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Molecular genetics of xanthophyll-dependent photoprotection in green algae and plants

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The involvement of excited and highly reactive intermediates in oxygenic photosynthesis inevitably results in the generation of reactive oxygen species. To protect the photosynthetic apparatus from oxidative damage, xanthophyll pigments are involved in the quenching of excited chlorophyll and reactive oxygen species, namely $^1Chl^*$, $^3Chl^*$, and $^1O_2^*$. Quenching of $^1Chl^*$ results in harmless dissipation of excitation energy as heat and is measured as non-photochemical quenching (NPQ) of chlorophyll fluorescence. The multiple roles of xanthophylls in photoprotection are being addressed by characterizing mutants of *Chlamydomonas reinhardtii* and *Arabidopsis thaliana*. Analysis of *Arabidopsis* mutants that are defective in $^1Chl^*$ quenching has shown that, in addition to specific xanthophylls, the *psbS* gene is necessary for NPQ. Double mutants of *Chlamydomonas* and *Arabidopsis* that are deficient in zeaxanthin, lutein and NPQ undergo photo-oxidative bleaching in high light. Extragenic suppressors of the *Chlamydomonas npq1 lor1* double mutant identify new mutations that restore varying levels of zeaxanthin accumulation and allow survival in high light.

Keywords: lutein; non-photochemical quenching; photoprotection; xanthophyll cycle; zeaxanthin

1. INTRODUCTION

The reactions of oxygenic photosynthesis involve the formation of excited and highly reactive intermediates in the thylakoid membranes of chloroplasts. These reactions fuel essentially all life on Earth, but they pose a major problem for plants and algae with respect to the generation of reactive oxygen species (Niyogi 1999). Chlorophyll (Chl), the essential pigment for light harvesting and photochemical energy conversion, can act in its triplet excited state ($^3Chl^*$) as a potent photosensitizer in the generation of singlet oxygen ($^1O_2^*$), one of several reactive oxygen species that are inevitable by-products of oxygenic photosynthesis. To prevent photo-oxidative damage that may result from reaction of reactive oxygen species with pigments, proteins and lipids, plants must produce antioxidants that are able to quench $^3Chl^*$ and/or scavenge reactive oxygen species. Carotenoid pigments in the chloroplast serve this essential protective function (see figure 1).

Carotenoids are C_{40} tetraterpenes that are widely distributed in living organisms. The polyene chain of carotenoids, consisting of conjugated double bonds, is responsible for the pigmentation of carotenoids and their ability to absorb photons in the visible wavelengths. Carotenoids are synthesized by all photosynthetic organisms, as well as by many non-photosynthetic bacteria and fungi. There are two main classes of naturally occurring carotenoids: the carotenes, which are hydrocarbons that are either linear or cyclized at one or both ends of the molecule (such as β-carotene and α-carotene); and the xanthophylls, which are oxygenated derivatives of carotenes (these include violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, lutein and loroxanthin; see figure 2).

Green plants have a remarkably similar carotenoid composition. The main carotenoids in photosynthetic tissue of land plants are lutein (45% of the total), β-carotene (25–30%), violaxanthin (10–15%) and neoxanthin (10–15%) (Britton 1993). Under high light conditions, chloroplasts also accumulate zeaxanthin and antheraxanthin. In contrast to land plants, the algae show much greater diversity in their pigment composition. In general, green algae (Chlorophyta) possess a carotenoid composition similar to that of land plants, although additional xanthophylls are sometimes found, such as loroxanthin in *Chlamydomonas reinhardtii* (figure 2).

In algae and plants, carotenoids accumulate in all chloroplast membranes. Some of the carotenoids, especially xanthophylls such as violaxanthin, can be found in the chloroplast envelope, where it is hypothesized that carotenoid synthesis takes place (Douce & Joyard 1996; Joyard *et al.* 1998). It is not clear yet whether carotenoids in the envelope are organized in pigment–protein complexes or free in the membrane lipid phase. However, the majority of the carotenoids are located, together with the chlorophylls, in functional pigment-binding proteins embedded in the thylakoid membrane.

In the thylakoid membrane, β-carotene is more abundant in the reaction centres of photosystem I (PS I) and photosystem II (PS II), whereas the xanthophylls are preferentially distributed in the light-harvesting complexes (LHCs) that surround the reaction centres and transfer excitation energy to them. The reaction centre core of PS I contains 14 β-carotene molecules. Although the arrangement of light-harvesting chlorophyll molecules

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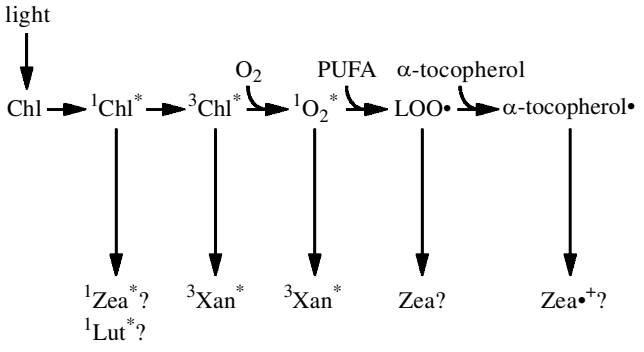

