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PHOTOPROTECTION REVISITED: *Genetic and Molecular Approaches*

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ABSTRACT

The involvement of excited and highly reactive intermediates in oxygenic photosynthesis poses unique problems for algae and plants in terms of potential oxidative damage to the photosynthetic apparatus. Photoprotective processes prevent or minimize generation of oxidizing molecules, scavenge reactive oxygen species efficiently, and repair damage that inevitably occurs. This review summarizes several photoprotective mechanisms operating within chloroplasts of plants and green algae. The recent use of genetic and molecular biological approaches is providing new insights into photoprotection, especially with respect to thermal dissipation of excess absorbed light energy, alternative electron transport pathways, chloroplast antioxidant systems, and repair of photosystem II.

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INTRODUCTION

Light is required for photosynthesis, yet plants need protection from light. Photosynthesis inevitably generates highly reactive intermediates and byproducts that can cause oxidative damage to the photosynthetic apparatus (16, 58). This photo-oxidative damage, if not repaired, decreases the efficiency and/or maximum rate of photosynthesis, termed "photoinhibition" (89; reviewed in 13, 98, 118, 129).

Oxygenic photosynthetic organisms have evolved multiple photoprotective mechanisms to cope with the potentially damaging effects of light, as diagrammed in Figure 1. Some algae and plants avoid absorption of excessive light by movement of leaves, cells (negative phototaxis), or chloroplasts. Within the chloroplast, regulation of photosynthetic light harvesting and electron transport

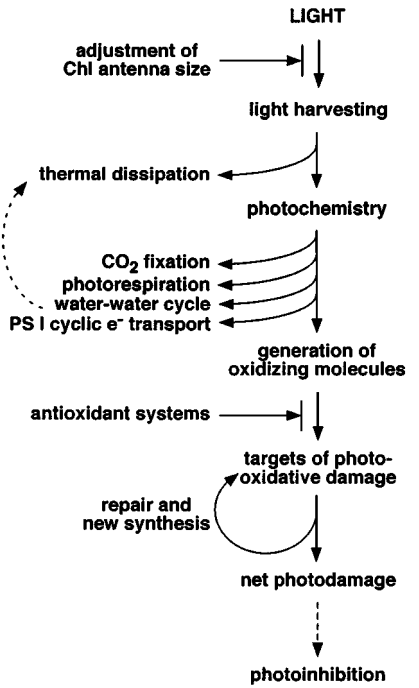

Figure 1 Schematic diagram of photoprotective processes occurring within chloroplasts.

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balances the absorption and utilization of light energy. For example, adjustments in light-harvesting antenna size and photosynthetic capacity can decrease light absorption and increase light utilization, respectively, during relatively long-term acclimation to excessive light. Alternative electron transport pathways and thermal dissipation can also help to remove excess absorbed light energy from the photosynthetic apparatus. Numerous antioxidant molecules and scavenging enzymes are present to deal with the inevitable generation of reactive molecules, especially reactive oxygen species. However, despite these photoprotective defenses, damage to the photosynthetic machinery still occurs, necessitating turnover and replacement of damaged proteins. The overall goal of photoprotection is, therefore, to prevent net damage from occurring.

Because of its importance for maintaining photosynthesis and ultimately for survival of photosynthetic organisms in many natural environments, photoprotection has long been a topic of considerable interest in plant physiology and biochemistry. The general subject of photoprotection, as well as several specific photoprotective processes, have been extensively reviewed (9, 13, 18, 31, 37, 47–49, 51, 57, 65, 76, 77, 98, 112, 118, 128), so one might ask why the topic needs to be "revisited." Although genetic methods have long been used in analysis of photosynthesis (95, 142), only recently has there been widespread use of genetic and molecular techniques to dissect specific processes involved in photoprotection. The roles of specific cloned genes are being tested through reverse genetics, and classical (forward) genetics is uncovering new mutants and providing insights into the complexity of photoprotection. This article reviews several photoprotective processes that occur within chloroplasts of eukaryotic photosynthetic organisms, with a particular emphasis on the use of molecular and genetic approaches in intact green algae and plants. Space constraints direct my focus to results obtained with the most commonly used model organisms with good molecular genetics, the green alga *Chlamydomonas reinhardtii* (Table 1) and the C3 vascular plants *Arabidopsis thaliana* and tobacco (Table 2).

PHOTO-OXIDATIVE DAMAGE TO THE PHOTOSYNTHETIC APPARATUS

Before discussing specific photoprotective mechanisms, I briefly summarize the types of oxidizing molecules that are involved in damaging the photosynthetic machinery.

Generation of Oxidizing Molecules in Photosynthesis

Because of the large differences in redox potential between reactants and products and the involvement of excited intermediates, oxygenic photosynthesis

Table 1 Summary of *Chlamydomonas* mutants affecting photoprotection in the chloroplast

Photoprotective system	Mutant	Description	Photoprotection phenotype	Reference
Thermal dissipation and xanthophylls	*npq1*	Lacks zeaxanthin in HL[a]	Partly NPQ-deficient; grows in HL	109
	npq2	Accumulates zeaxanthin; lacks antheraxanthin, violaxanthin, and neoxanthin	Faster induction of NPQ; grows in HL	109
	lor1	Lacks lutein and loroxanthin	Partly NPQ-deficient; grows in HL	38, 110
	npq1 lor1	Lacks zeaxanthin in HL; lacks lutein and loroxanthin	NPQ-deficient; sensitive to HL	110
	npq4	Normal xanthophylls	NPQ-deficient; grows in HL	D Elrad, KK Niyogi & AR Grossman, unpublished results
	npq2 lor1	Accumulates zeaxanthin; lacks antheraxanthin, violaxanthin, neoxanthin, lutein, and loroxanthin	Grows in HL	KK Niyogi, unpublished results
Water-water cycle and scavenging enzymes	PAR19	Overexpresses FeSOD	n.d.[b]	85
PS II repair	*sr/spr*	Deficient in chloroplast protein synthesis	Sensitive to HL	73
	ag16.2	Accumulates D1 protein	Sensitive to HL	158
	*psbT*Δ	Deficient in PS II in HL	Sensitive to HL	105

[a]High light.
[b]Not determined.

poses unique problems for algae and plants with respect to the generation of reactive oxygen species and other oxidizing molecules. Potentially damaging molecules are generated at three major sites in the photosynthetic apparatus: the light-harvesting complex (LHC) associated with photosystem (PS) II, the PS II reaction center, and the PS I acceptor side.

