10^6 cells was plated on HS agar medium and placed in HL. Suppressor colonies were detected 7 days after plating from a background of bleached cells. Colonies appeared with a frequency of $\sim 10^{-4}$.

Pigment Analysis and Quantification

For pigment analysis, cell samples were collected from agar plates at the growth light intensity and immediately frozen in liquid nitrogen. Pigments were extracted by vortexing in 300 μL of acetone at maximum speed for 2 min, and the extracts were filtered through a 2-μm nylon filter. Twenty microliters of each extract was separated by HPLC on a Waters Spherisorb S5 ODS1 4.6- × 250-mm cartridge column (Milford, MA) using a modification of the method developed by García-Plazaola and Becerril (1999). Pigments were eluted at a flow rate of 1.2 mL/min with a linear gradient from 100% solvent A (acetonitrile:methanol:0.1 M Tris-HCl, pH 8.0 [84:2:14]) to 100% solvent B (methanol:ethyl acetate [68:32]) for 15 min, followed by 3 min of solvent B. Pigments were detected at 445 nm with 550 nm as the reference wavelength. The concentration of individual carotenoids was determined using standard curves of purified pigments (purchased from VKI, Hørsholm, Denmark) at known concentrations. Carotenoid content is given as a molar fraction of Chl *a*, which was determined from the same HPLC run as the carotenoids, using a calibration curve constructed with Chlamydomonas cell extracts containing known amounts of spectrophotometrically determined chlorophyll (Porra et al., 1989). For total cellular chlorophyll content, the concentration of Chl *a* plus Chl *b* was determined spectrophotometrically in 90% acetone (Porra et al., 1989) using cells grown in liquid HS medium.

Measurements of Fluorescence and Oxygen Evolution

To measure fluorescence parameters and oxygen evolution, cells were grown in HS medium to the exponential phase. Chlorophyll fluorescence was measured using a pulse-amplitude modulation fluorometer (FMS2; Hansatech, King's Lynn, UK). Cells corresponding to 30 μg of Chl *a* were deposited onto 2.5-cm diameter, 12-μm pore size nitrocellulose filters by filtration and dark adapted in a moist Petri dish for 15 min before measurement. The cells were exposed to 8 min of illumination with weak far-red light (light-emitting diode source of 735 nm peak wavelength) before determination of the fluorescence parameter F_v/F_m (maximum photochemical efficiency of PSII in the dark-adapted state). Oxygen evolution of intact cells was measured with a polarographic, Clark-type oxygen electrode (Hansatech, Norfolk, UK) at 25°C. Cells (chlorophyll concentration of 2 to 5 μg/mL) were used in their original growth medium, with the addition of 4 mM $NaHCO_3$ as a terminal electron acceptor. Illumination with white light was provided by an LS2 lamp (Hansatech), and the intensity of the incident light was varied with neutral-density filters (Melles-Griot, Irvine, CA). The initial oxygen concentration in the sample was decreased to 30 to 40% saturation by bubbling with N_2 gas before measurement. Dark respiration was measured first, followed by measurements of the rate of photosynthesis at sequentially increasing irradiance levels. The linear rate of oxygen evolution was recorded for 2 to 3 min at each irradiance. The maximum photosynthetic rate was estimated from light saturation curves constructed by plotting the total (photosynthesis plus respiration) photosynthetic rate versus light intensities. The relative efficiency of photosynthesis was calculated as the slope of the linear portion of the light saturation curve (Björkman and Demmig, 1987).

Genetic Analysis

Genetic crosses and tetrad analysis were performed according to established methods (Harris, 1989). The suppressor strain *npq2-2 npq1 lor1* was isolated in the background of the Arg auxotrophic *arg7-1* mutation, and it was of mating type +. The other two suppressor strains described in this study, *npq2-3 npq1 lor1* and *npq2-4 npq1 lor1*, were in an *arg7-8 mt−* background. The allelic *arg7-1* and *arg7-8* mutations exhibit intragenic complementation, allowing the selection of stable diploid strains on medium lacking Arg for dominance and complementation tests (Harris, 1989).

Determination of Lipid Peroxides

The extent of lipid peroxidation in cells exposed to light treatments was estimated by measuring the formation of thiobarbituric acid–reactive substances as described (Vavilin et al., 1998), with minor modifications. Cells were grown in minimal medium in LL to the mid exponential phase and transferred to HL at time 0. Ten-milliliter culture aliquots were taken before and at intervals during exposure to HL. Butylated hydroxytoluene was added to the samples at a final concentration of 0.01% (w/v) to terminate lipid peroxidation chain reactions. The cells were harvested by centrifugation at 3000*g* for 10 min at 4°C and extracted with 1.8 mL of trichloroacetic acid/thiobarbituric acid reagent. After incubation at 95°C for 25 min, samples were cooled to room temperature and spun in a microcentrifuge for 5 min at maximum speed to obtain a clear supernatant. Thiobarbituric acid–reactive substances in the supernatant were determined by absorbance at 532 nm, with a correction for nonspecific absorbance at 440 and 600 nm (Hodges et al., 1999), using a molar extinction coefficient for the thiobarbituric acid–malondialdehyde complex of 155 mM^{-1} cm^{-1}.