Figure 1. Antioxidant interactions of xanthophylls (Xan) in photosynthetic membranes. Absorption of light by chlorophyll (Chl) results in singlet excitation (^{1}Chl*) that can drive photochemistry. In excessive light, zeaxanthin (Zea) and lutein (Lut) are involved in non-photochemical de-excitation of ^{1}Chl* that may involve direct singlet–singlet energy transfer between ^{1}Chl* and these xanthophylls (^{1}Zea* or ^{1}Lut*). The excited singlet carotenoid can return to the ground state by internal conversion, which results in harmless dissipation of excitation energy as heat. ^{1}Chl* can also yield triplet excited chlorophyll (^{3}Chl*) by intersystem crossing, and ^{3}Chl* may interact with ground state O_2 to generate singlet excited oxygen ($^1O_2$). Both ^{3}Chl* and $^1O_2$ can be quenched by xanthophylls, producing triplet excited xanthophylls (^{3}Xan*) that decay to ground state harmlessly by internal conversion. $^1O_2$ can react with polyunsaturated fatty acids (PUFA) in the thylakoid membrane to generate lipid peroxyl radicals (LOO·). Along with α-tocopherol, Zea may be important in terminating lipid peroxyl radical chain reactions. Zea may also be involved in regeneration of oxidized α-tocopherol. Question marks indicate interactions in which the mechanistic role of the xanthophyll is uncertain.

in the crystal structure of PS I of cyanobacteria has been described (Schubert *et al.* 1997), the organization of β-carotenes in PS I has not been resolved. The LHC of PS I comprises two copies of each of four polypeptides, Lhca1–Lhca4 (Simpson & Knoetzel 1996), which together bind 100 chlorophyll $a+b$ molecules, 24 luteins and nine violaxanthins (Siefermann-Harms 1985). The presence of β-carotene has not been reported in LHC I, but its concentration in PS I is decreased by half when LHC I is removed from PS I, suggesting that β-carotene may be loosely attached to LHC I (Yamamoto & Bassi 1996).

In PS II, one or two molecules of β-carotene are present per reaction centre (Gounaris *et al.* 1990; Kobayashi *et al.* 1990), whereas xanthophylls are restricted to the antenna polypeptides. The reaction centre of PS II (Barber & Kühlbrandt 1999) is associated with an inner antenna complex, composed of the pigment-binding proteins CP43 and CP47 that each bind approximately 25 chlorophyll *a* and two or three β-carotenes, with lutein probably present in substoichiometric amounts (Bassi *et al.* 1993). CP43 and CP47 are in turn surrounded by the so-called minor LHC II proteins: Lhcb4, Lhcb5, and Lhcb6, which each bind one or two molecules of lutein and the majority of the violaxanthin in the LHC II (Bassi *et al.* 1993; Ruban *et al.* 1994; Lee & Thornber 1995; Färber *et al.* 1997). The minor LHC II pigment–protein complexes make contact with trimers of the Lhcb1, Lhcb2 and Lhcb3 polypeptides that bind about 65% of all the chlorophyll in the antenna of PS II. A high resolution crystal structure of Lhcb1 (Kühlbrandt *et al.* 1994) shows the central position in the protein of two xanthophyll molecules, probably luteins. Occupancy of at least one of these sites is thought to be essential in maintaining the proper structure of the Lhcb1 protein (Croce *et al.* 1999).

Carotenoids can act as accessory light-harvesting pigments by absorbing in the range of 450–570 nm, where chlorophyll does not absorb efficiently. This absorption arises from the strongly allowed transition from the ground state (S_0) to the second excited singlet state (S_2) of the carotenoid. The S_2 state has a short lifetime of 200 fs (femto seconds) or less (Truscott 1990) and consequently a very low quantum yield of fluorescence. The transition from S_0 to the lowest excited singlet state of the carotenoids, S_1, is optically forbidden, but S_1 is readily populated by relaxation (internal conversion) from S_2. The S_1 state decays almost entirely by internal conversion (heat emission) or energy transfer. Fluorescence from S_1 is virtually undetectable, and intersystem crossing to the triplet state is unobserved. Because of the difficulty in observing the S_1 state, the S_1 energy level for only some of the carotenoids involved in photosynthesis is known in organic solvents (Frank *et al.* 2000; Polívka *et al.* 1999), and no measurements have been done in the pigment–protein complexes themselves. The excitation energy absorbed by carotenoids can be transferred to ground state chlorophyll by singlet–singlet resonance, probably via a Dexter-type interaction that involves the overlap of electron clouds from the two interacting partners (Siefermann-Harms 1985), and thus requires the close proximity of chlorophylls and the carotenoids acting in light harvesting. However, the relative contribution of the carotenoid S_1 and S_2 states in excitation energy transfer to chlorophyll in the LHCs is still under debate. Subpicosecond time-scale studies of energy transfer in the antenna of photosynthetic bacteria have suggested that energy donation to chlorophyll can proceed from the carotenoid S_2 state (Koyama *et al.* 1996; Ricci *et al.* 1996; Krueger *et al.* 1998), but more accurate estimates of the *in vivo* energies of the carotenoids are needed to clarify the roles of each of the carotenoid singlet excited states. Under optimal conditions, energy transfer from excited carotenoids to chlorophyll occurs with an efficiency of about 85% in the chloroplast (Owens 1996). Singlet excitation of chlorophyll (^{1}Chl*) can be transferred on a picosecond time-scale to neighbouring chlorophyll molecules in the LHC by resonance transfer (Van Grondelle *et al.* 1994), and excitation energy is ultimately trapped in the reaction centre of photosystems I and II by charge separation. The light-harvesting ability of carotenoids may be especially important in the aquatic environment, which is enriched in blue and blue–green light and depleted of red light absorbed by chlorophyll (Hiller 1999).

