Chapter 27

Oxygen Metabolism and Stress Physiology

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Summary

Plants in nearly all growth environments absorb more light energy than they can utilize in support of photosynthetic CO_2 assimilation. This "excess light" is problematic because it can lead to the formation of unstable forms of oxygen known as reactive oxygen species (ROS), including superoxide and singlet O_2. ROS damage to chloroplast macromolecules contributes to light-mediated decreases in photosynthetic capacity. The rate of ROS formation increases during exposure to environmental stresses such as chilling, since such conditions exacerbate the imbalance between light absorption and light use by inhibiting Calvin-Benson cycle activity. Plants minimize oxidative damage caused by ROS primarily via two mechanisms, antioxidation and energy dissipation. In this chapter, I review attempts to quantify the rate of ROS formation, the molecular mechanisms of antioxidation and energy dissipation as well as their acclimation to the growth environment. I also survey recent attempts to employ molecular genetic techniques to confer greater stress tolerance to plants via manipulation of the production of proteins involved in antioxidation and energy dissipation.

I. Introduction

It has been known for some time that molecular oxygen (O_2) is capable of accepting electrons from the photosynthetic electron transport chain (Mehler, 1951; Mehler and Brown, 1952). Photoreduction of O_2 in the so-called "Mehler reaction" yields superoxide, a species with considerable reactivity and the ability to damage cellular macromolecules (Halliwell and Gutterridge, 1999). Molecular oxygen can also be converted to singlet O_2, another highly reactive species, via interaction with long-lived, triplet-excited-state forms of chlorophyll (Chl) (Foote, 1976). Collectively, singlet O_2, superoxide and the two- and three-electron products of O_2 reduction (H_2O_2 and the hydroxyl radical, respectively) are referred to as "active" or "reactive" oxygen "intermediates" or "species" (abbreviated variously as: AOI, AOS, ROI, or the term I shall use throughout this chapter, ROS). A large body of evidence suggests that abiotic environmental stresses that perturb the balance between light absorption and photosynthetic light utilization in favor of excess light absorption increase the rate of chloroplastic ROS

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generation. Furthermore, molecular damage caused by ROS likely plays a role in slowly reversible, stress-induced loss of photosynthetic capacity, which is commonly referred to as photoinhibition (Allen, 1995; Niyogi, 1999).

Plants are not completely at the mercy of ROS and the damage they can render; they possess an integrated array of biochemical mechanisms that both proactively prevent the formation of ROS and also detoxify those ROS that are formed. These mechanisms fall under the general heading "photoprotection" and include energy dissipation, which safely converts absorbed light energy to heat (Demmig-Adams and Adams, 1996; Niyogi, 1999), and the low-molecular weight and enzymatic antioxidants that operate in concert to reduce superoxide to H_2O_2 and ultimately to water (Alscher and Hess, 1993; Asada, 1996, 1999; Logan *et al.*, 1999a). Research into photoprotective processes has flourished over the last two decades, revealing much about their molecular mechanics and ecophysiology. Furthermore, with the arrival of molecular genetic techniques, the discipline has expanded from examining wild-type plants to manipulating components of photoprotection in transgenic plants in an attempt to improve performance and stress tolerance.

In this chapter, I will describe the current state of knowledge of the mechanisms of energy dissipation and chloroplastic antioxidation, the role they play in the regulation of chloroplast metabolism, and the manner in which these processes acclimate to the growth environment. In addition, I will survey the results of recent attempts to enhance plant stress tolerance via transgenic upregulation of proteins involved in photoprotection.

II. The Size of the O_2 Photoreduction "Sink"

Molecular oxygen can be reduced by several components of the photosynthetic electron transport chain, however reduction by Fe-S clusters of photosystem (PS) I appears to predominate (Asada, 1999; Badger *et al.*, 2000). The overall size of the electron "sink" represented by O_2 reduction via the Mehler reaction is an area of active research, with different experimental approaches often yielding profoundly divergent results that range from negligible to ca. 30% of total photosynthetic electron transport (reviewed in Badger, 1985; Osmond and Grace, 1995; Badger *et al.*, 2000). Quantifying O_2 photoreduction via leaf O_2 uptake is complicated by the oxygenase activity of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) as well as by simultaneous O_2 production by the Oxygen Evolving Complex (OEC) of PSII. Some of the first attempts to quantify the Mehler reaction took advantage of the fact that O_2 uptake and efflux can be distinguished by mass spectrometry in the presence of isotopically labeled $^{18}O_2$ since the O_2 produced by the OEC derives from water. Under high [CO_2] to inhibit Rubisco oxygenase activity, significant electron flow to O_2 has been observed, often between 10 and 30% of total electron transport (e.g. Canvin *et al.,* 1980; Furbank *et al.*, 1982). The magnitude of the Mehler reaction also can be quantified from the relationship between rates of photosynthetic electron transport estimated from Chl fluorescence versus those estimated from O_2 evolution. O_2 uptake via the Mehler reaction will "silence" a portion of photosynthetic O_2 evolution and thereby influence the slope of this relationship. Using this method the Mehler reaction has been shown to account for as much as 30% of total electron transport at light saturation in tropical trees (Lovelock and Winter, 1996).