Chlorophyll (Chl) molecules are critical participants in light-harvesting and electron transfer reactions in photosynthesis, but Chl can act as a potent endogenous photosensitizer in algae and plants. Absorption of light causes Chl to enter the singlet excited state (^{1}Chl), and the excitation energy is rapidly transferred (<ps time scale) between neighboring Chls in the LHC by resonance transfer. Before excitation energy is trapped in the reaction center, triplet Chl (^{3}Chl) can be formed from ^{1}Chl through intersystem crossing. This is an inherent physical property of ^{1}Chl (121), and the yield of ^{3}Chl formation

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depends on the average lifetime of ^{1}Chl (∼ns time scale) in the antenna (58). In contrast to ^{1}Chl, ^{3}Chl is relatively long-lived (∼ms time scale) and can interact with O_2 to produce singlet oxygen ($^1O_2$) (56). Because the average lifetime of ^{1}Chl in the PS II LHC is several times longer than in the PS I LHC, the potential for generation of $^1O_2$ is greater in the PS II LHC.

In the PS II reaction center, trapping of excitation energy involves a primary charge separation between a Chl dimer (P680) and a pheophytin molecule (Pheo) that are bound to the D1 reaction center protein. The $P680^+/Pheo^-$ radical pair is reversible (44, 134), and the charge recombination (and other backreactions in PS II) can generate triplet P680 (^{3}P680) (11, 13, 115, 148). As in the LHC, energy exchange between ^{3}P680 and O_2 results in formation of $^1O_2$. The $P680^+/Pheo^-$ charge separation can be stabilized upon electron transfer from $Pheo^-$ to the quinone acceptor Q_A, and $P680^+$ is subsequently reduced by an electron derived from the oxidation of H_2O via the secondary donor Y_Z (tyrosine 161 of the D1 protein). Although the very high oxidizing potential of $P680^+$ and Y_Z^+ enables plants to use H_2O as an electron donor, $P680^+$ and Y_Z^+ themselves are also capable of oxidizing nearby pigments and proteins (11, 13).

In PS I, charge separation occurring between P700 and the primary acceptor Chl A_0 is stabilized by subsequent electron transfer to secondary acceptors, the phylloquinone A_1 and three iron-sulfur clusters (F_X, F_A, and F_B). In contrast to $P680^+$, $P700^+$ is less oxidizing and relatively stable; in fact, $P700^+$ is a very efficient quencher of excitation energy from the PS I LHC (44, 121). However, the acceptor side of PS I, which has a redox potential low enough to reduce $NADP^+$ via ferredoxin, is also capable of reducing O_2 to the superoxide anion radical (O_2^-) (102). O_2^- can be metabolized to H_2O_2, and a metal-catalyzed Haber-Weiss or Fenton reaction can lead to production of the hydroxyl radical (OH·), an extremely toxic type of reactive oxygen species (16).

Generation of these oxidizing molecules occurs at all light intensities, but when absorbed light energy exceeds the capacity for light energy utilization through photosynthesis, the potential for photo-oxidative damage is exacerbated. In excessive light, accumulation of excitation energy in the PS II LHC will increase the average lifetime of ^{1}Chl, thereby increasing the yield of ^{3}Chl and $^1O_2$. Higher excitation pressure in PS II can increase the frequency of direct damage by $P680^+$ and formation of $^1O_2$ as a result of $P680^+/Pheo^-$ recombination. Furthermore, the high ΔpH that builds up in excessive light can inhibit electron donation to $P680^+$ from the oxygen-evolving complex, resulting in longer-lived $P680^+$ and/or Y_Z^+. Overreduction of the PS I acceptor side in excessive light favors direct reduction of O_2 to form O_2^-. Analysis of transgenic tobacco plants with reduced levels of cytochrome b_6/f complex has pointed to the involvement of lumen acidification and/or overreduction of the PS I acceptor side in photoinhibition (80).

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Table 2 Summary of Arabidopsis and tobacco mutants and transgenics affecting photoprotection in the chloroplast

Photoprotective system	Plant	Mutant/ transgenic	Description	Photoprotection phenotype	Reference
Thermal dissipation and xanthophylls	Arabidopsis	*npq1*	Lacks zeaxanthin in HL[a]	NPQ-deficient; sensitive to short-term HL	111
	Arabidopsis	*aba1* (*npq2*)	Accumulates zeaxanthin; lacks antheraxanthin, violaxanthin, and neoxanthin	Faster induction of NPQ	81, 111, 144
	Tobacco	*aba2*	Accumulates zeaxanthin; lacks antheraxanthin, violaxanthin, and neoxanthin	n.d.[b]	100
	Arabidopsis	*lut2*	Lacks lutein	Partly NPQ-deficient; grows in HL	127
	Arabidopsis	*lut1*	Accumulates zeinoxanthin; lacks lutein	Partly NPQ-deficient	127
	Arabidopsis	*npq1 lut2*	Lacks zeaxanthin in HL; lacks lutein	NPQ-deficient; older leaves sensitive to HL	O Björkman, C Shih, B Pogson, D DellaPenna & KK Niyogi, unpublished results
	Arabidopsis	*ch1*	Lacks Ch1 *b*	Partly NPQ-deficient	KK Niyogi, unpublished results
	Arabidopsis	*npq4*	Normal xanthophylls	NPQ-deficient	KK Niyogi, C Shih & O Björkman, unpublished results