However, the essential role of carotenoids in photosynthesis involves protection against photo-oxidative damage. Especially at photon flux densities that are saturating for photosynthesis, absorption of excessive light is potentially harmful because accumulation of excitation energy in the antenna can increase the yield of ^{3}Chl* via

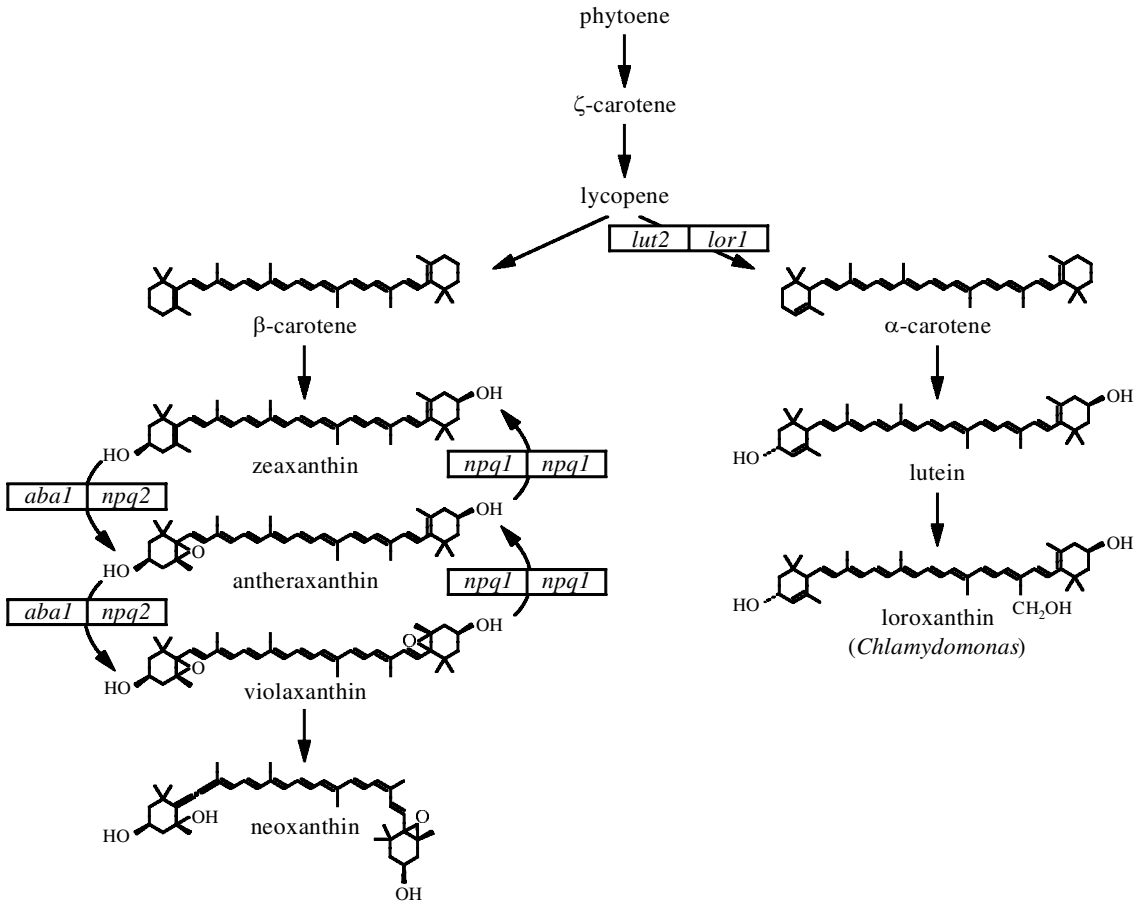

Figure 2. The xanthophyll biosynthetic pathway in green algae and plants. The steps that are blocked in mutants are indicated, with the *Arabidopsis* mutant name on the left and the *Chlamydomonas* mutant name on the right half of each box.

intersystem crossing from ^{1}Chl*. Even under optimal conditions, 10% of the absorbed light energy in PS II is lost to ^{3}Chl* formation (Owens 1996). Carotenoids can quench ^{3}Chl* by triplet–triplet energy transfer (figure 1). In isolated LHC II, this occurs with an efficiency of almost 100%; virtually no ^{3}Chl* can be observed at room temperature (Peterman *et al.* 1995). The resulting carotenoid triplets cannot react with O_2 to produce $^1O_2^*$, and they decay harmlessly by vibrational relaxation (thermal emission). However, if the rate of ^{3}Chl* formation is so high that it exceeds the quenching capacity of carotenoids, net formation of $^1O_2^*$ can occur, with associated oxidative damage. Formation of $^1O_2^*$ has been detected in leaves subjected to excessive light intensities (Hideg *et al.* 1998).

Carotenoids are also able to quench $^1O_2^*$ by energy transfer. As is the case with ^{3}Chl*, interaction with $^1O_2^*$ leads to the formation of a carotenoid triplet that decays to the ground state by thermal dissipation (figure 1). The energy level of the triplet state of the chloroplast carotenoids (which all contain nine or more conjugated double bonds) is well below that of $^1O_2^*$, making the quenching reaction irreversible. For carotenoids in general, the ability to quench $^1O_2^*$ increases with the number of conjugated double bonds. From measurements performed in benzene, violaxanthin, β-carotene and zeaxanthin (in decreasing order of effectiveness) appear as the best $^1O_2^*$ quenchers of all the carotenoids in the chloroplast, with lutein showing a rate of quenching that is about half of that of the xanthophyll cycle pigments (for a review, see Edge *et al.* 1997). Experiments with reconstituted LHC II pigment–protein complexes have shown that lutein, zeaxanthin and violaxanthin (in decreasing order of effectiveness) are involved in protecting against chlorophyll photobleaching by quenching both ^{3}Chl* and $^1O_2^*$, whereas neoxanthin functions mainly in $^1O_2^*$ quenching (Croce *et al.* 1999). The intrinsic β-carotene molecule(s) present in the reaction centre of PS II can quench $^1O_2^*$, and this quenching has been correlated with decreased photo-oxidative damage to PS II (Telfer *et al.* 1994, 1999).