Preparation of Protein Extracts, SDS-PAGE, and Immunoblot Analysis

To prepare whole-cell protein extracts for SDS-PAGE, cells were harvested from 20- to 40-mL culture aliquots during the mid exponential phase of growth (1×10^6 to 3×10^6 cells/mL) by centrifugation at 3000*g* for 10 min at 4°C. The cell pellets were resuspended in a small volume of distilled water to which the same volume was added of a 2× solubilization buffer containing 500 mM Tris-HCl, pH 6.8, 20% glycerol, 7% SDS, 2 M urea, and 20% β-mercaptoethanol. Samples were vortexed at medium speed for 5 min and incubated further at room temperature for 30 min to solubilize proteins. Samples then were centrifuged at 11,000*g* in a microcentrifuge for 5 min at room temperature to pellet unsolubilized material. Proteins in the supernatant were fractionated by electrophoresis in precast 10 to 20% polyacrylamide Tris-Gly gradient gels (Invitrogen,

Carlsbad, CA). Gel lanes were loaded with proteins from an equal number of cells. Proteins were blotted onto nitrocellulose membranes, and the blots were analyzed with anti-P17 polyclonal antibodies that recognize the major Chlamydomonas LHCII proteins (Bassi and Wollman, 1991). Cross-reaction with the antibodies was detected by enhanced chemiluminescence after incubation with peroxidase-conjugated anti-IgG secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ).

Isolation of Nucleic Acids, DNA Gel Blot Analysis, and DNA Sequencing

Nucleic acids were isolated from cells grown in Tris-acetate-phosphate medium until the late exponential phase (approximately 6×10^6 cells/mL) by lysing the cells in SDS-EB buffer containing 1% SDS, 200 mM NaCl, 20 mM EDTA, and 50 mM Tris-HCl, pH 8.0. The solution was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1), and the DNA was precipitated with ethanol. DNA (10 to 15 μg) was digested with PstI or BamHI-SpeI for 16 h. After separation on 1% agarose gels, the DNA was transferred to Hybond N^+ membranes (Amersham Pharmacia Biotech) by alkaline capillary blotting, cross-linked with UV light, and hybridized at 70°C with a 1.8-kb alkaline phosphatase–labeled probe. The probe was amplified by PCR from Chlamydomonas zeaxanthin epoxidase cDNA clone AV643111 (Asamizu et al., 2000) with primers 5′-CGACTTAAACGACCTACGTCG-3′ and 5′-GCTTGTCGCCCAGCGAGCAGT-3′. The probe corresponded to the 5′ untranslated region of the gene and approximately two-thirds of the coding sequence. Of the restriction enzymes used to digest the genomic DNA, PstI cuts twice in the 1.8-kb sequence of the probe and the pair BamHI and SpeI do not cut at all. Posthybridization washes were performed at 70°C, and hybridization was detected by chemiluminescence. Nonradioactive labeling of the probe and chemiluminescence detection were performed with the AlkPhos Direct kit (Amersham Pharmacia).

The sequence of the Chlamydomonas strain W80 zeaxanthin epoxidase cDNA was obtained by completing the sequence of EST clone AB009147 (Miyasaka et al., 2000). Similarly, the entire Chlamydomonas zeaxanthin epoxidase cDNA sequence was obtained by completing the sequence of EST clone AV643111 (Asamizu et al., 2000). To sequence the zeaxanthin epoxidase gene from the Chlamydomonas wild type and *npq2* mutant alleles, a series of synthetic primers were designed based on the cDNA sequence and used to amplify overlapping fragments ranging in length from 0.4 to 2.0 kb from genomic DNA. Sequencing of the PCR fragments on both strands was performed with the Big-Dye Terminator Version 3 Kit (Applied Biosystems, Foster City, CA) and an ABI 3100 automated DNA sequencer (Applied Biosystems), and the sequences were assembled into a contig using the Lasergene MegAlign software package (DNASTAR, Madison, WI). Each region containing a putative point mutation was amplified in at least three independent PCR procedures and sequenced on both DNA strands. The Chlamydomonas zeaxanthin epoxidase genomic DNA sequence was deposited in the GenBank database. The zeaxanthin epoxidase protein sequences were aligned using the T-COFFEE algorithm (www.ch.embnet.org/software/TCoffee.html), and the alignment figure (Figure 5) was created with Boxshade software (www.ch.embnet.org/software/BOX_form.html).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Numbers

The GenBank accession numbers for the new sequences reported in this article are AY211268 (strain W80 zeaxanthin epoxidase complete cDNA), AY212923 (Chlamydomonas zeaxanthin epoxidase complete cDNA), and AY211267 (Chlamydomonas zeaxanthin epoxidase genomic DNA).

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