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	Arabidopsis	*aba1 lut2*	Accumulates zeaxanthin; lacks antheraxanthin, violaxanthin, neoxanthin, and lutein	Partly NPQ-deficient; impaired growth	127
Photorespiration	Tobacco	35S-GS2	Overexpresses glutamine synthetase in chloroplast	Resistant to HL	91
Water-water cycle and scavenging enzymes	Arabidopsis	Cu/ZnSOD-deficient mutant	Lacks Cu/ZnSOD in chloroplast and cytosol	n.d.	DJ Kliebenstein & RL Last, unpublished results
	Arabidopsis	FeSOD-deficient mutant	Lacks FeSOD in chloroplast	n.d.	DJ Kliebenstein & RL Last, unpublished results
	Tobacco	35S-Cu/ZnSOD	Overexpresses Cu/ZnSOD in chloroplast	Resistant to HL + cold	137, 138
	Tobacco	35S-FeSOD	Overexpresses FeSOD in chloroplast	Same as wild type	147
PS I cyclic electron transport	Tobacco	*ndhC, ndhK, ndhJ*	Lacks thylakoid NDH complex	Slower induction of NPQ in water-stressed plants	36
	Tobacco	*ndhB*	Lacks thylakoid NDH complex	n.d.	139
Ascorbate	Arabidopsis	*vtc1*	Ascorbate-deficient	n.d.	40, 41
Glutathione	Arabidopsis	*cad2*	Glutathione-deficient	n.d.	39, 78
PS II repair	Tobacco	35S-glycerol-3-P acyltransferase	Decreased unsaturation of chloroplast lipids	Sensitive to HL + cold	106

[a]High light.
[b]Not determined.

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Targets of Photo-Oxidative Damage

Although the exact mechanism(s) of damage has not been determined, PS II is a major target of photo-oxidative damage (10, 11, 13, 115). Interestingly, photoinhibition of PS II, measured as a decrease in either the flash yield of O_2 evolution or the Chl fluorescence parameter F_v/F_m, seems to depend on the number of absorbed photons rather than the rate of photon absorption. This implies that there is a constant probability of photodamage for each absorbed photon during conditions of steady-state photosynthesis (122, 123). However, the probability of photodamage can be modulated by changes in Chl antenna size and rate of electron transport (20, 124), which alter the excitation pressure on PS II (79) at a given light intensity.

Generation of $^1O_2$ within the LHCs can potentially lead to oxidation of lipids, proteins, and pigments in the immediate vicinity (87). Thylakoid membrane lipids are especially susceptible to damage by $^1O_2$ because of the abundance of unsaturated fatty acid side chains. Reaction between $^1O_2$ and these lipids produces hydroperoxides and initiates peroxyl radical chain reactions in the thylakoid membrane. Generation of $^1O_2$ and/or $P680^+$ in the PS II reaction center can result in damage to lipids, critical pigment cofactors, and protein subunits associated with PS II, especially the D1 protein, resulting in photo-oxidative inactivation of entire reaction centers (13, 18).

On the acceptor (stromal) side of PS I, the targets of oxidative damage by O_2^-, H_2O_2, and OH· include key enzymes of photosynthetic carbon metabolism such as phosphoribulokinase, fructose-1,6-bisphosphatase, and NADP-glyceraldehyde-3-phosphate dehydrogenase (16, 84). Photoinhibition due to damage to the PS I reaction center itself can be observed under some circumstances, especially during chilling stress (reviewed in 143).

ADJUSTMENT OF LIGHT-HARVESTING ANTENNA SIZE

Changes in the sizes of the Chl antennae associated with PS II and PS I are involved in balancing light absorption and utilization (reviewed in 7, 103). During long-term acclimation to growth in different light intensities, changes in antenna size are due to changes in LHC gene expression (54, 101, 150) and/or LHC protein degradation (96). No mutants affecting acclimatory changes in Chl antenna size have yet been reported.

Short-term alteration of the relative antenna sizes of PS II and PS I can occur because of state transitions. According to the state transition model (reviewed in 2), overexcitation of PS II relative to PS I reduces the plastoquinone pool and activates a kinase that phosphorylates the peripheral LHC associated with PS II. Subsequent detachment of phospho-LHC from PS II decreases the effective size

of the PS II antenna, and the phospho-LHC may then transfer excitation energy to PS I. Although the state transition is often suggested to be photoprotective (for example, see 8, 13), there is no convincing evidence for its role in photoprotection, at least in excessive light. In fact, the LHC kinase system seems to be inactivated in high light (46, 130, 136). Mutants affecting the state transition would be useful to test the hypothesis.

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THERMAL DISSIPATION OF EXCESS ABSORBED LIGHT ENERGY

In excessive light, an increase in the thylakoid ΔpH regulates PS II light harvesting by triggering the dissipation of excess absorbed light energy as heat (reviewed in 31, 47, 48, 49, 51, 65, 76, 77). Over 75% of absorbed photons can be eliminated by this process of thermal dissipation (50), which involves de-excitation of ^{1}Chl and which is measured (and often referred to) as nonphotochemical quenching of Chl fluorescence (NPQ). Thermal dissipation is thought to protect photosynthesis by (*a*) decreasing the lifetime of ^{1}Chl to minimize generation of $^1O_2$ in the PS II LHC and reaction center, (*b*) preventing overacidification of the lumen and generation of long-lived $P680^+$, and (*c*) decreasing the rate of O_2 reduction by PS I. Although thermal dissipation is usually reversible within seconds or minutes, sustained thermal dissipation (also called qI) can be manifested as photoinhibition, which may actually be due to a photoprotective mechanism (52, 77, 82, 92, 116). I focus on the rapidly reversible (pH-dependent) type of thermal dissipation (also called qE) that predominates under most circumstances.