In addition to the photosensitized generation of $^1O_2^*$ by the transfer of excitation energy from ^{3}Chl*, photosynthetic activity in the chloroplast can lead to sequential one-electron transfers to ground state O_2, resulting in the formation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH). These states of oxygen are also very reactive and readily form peroxides and ketones with oxidizable molecules, such as lipids and proteins. Of particular importance in the thylakoid membrane is the initiation of peroxidation chain reactions by attack of reactive oxygen species on the acyl chains of polyunsaturated fatty acids (figure 1), which are highly abundant in thylakoid lipids. Once initiated, these reactions become autocatalytic and result in massive membrane photodestruction. It is thought that tocopherols (vitamin E) are the major antioxidants responsible for breaking lipid peroxidation chain reactions (Fryer 1992), but carotenoids have also been implicated in

protection at this level (Edge *et al.* 1997). In this case the end-product of the reaction of the carotenoid with free radicals is not energy dissipation as heat but rather the transfer of an electron or an addition reaction (figure 1). Although there is evidence for the interaction of several naturally occurring carotenoids with free radicals *in vitro*, these reactions have not yet been documented *in vivo*. In artificial lipid membranes, both lutein and zeaxanthin were recently shown to terminate lipid peroxidation reactions with the same efficacy (Sujak *et al.* 1999). A relatively small pool of free xanthophylls may exist in the lipid phase of the thylakoid membrane, and these xanthophylls have been implicated in the protection of the thylakoid lipids against peroxidation chain reactions triggered by photo-oxidation (Havaux 1998).

β-carotene and α-tocopherol can act synergistically as radical scavengers in a mammalian membrane system (Palozza & Krinsky 1992). The mechanism for this interaction is not clear at present, but it has been suggested that the carotenoids would actually regenerate the α-tocopheroxyl radical (figure 1), which is produced when α-tocopherol scavenges any oxy-radical, and that the carotenoid radical cation formed in this reaction would be regenerated by ascorbic acid (Böhm *et al.* 1997). A combined action of the carotenoids lutein and zeaxanthin with α-tocopherol has also been hypothesized to aid in the prevention of macular degeneration in the human retina (Snodderly 1995).

Specific xanthophylls are also involved in quenching of ^{1}Chl*, the same excited state that initiates photochemical charge separation in photosynthesis. An increase in the transthylakoid ΔpH in excessive light leads to the thermal dissipation of excess absorbed light energy in the PS II antenna. This non-radiative dissipation is due to non-photochemical de-excitation of ^{1}Chl*, which can be measured as a lowering in the yield of chlorophyll fluorescence. It is therefore commonly referred to as non-photochemical quenching (NPQ) or energy-dependent quenching (q_E). NPQ can dissipate over 75% of the absorbed light energy (Demmig-Adams *et al.* 1996), thereby decreasing the yield of ^{3}Chl* and $^1O_2^*$ and decreasing the excitation pressure on the PS II reaction centre, which is subject to photo-oxidation and turnover of damaged proteins (Aro *et al.* 1993).

Demmig *et al.* (1987) first observed a correlation between NPQ and zeaxanthin formation in intact leaves. In low light, violaxanthin was present and no quenching was observed, whereas in high light, violaxanthin was stoichiometrically converted to zeaxanthin, in parallel with the development of NPQ. Later this correlation was found also in many other plant species and under a wide range of environmental stresses (Demmig-Adams & Adams 1994, 1996*b*). A high transthylakoid ΔpH (acting either directly on the enzyme or indirectly by increasing the amount of one of its substrates, ascorbic acid) stimulates the sequential conversion of violaxanthin to antheraxanthin and zeaxanthin by a de-epoxidase present in the thylakoid lumen. This enzymatic carotenoid interconversion is called the xanthophyll cycle (see figure 2), and the two participating enzymes, violaxanthin de-epoxidase and zeaxanthin epoxidase, have been extensively studied (Yamamoto 1979; Pfündel & Bilger 1994; Eskling *et al.* 1997; Bugos *et al.* 1998).