Recently, transgenic tobacco expressing an antisense construct against the small subunit of Rubisco has been employed to examine photosynthetic O_2 reduction (Badger *et al.,* 2000; Ruuska *et al.*, 2000a). These plants possess reduced Rubisco activities and greatly depressed steady-state rates of CO_2 assimilation without concomitant reductions in electron transport capacity. Since reductions in Rubisco oxygenase activity parallel reductions in carboxylase activity in the transgenic plants, comparison with wild-type plants enables one to parse photorespiratory O_2 consumption from contributions of the Mehler reaction. Using simultaneous measurements of Chl fluorescence and gas exchange, the authors showed that the relationship between the rate of photosynthetic electron transport and the rate of CO_2 assimilation was linear across a range of CO_2 and O_2 concentrations and that this relationship was similar in transgenic and wild-type plants (Ruuska *et al.*, 2000a). Therefore, electron flow to the Mehler reaction was not appreciable, even in transgenic plants with strongly reduced photosynthetic light utilization. These observations were confirmed with measurements of $^{18}O_2$ uptake (Ruuska *et al.*, 2000a).

Abbreviations: APX – ascorbate peroxidase; Asc – ascorbate; DHA – dehydroascorbate; DHAR – dehydroascorbate reductase; Fd – ferredoxin; GR – glutathione reductase; GSH – reduced glutathione; GSSG – oxidized glutathione; MDA – monodehydroascorbate radical; MDAR – monodehydroascorbate reductase; OEC – oxygen evolving complex; SOD – superoxide dismutase.

All of the techniques used to date to quantify photosynthetic O_2 reduction via the Mehler reaction come with attendant complications. Isotopic labeling/mass spectrometry and determinations of photosynthetic O_2 evolution require that measurements be performed under physiologically unrealistic concentrations of O_2 and/or CO_2. Estimates of whole leaf electron transport from Chl fluorescence suffer from the possibility that fluorescence emission overemphasizes the response of the upper layer of photosynthetic cells, which may not be representative of the leaf as a whole. Antisense transgenic plants might exhibit pleiotropic effects that could alter chloroplastic function or regulation. Whether superoxide formation via the Mehler reaction is, itself, a large sink for reducing equivalents (and hence, absorbed light energy) under steady-state conditions remains to be resolved. Multiple methods do agree, however, that significant electron flow to O_2 occurs during photosynthetic induction after a period of prolonged darkness (Neubauer and Yamamoto, 1992; Ruuska *et al.*, 2000b). Even if the Mehler reaction is ultimately shown to be a relatively minor sink at steady state, superoxide production and detoxification have profound effects on the balance between reduction and oxidation of key chloroplast constituents (i.e. the "redox state" of the chloroplast) and the response to stress. An overwhelming body of evidence, albeit indirect, of the induction of superoxide-scavenging antioxidants under conditions of stress strongly suggests that environmental conditions influence the rate of chloroplastic superoxide production and that protecting against ROS-induced molecular damage is of paramount importance for stress tolerance.

III. The Water-Water Cycle

In the chloroplast, superoxide is detoxified by a complex and, in places, redundant series of reactions that leads to the formation of water using reducing power derived from photosynthetic electron transport. This series of reactions has acquired several different names, some of which honor important contributors to its understanding, others recognize important components, still others attempt to do both. The "Mehler-peroxidase pathway," "Ascorbate-glutathione cycle," "Halliwell-Asada pathway (or cycle)," "Foyer-Halliwell-Asada cycle" and the name I shall use through this chapter, the "Water-Water cycle" are among those commonly used. Some authors restrict the use of some of these names to narrowly defined portions of the reaction series (e.g. Asada, 1999; Mittler, 2002), however this practice is by no means uniformly applied across the literature. The name "Water-Water cycle" was coined by Kozi Asada (Asada, 1999) and recognizes the fact that water is both the original reducing agent (via water-splitting by the Oxygen Evolving Complex associated with PSII) as well as the final product of superoxide reduction. Hence, the Water-Water cycle is a series of reactions that produces nothing except a sink for photosynthetically-generated reducing equivalents (Fig. 1).