pH-dependent thermal dissipation occurs in the PS II antenna pigment bed and involves specific de-epoxidized xanthophyll pigments (31, 47–49, 51; 65, 76, 77). The increase in the thylakoid ΔpH in excessive light is thought to result in protonation of specific LHC polypeptides associated with PS II, namely LHCB4 (CP29) and LHCB5 (CP26) (42, 75, 151, 152). The ΔpH also activates the enzyme violaxanthin de-epoxidase, which converts violaxanthin associated with the LHCs to zeaxanthin (and antheraxanthin) via the so-called xanthophyll cycle (see Figure 2) (55, 125). Binding of zeaxanthin and protons to the LHC may cause a conformational change, monitored by an absorbance change at 535 nm (23–25, 132), that is necessary for thermal dissipation. The actual mechanism of ^{1}Chl de-excitation may involve a direct transfer of energy from Chl to zeaxanthin (37, 62, 120). Alternatively, xanthophylls (and protons) may act as allosteric effectors of LHC structure, leading to "concentration quenching" by Chls (77) or quenching via Chl dimer formation (42).

Mutants of *Chlamydomonas* and Arabidopsis that affect thermal dissipation have been isolated by video imaging of Chl fluorescence quenching (109,

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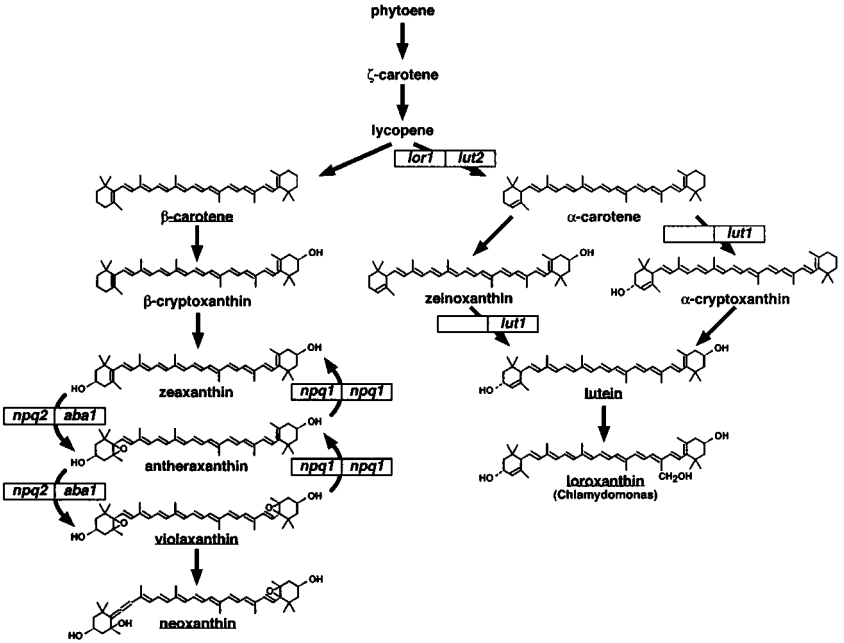

Figure 2 The pathway for carotenoid biosynthesis in green algae and plants. The steps blocked in mutants of *Chlamydomonas* and Arabidopsis are designated by the boxes, with the names of the mutants shown in the left half of the box for *Chlamydomonas* and the right half for Arabidopsis. Strains defective in some of these reactions have also been identified in *Scenedesmus obliquus* (26, 28). The xanthophyll cycle that operates in the β-carotene branch of the pathway involves zeaxanthin, antheraxanthin, and violaxanthin. The carotenoids that normally accumulate in the chloroplast are underlined; zeaxanthin accumulates in excessive light.

111, 140). Characterization of mutants affected in xanthophyll metabolism (Figure 2) has confirmed a role for zeaxanthin in thermal dissipation (81, 109, 111, 144). The *npq2* mutants of *Chlamydomonas* and Arabidopsis, together with the existing *aba1* mutant of Arabidopsis (90) and *aba2* mutant of tobacco (100), are defective in zeaxanthin epoxidase activity; they accumulate zeaxanthin and contain only trace amounts of antheraxanthin, violaxanthin, and neoxanthin (53, 100, 109, 111, 131). The constitutive presence of zeaxanthin in the PS II LHCs of *npq2* and *aba1* mutants is not sufficient for thermal dissipation, which also requires the ΔpH. However, induction of NPQ by illumination with high light is more rapid in the mutants compared to wild type (81, 109, 111, 144), presumably because it is driven solely by the build-up of the ΔpH. During short-term illumination with high light, some *aba1* mutants exhibited the same sensitivity to photoinhibition as the wild type

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(81), whereas an *aba1* mutant with a different allele appeared more sensitive (144).

The *Chlamydomonas npq1* mutant, which is unable to convert violaxanthin to zeaxanthin, is partially defective in NPQ but retains substantial, pH-dependent NPQ, which suggests that some but not all thermal dissipation depends on operation of the xanthophyll cycle (109). Characterization of the *lor1* mutant, which lacks xanthophylls derived from α-carotene (38, 110), indicates a possible role for lutein in thermal dissipation (110). Neither *npq1* nor *lor1* is particularly sensitive to photoinhibition during growth in high light. However, an *npq1 lor1* double mutant lacks almost all pH-dependent NPQ and is very susceptible to photo-oxidative bleaching in high light. Although this result seems to provide evidence for the importance of thermal dissipation for photoprotection in vivo, the phenotype is complicated by the fact that xanthophylls are also involved in quenching of ^{3}Chl and $^1O_2$ and inhibition of lipid peroxidation, as described below. Another mutant, *npq4*, lacks NPQ but has normal xanthophyll composition. It is able to survive in high light, suggesting that thermal dissipation itself is not required for photoprotection (D Elrad, KK Niyogi & AR Grossman, unpublished results).