Binding of protons and zeaxanthin to proteins in the PS II antenna is suggested to cause a conformational change that results in quenching of ^{1}Chl* (Horton *et al.* 1996; Gilmore 1997). The exact mechanism of de-excitation of ^{1}Chl* is still unknown, but it could occur in principle by singlet–singlet energy transfer from chlorophyll to zeaxanthin or by internal conversion of ^{1}Chl* to the ground state. The addition of two conjugated double bonds to the delocalized π electrons in the carotenoid chromophore by the de-epoxidation of violaxanthin to zeaxanthin is expected to decrease the energy level of the S_1 excited state. By using femtosecond time-resolved optical spectroscopy and the energy gap law to estimate the energy of lowest S_1 excited state of violaxanthin and zeaxanthin, Frank *et al.* (1994) proposed a 'molecular gear shift' mechanism for xanthophyll-mediated NPQ in which violaxanthin, with an S_1 state energy above that of chlorophyll *a*, would funnel excitation energy to chlorophyll, whereas zeaxanthin, with an S_1 state energy lower than that of chlorophyll *a*, would accept and thermally dissipate excitation energy from chlorophyll *a*, thus acting as a trap for excess excitation. Recently, more direct measurements of the S_1 states of violaxanthin and zeaxanthin have shown that ^{1}Chl* quenching by either of these xanthophylls is energetically feasible (Polívka *et al.* 1999; Frank *et al.* 2000), thus arguing against the molecular gear shift as the only mechanism controlling xanthophyll-mediated ^{1}Chl* quenching. However, as discussed above, no measurement of the energetics of these xanthophyll molecules has been done yet in the native protein environment. Alternatively, a more indirect mechanism has been suggested in which the conversion of violaxanthin to zeaxanthin could cause changes in the conformation or aggregation state of the LHC proteins in PS II, and these changes would result in increased rates of ^{1}Chl* de-excitation in the antenna (Horton *et al.* 1996). In isolated LHC proteins, an inhibition and stimulation of fluorescence quenching mediated by violaxanthin and zeaxanthin, respectively, has been observed, especially in the minor LHC II proteins, Lhcb4 and Lhcb5, and to a lesser extent in trimeric LHC II (reviewed by Horton *et al.* 1996). These proteins have been implicated in NPQ also because of their relatively high levels of bound xanthophyll cycle carotenoids (Bassi *et al.* 1993).

The essential nature of the protective functions of carotenoids has been demonstrated using mutants and inhibitors that block the synthesis of carotenoids. When wild-type plants are treated with carotenoid synthesis inhibitors and then exposed to light that is absorbed by chlorophyll, there is widespread destruction of photosynthetic membranes and extensive pigment bleaching (Oelmüller 1989). By 1960, mutants lacking carotenoids had been isolated from purple photosynthetic bacteria (Griffiths *et al.* 1955), maize (Anderson & Robertson 1960) and green algae (Sager & Zalokar 1958). A common characteristic of all these mutants is that they are unable to grow aerobically under normal illumination, and even though chlorophyll synthesis *per se* is not inhibited, they fail to accumulate normal photosynthetic membranes when exposed to light, suggesting that carotenoids are necessary for survival of photosynthetic organisms in the presence of light and oxygen (Stanier 1959). These mutants probably have defects early in the

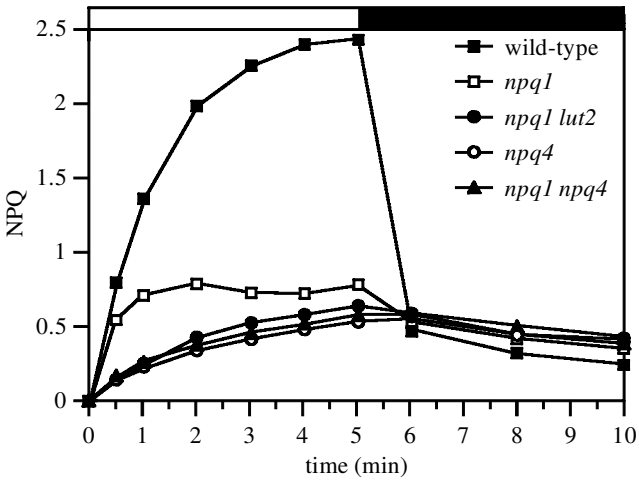

Figure 3. Induction of non-photochemical ^{1}Chl* quenching (NPQ) by high light in several *Arabidopsis* mutants. Plants grown in low light were dark adapted overnight and then transferred to illumination with excessive light of 1250 μmol photons $m^{-2} s^{-1}$ at a time of 0 min. The white bar above the graph indicates high light illumination, and the black bar indicates darkness. NPQ was calculated as $(F_m - F'_m)/F'_m$.

carotenoid biosynthetic pathway, resulting in a lack of all xanthophylls that makes it impossible to determine if specific xanthophylls have different functions. However, in the past few years, new mutants have been isolated with specific defects in xanthophyll biosynthesis, and this is allowing for a dissection of the roles of individual xanthophylls in photoprotection. Recent results from our laboratory are summarized in § 2.

2. MOLECULAR GENETICS OF THE XANTHOPHYLL CYCLE AND ^{1}Chl* QUENCHING

To identify factors that are involved in quenching of ^{1}Chl*, mutants of *Chlamydomonas* and *Arabidopsis* that are defective in NPQ have been isolated by video imaging of chlorophyll fluorescence quenching (Niyogi *et al.* 1997*a*, 1998; Shikanai *et al.* 1999; Peterson & Havir 2000). The screen for *Arabidopsis* mutants targeted specifically mutants with defects in the rapidly reversible, ΔpH-dependent component of NPQ (q_E) (Niyogi *et al.* 1998). The *npq1* mutant of *Arabidopsis* is defective in violaxanthin de-epoxidase activity (figure 2), and it is therefore unable to convert violaxanthin to zeaxanthin when exposed to high light. Genetic mapping, sequencing of a mutant allele and complementation of mutations with a wild-type transgene demonstrated that the *npq1* mutants are defective in the single-copy *Arabidopsis* gene encoding violaxanthin de-epoxidase (Niyogi *et al.* 1998). The de-epoxidase gene had been identified originally from lettuce after purification of the enzyme (Bugos & Yamamoto 1996) and was subsequently cloned from tobacco and *Arabidopsis* (Bugos *et al.* 1998). Induction of NPQ in the *npq1* mutant is severely impaired (figure 3), indicating that de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin is necessary for most of the NPQ in *Arabidopsis*. Approximately 70% of the total NPQ and 80% of the q_E that normally occurs during a 5 min illumination with high light are eliminated in *npq1*, confirming the results of numerous physiological studies (Demmig-Adams & Adams 1996*a*).

The small but significant amount of q_E that is retained in the *Arabidopsis npq1* mutant identifies a component of q_E that is not related to operation of the xanthophyll cycle. Analyses of ^{1}Chl* quenching in lutein-deficient mutants of *Chlamydomonas* (Niyogi *et al.* 1997*b*) and *Arabidopsis* (Pogson *et al.* 1998) have suggested the possible involvement of lutein in NPQ. For example, induction of NPQ in the *Arabidopsis lut2* mutant (figure 2), which lacks lutein, is slower and reaches a lower maximum extent than in the wild-type (Pogson *et al.* 1998). Construction of a double mutant between *npq1* and *lut2* revealed that the residual q_E in the *npq1* single mutant is abolished in the double mutant (figure 3), which lacks both lutein and the high-light-induced synthesis of antheraxanthin and zeaxanthin (Niyogi *et al.* 2000). An identical phenotype was observed for the analogous *npq1 lor1* double mutant (figure 2) of *Chlamydomonas* that is deficient in zeaxanthin, lutein and loroxanthin (Niyogi *et al.* 1997*b*). Even in low light, the *npq1 lor1* and *npq1 lut2* mutants exhibit a stable increase in the xanthophyll cycle pool size and an elevated de-epoxidation state, suggesting that lutein in the LHCs can be replaced by xanthophylls derived from β-carotene but that some sites may prefer antheraxanthin or zeaxanthin rather than violaxanthin (Pogson *et al.* 1998). The exact LHC protein composition of mutants that lack lutein, and double mutants that lack both lutein and zeaxanthin, is not known. In *Arabidopsis*, oxygen flash yield measurements revealed a slightly smaller PS II antenna size in the absence of lutein, although the ratio of chlorophyll *a* to chlorophyll *b* remains nearly unchanged (Niyogi *et al.* 2000). In contrast, in lutein- and loroxanthin-deficient mutants of *Chlamydomonas* and another green alga, *Scenedesmus obliquus*, the lack of lutein and loroxanthin results in a considerable change in the organization of the LHC as reflected by a marked increase in the chlorophyll *a* to chlorophyll *b* ratio (Bishop 1996; Niyogi *et al.* 1997*b*). Therefore, the apparent involvement of lutein in ^{1}Chl* quenching may be an indirect consequence of a perturbation of LHC structure or a sequestration of antheraxanthin and zeaxanthin in binding sites that are normally occupied by lutein (Niyogi *et al.* 2000). Alternatively, it is possible that one or more lutein molecules bound to a specific site in PS II may have a direct involvement in ^{1}Chl* quenching (Niyogi *et al.* 1997*b*). The photophysical properties of lutein are predicted to be similar to those of antheraxanthin (Frank & Cogdell 1996), for which a role in NPQ has been demonstrated (Gilmore & Yamamoto 1993; Goss *et al.* 1998). In summary, the data from the *Arabidopsis* and *Chlamydomonas* mutants support the idea that xanthophylls are necessary for ΔpH-dependent NPQ *in vivo*, but the mechanism(s) by which xanthophylls promote NPQ is still undefined.

Synthesis of zeaxanthin is not sufficient for quenching of ^{1}Chl*. *Arabidopsis* mutants that accumulate zeaxanthin constitutively do not exhibit constitutive quenching of ^{1}Chl*, but induction of NPQ depends solely on the build up of the transthylakoid ΔpH and therefore occurs more rapidly than in the wild-type. Although the extent of NPQ is unaffected, zeaxanthin-accumulating mutants were isolated in the screen for *npq* mutants, because they also exhibit slower relaxation of NPQ in darkness (Niyogi

et al. 1998). These mutants, named *npq2*, were found to be in the same complementation group as the *aba1* mutants that were originally isolated as abscisic acid-deficient mutants (Koorneef *et al.* 1982). These mutants are defective in the structural gene encoding zeaxanthin epoxidase (Marín *et al.* 1996). The *aba1* mutations (figure 2) almost completely eliminate the accumulation of antheraxanthin, violaxanthin and neoxanthin (Duckham *et al.* 1991; Rock & Zeevaart 1991), which are replaced in the LHC by equimolar amounts of zeaxanthin, even under non-stress conditions (Tardy & Havaux 1996). Although a decreased LHC stability has been reported for *aba1* (Hurry *et al.* 1997), the constitutive presence of zeaxanthin and the lack of the presumed light-harvesting pigments violaxanthin and neoxanthin did not affect photosynthetic performance significantly under either light-limited or light-saturated conditions, and the mutant was not particularly resistant to illumination with excess light (Tardy & Havaux 1996; Hurry *et al.* 1997; Niyogi *et al.* 1998).