Results with Arabidopsis xanthophyll mutants, although generally similar to results with *Chlamydomonas*, revealed that the relative contributions of different xanthophylls to thermal dissipation vary in different organisms. Like *npq1* of *Chlamydomonas*, Arabidopsis *npq1* mutants are unable to convert violaxanthin to zeaxanthin in high light. Genetic and molecular analyses (111) demonstrated that the phenotype of *npq1* is due to a recessive mutation in the Arabidopsis violaxanthin de-epoxidase gene (35). In contrast to the results with *Chlamydomonas*, induction of pH-dependent thermal dissipation in the Arabidopsis *npq1* mutant is almost completely inhibited, suggesting that most of the thermal dissipation in Arabidopsis depends on de-epoxidation of violaxanthin (111). Leaves of *npq1* plants sustain more photoinhibition than wild type following short-term illumination with high light, in agreement with experiments that used dithiothreitol as an inhibitor of violaxanthin de-epoxidase in detached leaves (23, 156). However, growth of *npq1* plants that are acclimated to high light is not noticeably different from that of the wild type, which suggests that, in the long term, other photoprotective processes can compensate for the defect in *npq1* (111).

As in *Chlamydomonas*, analysis of Arabidopsis mutants lacking lutein suggests that the residual pH-dependent NPQ in *npq1* may be attributable to a contribution of lutein-dependent NPQ (127). The *lut2* mutant, which lacks lutein owing to a mutation in the lycopene ε-cyclase gene (126), exhibits slower induction and a lower maximum extent of NPQ (127), and an *npq1 lut2* double mutant lacks almost all pH-dependent NPQ (O Björkman, C Shih, B Pogson,

D DellaPenna & KK Niyogi, unpublished results). Lutein may have a direct role in thermal dissipation, or the lack of lutein in *lor1* and *lut2* mutants may have an indirect effect. In mutants lacking lutein, zeaxanthin may be sequestered in binding sites that are normally occupied by lutein but inactive in thermal dissipation, thereby lowering the relative effectiveness of zeaxanthin (AM Gilmore, KK Niyogi & O Björkman, unpublished results).

The requirement for different proteins in the PS II LHC for thermal dissipation has been addressed using mutants lacking Chl *b*. These mutants, such as barley *chlorina f2* and Arabidopsis *ch1*, are impaired in the synthesis and/or assembly of LHC proteins, especially the peripheral LHC of PS II, due to the lack of Chl *b* (32, 93, 108). Measurements of NPQ and Chl fluorescence lifetime components in these mutants suggest that only the minor LHC proteins, such as LHCB4 and LHCB5, are necessary for thermal dissipation, although maximal NPQ requires the presence of the entire LHC (12, 34, 66, 68, 69, 97; KK Niyogi, unpublished results). To test the role of specific LHC proteins in thermal dissipation, mutants or antisense plants affecting individual genes will be very useful.

Several other *npq* mutants exhibit normal pigment composition and xanthophyll interconversions. These mutants presumably identify factors besides the ΔpH and xanthophylls (perhaps LHC components) that are required for thermal dissipation in *Chlamydomonas* (109; D Elrad, KK Niyogi & AR Grossman, unpublished results) and Arabidopsis (KK Niyogi, C Shih & O Björkman, unpublished results). For example, the Arabidopsis *npq4* mutant exhibits the same lack of NPQ as an *npq1 lut2* double mutant, which suggests that it is defective in all pH- and xanthophyll-dependent thermal dissipation, yet it is indistinguishable from wild type in terms of xanthophyll interconversion and growth in low light. In addition, *npq4* lacks the absorbance change that presumably reflects the conformational change that is necessary for thermal dissipation (KK Niyogi, C Shih & O Björkman, unpublished results). Determination of the molecular basis for mutations such as *npq4* will likely provide new insights into the molecular mechanism of thermal dissipation.

PHOTOPROTECTION THROUGH PHOTOCHEMISTRY

Assimilatory Linear Electron Transport

Much of the light energy absorbed by the LHCs is utilized through photochemistry that drives linear electron transport from H_2O to NADPH, resulting in O_2 evolution and reduction of CO_2, NO_3^-, and SO_4^{2-}. The maximum rate of photosynthesis is a dynamic parameter that can be altered during acclimation to growth in different light environments (reviewed in 30, 37) through changes in enzyme activities and gene expression. These acclimation responses generally occur during a period of several days, and there are few or no molecular

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genetic data that address the importance of long-term acclimation responses for photoprotection.

Oxygen-Dependent Electron Transport

There is abundant evidence that nonassimilatory electron transport to oxygen plays an important role in consuming excess excitation energy. Oxygen can function as an electron acceptor either through the oxygenase reaction catalyzed by Rubisco (photorespiration) or by direct reduction of oxygen by electrons on the acceptor side of PS I (102), and there is much debate about which process is more important for photoprotection (for example, see 22, 71, 119, 124, 155).

PHOTORESPIRATION In C3 plants, especially under conditions of CO_2 limitation, photorespiratory oxygen metabolism is capable of maintaining considerable linear electron transport and utilization of light energy (reviewed in 117). The role of photorespiration in photoprotection can be conveniently assessed by varying the gas composition to inhibit the oxygenation reaction of Rubisco. Blocking photorespiration with mutations or inhibitors leads to inhibition of photosynthesis and photo-oxidative damage, and the cause of inhibition varies (14). Accumulation of photorespiratory metabolites or depletion of carbon intermediates can inhibit the Calvin cycle and shut down the photochemical sink for excitation energy. To assess the importance of photorespiration without the complications of accumulating toxic intermediates, mutants of Rubisco that lack oxygenase activity would be required.