In addition to mutants affecting xanthophyll metabolism, the fluorescence video imaging screen also identified *npq* mutants with normal pigment composition that are defective in quenching of ^{1}Chl*. Several mutants exhibit high chlorophyll fluorescence in low light and are obviously defective in photosynthetic electron transport (photochemical quenching); such mutants affect the generation and/or maintenance of the ΔpH that is required for q_E. In contrast, the *Arabidopsis npq4* mutant has a severe lack of q_E (figure 3), similar to that of the *npq1 lut2* double mutant (Niyogi *et al.* 2000), but *npq4* plants appear to perform photosynthesis and grow normally in low light (Li *et al.* 2000). The *npq4* mutant has the same quantum yield and maximum rate of photosynthetic oxygen evolution as wild-type. However, the ΔpH- and xanthophyll-dependent conformational change associated with q_E (monitored by a light-induced absorbance change, ΔA_{535}) is absent in *npq4* leaves. Map-based cloning revealed that the *npq4* mutant is defective in the single nuclear gene encoding PsbS (Li *et al.* 2000), a pigment-binding PS II subunit of previously unknown function that is related to typical LHC proteins (Funk *et al.* 1995). The *psbS* gene is completely deleted in the *npq4-1* allele, and several other *npq4* alleles have single mis-sense mutations in the gene. Complementation of *npq4-1* and one of the point mutations by transformation with a wild-type copy of the *psbS* gene verified that the *npq4* gene is *psbS*. Immunoblot analyses confirmed that the PsbS protein was absent in *npq4-1* plants, whereas the levels of LHC proteins previously implicated in q_E (i.e. Lhcb4 and Lhcb5) were unaffected (Li *et al.* 2000). Therefore, the PsbS protein appears to function in ^{1}Chl* quenching rather than photosynthetic light harvesting. It is possible that binding of protons and/or xanthophylls to PsbS is involved in ^{1}Chl* quenching.

3. THE PHOTOPROTECTIVE ROLES OF ZEAXANTHIN

The *Arabidopsis npq1* mutant has been used to study the importance of zeaxanthin synthesis for photoprotection. During short-term exposure (time-scale of hours) of low-light grown plants to high light, *npq1* mutants are more sensitive than the wild-type to 'photoinhibition', as assessed by chlorophyll fluorescence measurements (Niyogi *et al.* 1998). For example, after 1 h in full sunlight, leaves of *npq1* exhibit sustained depressions in PS II efficiency that are associated with both a decreased maximum fluorescence (F_m) and an elevated minimum fluorescence (F_o) (Niyogi *et al.* 1998). Increasing the time of illumination with high light up to several hours enhances the difference in PS II efficiency between *npq1* and the wild-type, whereas the extent of thylakoid lipid peroxidation is indistinguishable (Havaux & Niyogi 1999).

During longer periods of illumination with high light (time-scale of days) following transfer from low light, *npq1* plants exhibit pronounced photo-oxidative bleaching, especially of older leaves, compared to the wild-type (Havaux & Niyogi 1999). The bleaching of pigments is associated with marked increases in thylakoid lipid peroxidation, as measured by thermoluminescence, ethane production and direct quantification of hydroperoxy fatty acids (Havaux & Niyogi 1999). However, during the second and third days in high light, the PS II efficiency of *npq1* recovers and approaches that of the wild-type, suggesting that PS II is able to acclimatize to high light during long-term experiments. The *npq1 lut2* double mutant shows a bleaching phenotype that is more severe than that of the *npq1* single mutant (Niyogi *et al.* 2000), indicating an antioxidant role for lutein *in vivo*, possibly in quenching of ^{3}Chl* and/or $^1O_2^*$ in the LHCs. Like the *npq1* single mutant, developing leaves of *npq1 lut2* plants are able to acclimate to high light conditions by an as yet unknown mechanism (Niyogi *et al.* 2000). These results are consistent with the observation that well-watered *npq1* or *npq1 lut2* plants can survive and grow even in full natural sunlight (Niyogi *et al.* 1998, 2000).

The increased lipid peroxidation in *npq1* leaves after three days in high light is mainly due to the lack of zeaxanthin rather than the lack of ^{1}Chl* quenching. This was demonstrated by comparing the extent of lipid peroxidation in the *npq4* mutant and an *npq4 npq1* double mutant (Havaux & Niyogi 1999). These mutant strains exhibit the same lack of ^{1}Chl* quenching in high light (figure 3; Li *et al.* 2000), but they differ in their ability to accumulate zeaxanthin in high light. The *npq4* mutant converts violaxanthin into zeaxanthin (like the wild-type), whereas the *npq4 npq1* double mutant is defective in the xanthophyll cycle (like the *npq1* single mutant). After exposure to high light for three days, the extent of lipid peroxidation in the *npq4 npq1* double mutant was indistinguishable from that in the *npq1* single mutant, as expected. However, lipid peroxidation in the *npq4* single mutant was more similar to that observed in the wild-type, indicating that zeaxanthin synthesis via the xanthophyll cycle is involved in protection against photo-oxidative damage, whereas only limited long-term protection is attributable to ^{1}Chl* quenching. A major antioxidant function of zeaxanthin therefore occurs by a mechanism that is distinct from ^{1}Chl* quenching (Havaux & Niyogi 1999).

Protection against lipid peroxidation by zeaxanthin could involve quenching of ^{3}Chl* or $^1O_2^*$, termination of peroxidation chain reactions, regeneration of tocopherol, or some other antioxidant function (figure 1). The presence of neoxanthin, violaxanthin and lutein is apparently not sufficient to prevent photo-oxidation in

the absence of zeaxanthin, despite the expectation that they would be very efficient quenchers of ^{3}Chl* or $^1O_2^*$ in the LHCs (Croce *et al.* 1999). It is possible that zeaxanthin is able to function in photoprotection at a site other than the LHCs, perhaps in the lipid matrix of the thylakoid membrane, where it could act synergistically with other antioxidants, for example in regeneration of oxidized α-tocopherol. Consistent with this idea, the *npq1* mutant of *Arabidopsis* shows lower levels of tocopherols than the wild-type after prolonged exposure to high light (Havaux & Niyogi 1999).