Manipulation of glutamine synthetase activity, a rate-limiting step in photorespiratory metabolism, in transgenic tobacco plants has provided additional evidence for the role of photorespiration in photoprotection (91). Antisense plants with less glutamine synthetase are more sensitive to photo-oxidation under conditions of CO_2 limitation because of accumulation of photorespiratory NH_3, in agreement with previous results with mutant barley plants lacking glutamine synthetase (149). In contrast, plants that overexpress glutamine synthetase have a higher capacity for photorespiration and are more resistant to photoinhibition and photo-oxidative damage (91).

PHOTOREDUCTION OF OXYGEN BY PHOTOSYSTEM I Direct reduction of O_2 by PS I is the first step in an alternative electron transport pathway that has been variously termed pseudocyclic electron transport, the Mehler-ascorbate peroxidase reaction, and the water-water cycle. Because a comprehensive review of this pathway appears in this volume (17), it is only briefly outlined here. The O_2^- produced on the acceptor side of PS I by reduction of O_2 is efficiently metabolized by thylakoid-bound isozymes of superoxide dismutase (SOD) and ascorbate peroxidase (APX) to generate H_2O and monodehydroascorbate, which can itself be reduced directly by PS I to regenerate ascorbate (15–17). Thus the four

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electrons generated by oxidation of H_2O by PS II are consumed by reduction of O_2 to H_2O by PS I. This pseudocyclic pathway generates a ΔpH for ATP synthesis, but neither NADPH nor net O_2 is produced.

Like photorespiration, the water-water cycle may help to dissipate excitation energy through electron transport. However, the capacity of this pathway to support electron transport is unclear; estimates range between 10% and 30% of normal linear electron transport in algae and C3 plants (22, 99, 119). The pathway may also be involved in maintaining a ΔpH necessary for thermal dissipation of excess absorbed light energy (135).

Genes encoding SOD and APX have been identified from several plants, including Arabidopsis (83, 86), and this should enable dissection of the water-water cycle by analysis of antisense plants or mutants. However, the mutant analysis will be complicated by the dual roles of these enzymes in scavenging reactive oxygen intermediates and in electron transport.

Cyclic Electron Transport

Within PS II, cyclic electron transport pathways, possibly involving cytochrome b_{559}, have been suggested to dissipate excitation energy (reviewed in 37, 154), but convincing evidence for their occurrence in vivo is lacking. Cytochrome b_{559} may function to oxidize $Pheo^-$ or reduce $P680^+$ to protect against photodamage to PS II (154). Site-directed mutagenesis of the chloroplast genes encoding the α and β polypeptides of cytochrome b_{559} in *Chlamydomonas* (1, 107) will be useful to dissect the possible role of this cytochrome in cyclic electron transport and photoprotection.

Cyclic electron transport around PS I is also suggested to have an important role in photoprotection. In addition to dissipating energy absorbed by PS I, cyclic electron transport may be involved in generating or maintaining the ΔpH that is necessary for downregulation of PS II by thermal dissipation of excess absorbed light energy (72). Biochemical approaches have led to the conclusion that there are at least two pathways of PS I cyclic electron transport, one involving a ferredoxin-plastoquinone oxidoreductase (FQR) and the other involving an NADPH/NADH dehydrogenase (NDH) complex (reviewed in 21). The FQR has not yet been identified, although the PsaE subunit of PS I is possibly involved. The NDH pathway involves a protein complex bound to the thylakoid membrane that is homologous to the NADH dehydrogenase complex I of mitochondria. Several subunits of this complex are encoded by genes on the chloroplast genome of many plants (reviewed in 63).

Mutants affecting the NDH complex have been generated by disrupting *ndh* genes in the chloroplast genome of tobacco by homologous recombination (36, 88, 139). These mutants have no obvious phenotype under normal growth conditions. However, measurements of Chl fluorescence and PS I reduction

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kinetics revealed that cyclic electron transport is partially impaired (36, 139). Induction of thermal dissipation upon sudden illumination was slightly delayed in mutants subjected to water stress, consistent with the idea that PS I cyclic electron transport is involved in maintaining a ΔpH for thermal dissipation (36). Thorough examination of photoprotection in these mutants under less favorable growth conditions should be very informative, although the existence of other cyclic pathways may complicate interpretations.

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SCAVENGING OF REACTIVE OXYGEN SPECIES

Several antioxidant systems in the chloroplast can scavenge reactive oxygen species that are inevitably generated by photosynthesis (15, 57, 128). In many cases, increases in antioxidant molecules and enzymes have been observed during acclimation to excessive light (for example, see 67), and the specific roles of these molecules are starting to be tested using mutant and transgenic organisms (3–5, 57).

Antioxidant Molecules

CAROTENOIDS Carotenoids, including the xanthophylls, are membrane-bound antioxidants that can quench ^{3}Chl and $^1O_2$, inhibit lipid peroxidation, and stabilize membranes (reviewed in 51, 60, 61, 70). The genes and enzymes involved in their biosynthesis have been reviewed recently in this series (43). Xanthophylls bound to the LHC proteins are located in close proximity to Chl for efficient quenching of ^{3}Chl and $^1O_2$ (94). β-carotene in the PS II reaction center quenches $^1O_2$ produced from interaction of ^{3}P680 and O_2 but is not thought to quench ^{3}P680 itself (145). As discussed above, specific xanthophylls are also involved in quenching of ^{1}Chl during thermal dissipation.

Carotenoids in general have essential functions in photosynthesis and photoprotection, as demonstrated by the bleached phenotypes of algae and plants that are unable to synthesize any carotenoids owing to mutations affecting early steps of carotenoid biosynthesis (6, 133). However, characterization of xanthophyll mutants blocked in later steps in the carotenoid pathway (Figure 2) has demonstrated that no single xanthophyll is absolutely required for photoprotection (109–111, 126). For example, *Chlamydomonas* mutants lacking lutein and loroxanthin (*lor1*), antheraxanthin, violaxanthin, and neoxanthin (*npq2*), or zeaxanthin (*npq1*) are all able to grow as well as wild type in high light (109, 110).

Eliminating combinations of xanthophylls in double mutants has revealed a redundancy among xanthophylls in terms of photoprotection. Construction of an *npq1 lor1* double mutant has demonstrated that accumulation of either zeaxanthin or lutein is necessary for photoprotection and survival of

Chlamydomonas in high light (110). The light sensitivity of the *npq1 lor1* strain probably reflects the multiple roles of specific xanthophylls in photoprotection; quenching of ^{1}Chl, $^1O_2$, and possibly also inhibition of lipid peroxidation are impaired in the absence of both zeaxanthin and lutein (110). A similar sensitivity to very high light is observed in older leaves of the Arabidopsis *npq1 lut2* double mutant, but the photo-oxidative damage is not lethal to the entire plant (O Björkman, C Shih, B Pogson, D DellaPenna & KK Niyogi, unpublished results).

Zeaxanthin is sufficient for photoprotection. Double mutants of *Chlamydomonas* (*npq2 lor1*) and Arabidopsis (*aba1 lut2*), as well as the C-2A′-67,3b strain of *Scenedesmus obliquus* (26), that contain zeaxanthin as the only chloroplast xanthophyll are viable, although the photosynthetic efficiency of the *Scenedesmus* strain is decreased (27), and growth of the Arabidopsis *aba1 lut2* is impaired (127). In the case of *Chlamydomonas npq2 lor1*, the mutant grows as well as wild type in high light (KK Niyogi, unpublished results). Selective elimination of all the xanthophylls derived from β-carotene to test whether lutein is sufficient for photoprotection may be difficult using mutants, because the same β-hydroxylase seems to be involved in synthesis of both zeaxanthin and lutein (43).

TOCOPHEROLS Another important thylakoid membrane antioxidant is α-tocopherol (vitamin E), which can physically quench or chemically scavenge $^1O_2$, O_2^- and OH· in the membrane to prevent lipid peroxidation (57). Whereas the xanthophylls are largely bound to proteins, α-tocopherol is free in the lipid matrix of the membrane and appears to have a role in controlling membrane fluidity and stability (64). In addition, α-tocopherol participates in the efficient termination of lipid peroxidation chain reactions with concomitant formation of its α-chromanoxyl radical (64).

Although α-tocopherol is the most abundant tocopherol in the chloroplast, other tocopherols such as β- and γ-tocopherols are also present at low levels. The minor tocopherols are intermediates in the synthesis of α-tocopherol that differ in the number of methyl groups on the chromanol head group. The relative abundance of the tocopherols ($\alpha > \beta > \gamma$) parallels their effectiveness as chemical scavengers of reactive oxygen species and as chain reaction terminators (64).

Unfortunately, there are few genetic data that address the importance of specific tocopherols, or tocopherols in general, in photoprotection. A *Scenedesmus* mutant lacking all tocopherols has been reported (29), but the lesion in the mutant appeared to affect the synthesis of phylloquinones and phytylbenzoquinones in addition to tocopherols (74). The Arabidopsis *pds1* and *pds2* mutants lack both tocopherols and quinones due to blocks in the biosynthetic

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pathway common to both types of molecules (113). Interestingly, the mutants also lacked carotenoids, uncovering a role for plastoquinone in the desaturation of phytoene (113). A gene encoding one of the later steps of tocopherol biosynthesis has recently been identified in cyanobacteria and Arabidopsis (140a), and application of reverse genetics promises insights into the photoprotective role of specific tocopherols in the near future.

ASCORBATE The soluble antioxidant ascorbate (vitamin C) has a central role in preventing oxidative damage through direct quenching of $^1O_2$, O_2^- and OH·, in regeneration of α-tocopherol from the α-chromanoxyl radical, and as a substrate in both the violaxanthin de-epoxidase and APX reactions (112, 141). Although ascorbate is very abundant in chloroplasts (~25 mM), the biosynthetic pathway for ascorbate in plants was elucidated only very recently (153), and the importance of ascorbate in photoprotection has not been determined. Ascorbate-deficient mutants affecting the biosynthetic pathway have been identified recently in a screen for ozone-sensitive mutants in Arabidopsis (40, 41). One mutant, originally called *soz1* but renamed *vtc1*, accumulates approximately 30% as much ascorbate as wild type. In addition to its ozone sensitivity, the *vtc1* mutant is more sensitive to exogenous H_2O_2, SO_2, and illumination with UV-B, but sensitivity to high light has not yet been examined.

GLUTATHIONE Another important soluble antioxidant in the chloroplast is glutathione, which is capable of detoxifying $^1O_2$ and OH·. Glutathione protects thiol groups in stromal enzymes, and it is also involved in α-tocopherol regeneration and ascorbate regeneration through the glutathione-ascorbate cycle (16, 59). Biosynthesis of glutathione proceeds by the reaction of glutamine and cysteine to form γ-glutamylcysteine, followed by the addition of glycine catalyzed by glutathione synthetase. The Arabidopsis *cad2* mutant is deficient in glutathione (78) owing to a defect in the gene encoding γ-glutamylcysteine synthetase (39). The level of glutathione in *cad2* leaves is approximately 30% of that in the wild type. As in the case of the ascorbate-deficient mutant, the sensitivity of *cad2* to photoinhibition remains to be determined.

Scavenging Enzymes

The enzymes SOD and APX are involved in scavenging reactive oxygen species in the chloroplast. As discussed above, O_2^- generated by reduction of O_2 by PS I is metabolized enzymatically by SOD to produce H_2O_2. The subsequent reduction of H_2O_2 by APX produces the monodehydroascorbate radical, which can be directly reduced by PS I (via ferredoxin) in the water-water cycle (17). Ascorbate can also be regenerated in the stroma by the set of enzymes comprising the glutathione-ascorbate cycle (16, 59).