Unlike the *Arabidopsis npq1 lut2* double mutant, the corresponding double mutant of *Chlamydomonas*, *npq1 lor1*, is unable to acclimate to high light and is therefore susceptible to lethal photo-oxidation (Niyogi *et al.* 1997*b*). After transfer from low light to high light, pigment bleaching in *npq1 lor1* cells is accompanied by dramatic increases in lipid peroxidation, assayed as thiobarbituric acid-reactive substances, followed by eventual destruction of thylakoid membranes (I. Baroli and K. K. Niyogi, unpublished data). Bleaching and death of cells is blocked under anaerobic conditions, but lethality is not prevented by photoheterotrophic growth on acetate, indicating that photo-oxidation affects essential cellular processes in addition to photosynthesis (I. Baroli and K. K. Niyogi, unpublished data). Acclimation to high light in the *Arabidopsis npq1 lut2* double mutant may be possible due to production of new leaves from the shoot apical meristem and consequent shading of young leaves during early stages of development (Niyogi *et al.* 2000). Photo-oxidative destruction of *npq1 lor1* cells of *Chlamydomonas* may therefore be analogous to the bleaching of mature leaves of *Arabidopsis npq1 lut2*.

To identify new mutations affecting xanthophyll-dependent photoprotection, we have isolated extragenic suppressors of the conditional lethality of the *Chlamydomonas npq1 lor1* double mutant (A. Do, I. Baroli and K. K. Niyogi, unpublished data). These suppressor mutations, which arise both spontaneously and after mutagenesis, allow survival of *npq1 lor1* cells in high light. Several suppressors are defective in zeaxanthin epoxidase activity, resulting in accumulation of zeaxanthin (and in some cases antheraxanthin); these suppressors are allelic to the previously identified *npq2* mutation of *Chlamydomonas* (figure 2; Niyogi *et al.* 1997*a*). In the most extreme examples, zeaxanthin is the only xanthophyll that the cells contain, demonstrating that zeaxanthin is sufficient for photoprotection in *Chlamydomonas*. In weaker *npq2* alleles, only small amounts of zeaxanthin accumulate, suggesting that surprisingly low levels of zeaxanthin are sufficient for photoprotection. It seems unlikely that such low levels of zeaxanthin could prevent lethal photo-oxidation by quenching ^{3}Chl* and/or $^1O_2^*$ while bound to LHC proteins. Instead, we hypothesize that zeaxanthin exerts its antioxidant effects in the thylakoid membrane, perhaps by terminating lipid peroxidation chain reactions that are initiated by $^1O_2^*$ (figure 1). Instead, or in addition, zeaxanthin could be important in regenerating α-tocopherol in the thylakoid membrane (Böhm *et al.* 1997).

4. CONCLUSIONS

Through work with mutants deficient in q_E, it appears that xanthophylls are essential but not sufficient for most of the non-photochemical quenching of ^{1}Chl* in high light. Efficient quenching also requires the presence of the PS II intrinsic protein PsbS. The mechanism by which PsbS and xanthophylls, especially zeaxanthin, work together in the thylakoid membrane to bring about quenching is still unresolved. The PsbS protein has been reported to bind chlorophyll and xanthophylls (Funk *et al.* 1995), and parts of the protein that are exposed to the thylakoid lumen contain several acidic amino-acid residues. Protonation of PsbS by the light-driven acidification of the lumen could change the conformation of the LHC in a way that allows for quenching of ^{1}Chl*, either by direct energy transfer to zeaxanthin or by somehow promoting internal conversion of ^{1}Chl* to the ground state. The quenching of ^{1}Chl* could occur in the PsbS protein itself or, mediated by PsbS, in other components of the LHC. In *Arabidopsis*, the lack of ΔpH-dependent ^{1}Chl* quenching does not affect plant growth in relatively high light under controlled laboratory conditions, suggesting that during acclimation to high light the photosynthetic apparatus is able to compensate for a lack of q_E in mutants like *npq4*.

Zeaxanthin and lutein protect thylakoid lipids from peroxidation, but it is not yet clear whether this happens as a consequence of the physical quenching of $^1O_2^*$ by the xanthophylls or if zeaxanthin and lutein act as chemical antioxidants, perhaps by regenerating tocopherols. This poorly understood antioxidant action of xanthophylls is in need of further clarification. The findings will also have significance for human health, for example in the prevention and treatment of macular degeneration in the retina where an interaction of lutein and zeaxanthin with other antioxidants has been implicated (Snodderly 1995).

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Discussion

J. Barber (*Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, UK*). How do the light-induced scattering changes vary between *npq4* and wild-type and what can be deduced from the difference in terms of the mechanism of NPQ?

K. K. Niyogi. The light-induced absorbance change at 535 nm is associated with NPQ and is absent in the *npq4-1* mutant that lacks the PsbS protein. At this point, the exact mechanistic connection between the absorbance change and NPQ is unclear, but it has been suggested that the absorbance change reflects a pH- and xanthophyll-dependent conformational change in the thylakoid membrane that is necessary for NPQ. We are currently testing the hypothesis that binding of protons and/or xanthophylls to the PsbS protein is necessary for the absorbance change and for NPQ.

P. Heifetz (*Novartis Agribusiness Biotech Research, Inc., NC, USA*). What is the effect on wild-type *Chlamydomonas* light sensitivity of the various *npq2* alleles isolated as suppressors of the *npq1 lor1* high light sensitivity phenotype (e.g. in a non-*npq1 lor1* background)?

K. K. Niyogi. We have not yet crossed these new *npq2* alleles into a wild-type background, but some properties of the original *npq2-1* allele (in an otherwise wild-type strain) have been described previously (Niyogi *et al.* 1997*a*). The growth of the *npq2* mutant was comparable to that of the wild-type under our usual high light conditions, but we have not yet tested more extreme conditions to determine if the mutant is perhaps more resistant to high light.