Both SOD and APX exist in multiple isoforms within the chloroplast. The SOD isozymes are generally classified according to their active site metal (33): copper/zinc (Cu/ZnSOD), iron (FeSOD), or manganese (MnSOD). Most plants have FeSOD and Cu/ZnSOD in their chloroplasts, whereas most algae appear to lack the Cu/ZnSOD completely (45). Thylakoid-bound forms of SOD (114) and APX (104) may efficiently detoxify O_2^- and H_2O_2 at their site of production (16, 58) and prevent inactivation of Calvin cycle enzymes (84). Soluble forms of SOD and APX react with O_2^- and H_2O_2 that diffuse into the stroma from the thylakoid membrane.

Mutants or antisense plants have the potential to reveal roles of different SODs and APXs in photoprotection. Arabidopsis has at least seven SOD genes: three encoding Cu/ZnSOD, three encoding FeSOD, and one encoding MnSOD (86). One of the Cu/ZnSOD and all three of FeSOD gene products are located in the chloroplast. Mutants that are deficient in expression of plastidic and cytosolic Cu/ZnSODs or plastidic FeSODs have been isolated (DJ Kliebenstein & RL Last, unpublished results), but the phenotypes of these mutants in high light have not been tested. Genes encoding the stromal and thylakoid-bound forms of APX have been cloned from Arabidopsis (83), but mutants or transgenic plants with altered expression of chloroplast APX have not yet been reported.

Transgenic plants overexpressing SOD have been generated by several groups, and the results of these experiments were recently reviewed (3–5, 57). Photoprotection has been examined specifically in only a few cases. An enhancement of tolerance to a combination of high light and chilling stress has been observed in tobacco plants overexpressing chloroplast Cu/ZnSOD (137, 138) but not in plants overexpressing chloroplast FeSOD (147). This is consistent with the localization of Cu/ZnSOD at the thylakoid membrane where O_2^- is reduced by PS I (114). However, chloroplast APX activity is induced in the Cu/ZnSOD transgenic plants; the combination of increased Cu/ZnSOD and APX activities may result in enhanced photoprotection (138). The photoprotection phenotype of a *Chlamydomonas* mutant that overexpresses chloroplast-localized FeSOD has not been reported (85).

REPAIR OF PHOTODAMAGE

Despite multiple lines of defense, damage to the photosynthetic apparatus is an inevitable consequence of oxygenic photosynthesis, and the PS II reaction center is especially susceptible to photo-oxidative damage. Therefore, oxygenic photosynthetic organisms have evolved an elaborate but efficient system for repairing PS II that involves selective degradation of damaged proteins (primarily D1) and incorporation of newly synthesized proteins to reconstitute

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functional PS II (13). Under some circumstances, damaged PS II reaction centers may also be sites of thermal dissipation (qI) (92, 116).

The repair of PS II is an important photoprotective mechanism, because the rate of repair must match the rate of damage to avoid photoinhibition resulting from net loss of functional PS II centers. Therefore, continual new synthesis of chloroplast-encoded proteins, especially D1, is critical for photoprotection at all light intensities. Blocking chloroplast protein synthesis with inhibitors during exposure of algae or plants to light results in photoinhibition and net loss of D1 protein (for example, see 19, 146). Similarly, attenuation of chloroplast protein synthesis in rRNA mutants of *Chlamydomonas* leads to chronic photoinhibition and lower levels of D1 during growth in high light (73). To analyze the effect of a specific limitation of D1 synthesis, rather than a general inhibition of all chloroplast protein synthesis, it may be possible to isolate partial loss-of-function alleles of mutations such as *Chlamydomonas* F35, which specifically affects expression of D1 (157).

Other factors involved in repair of PS II are being identified by various genetic approaches. A screen for *Chlamydomonas* mutants that are more sensitive to PS II photodamage has uncovered mutants that may be defective in PS II repair, including a mutant that accumulates more D1 protein than wild type (158). A possible role for the *psbT* gene (originally known as *ycf8*) in the chloroplast genome of *Chlamydomonas* in protection of PS II in high light has been suggested by characterization of a *psbT* disruption mutant (105). In transgenic tobacco plants, decreasing the unsaturation of chloroplast glycerolipids results in greater sensitivity to low-temperature photoinhibition, apparently because of inhibition of PS II repair (106).

CONCLUSIONS AND PROSPECTS

Photoprotection of photosynthesis is a balancing act. Thermal dissipation and alternative electron transport pathways, together with changes in antenna size and overall photosynthetic capacity, help to balance light absorption and utilization in the constantly changing natural environment. The generation of reactive oxygen species is balanced by the capacity of antioxidant systems. The capacity for repair must match the damage that is not prevented by other photoprotective processes.

Multiple, partially redundant mechanisms acting in concert help to prevent net photo-oxidative damage that results from generation of reactive molecules through photosynthesis. The redundancy is not surprising given how critical photoprotection is for fitness and survival of algae and plants in most environments. Indeed, many of the molecules and enzymes involved in photoprotection have roles in more than one photoprotective mechanism. For

example, xanthophylls are involved in both thermal dissipation and quenching of $^1O_2$, whereas SOD and APX enzymes scavenge reactive oxygen species while maintaining electron transport via the water-water cycle.

The use of molecular genetic approaches to study photoprotection is just beginning, but the potential for rapid progress is obvious. Mutants affecting many chloroplast processes are now available, and assessment of their relative importance for photoprotection is under way in several laboratories. Because photoprotective processes comprise several lines of defense against the damaging effects of light, construction of double and perhaps even triple mutants in some cases may be necessary in order to obtain clear phenotypes. In the future, other genetic approaches, such as screening for suppressors or enhancers of these phenotypes, may uncover previously unrecognized mechanisms of photoprotection.

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