The first step in the detoxification of superoxide is its disproportionation (i.e. dismutation) to H_2O_2 and O_2. This reaction can occur non-enzymatically, but is greatly accelerated by the enzyme superoxide dismutase (SOD; EC 1.15.1.1) (McCord and Fridovich, 1969), for which there are stromal and thylakoid-associated isoforms employing various metal cofactors, including a thylakoid-associated CuZn-SOD and a stromal Fe-SOD (Kurepa *et al.*, 1997; Asada, 1999). Hydrogen peroxide, although less reactive than superoxide, must be removed from the chloroplast nonetheless, as it may disrupt photosynthesis by deactivating certain Calvin-Benson cycle enzymes, such as the reductively-activated *bis*phosphatases (Charles and Halliwell, 1981). In addition, H_2O_2 can decompose into the hydroxyl radical via the Fenton reaction, if superoxide is available to reduce local transition-metal cations, such as Fe^{3+}. The hydroxyl radical is a very powerful oxidizing agent and the most reactive ROS (Halliwell and Gutteridge, 1999); it has been described as reacting at "diffusion-controlled" rates. The area around PSI would seem acutely vulnerable to hydroxyl radical attack, as PSI binds several Fe-S clusters and is also the principal site for superoxide generation. Catalase, the antioxidant enzyme principally responsible for H_2O_2 detoxification in peroxisomes and other cellular compartments (Halliwell and Gutteridge, 1999), is not found in chloroplasts at appreciable activities. Instead chloroplasts dispose of H_2O_2 via ascorbate-specific peroxidases (APX; EC 1.11.1.11) (Jablonski and Anderson, 1982). APX catalyzes the two-electron reduction of H_2O_2 to water using ascorbate as a reductant and generating two monodehydroascorbate radicals, the one-electron oxidation product of ascorbate, as by-products. Several isoforms of APX are found in the chloroplast, including stromal and thylakoid-associated forms and perhaps also a lumenal isoform that has been putatively identified via analysis of the arabidopsis chloroplast proteome (Peltier *et al.*, 2002).

CuZn-SOD and APX are found in the chloroplast at approximately equimolar concentrations with P700 of PSI (Miyake and Asada, 1992; Asada, 1996).

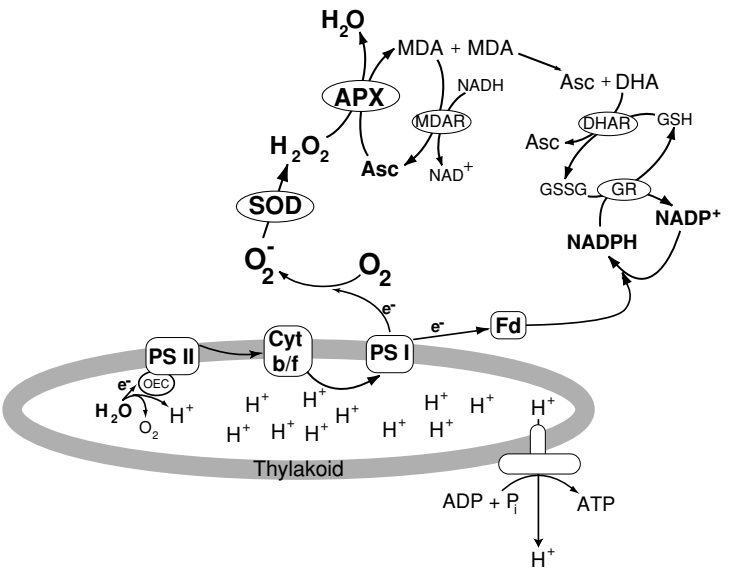

Fig. 1. A schematic depiction of electron flow through the water-water cycle. APX = ascorbate peroxidase; Asc = ascorbate, DHA = dehydroascorbate, DHAR = dehydroascorbate reductase, Fd = ferredoxin, GR = glutathione reductase, GSH = reduced glutathione, GSSG = oxidized glutathione, MDA = monodehydroascorbate radical, MDAR = monodehydroascorbate reductase, OEC = oxygen evolving complex, SOD = superoxide dismutase.

Immunogold labeling experiments suggests that thylakoid-associated and even stromal forms of APX are found predominantly in close association with PSI. These observations led Asada to propose the existence of a thylakoid super-enzyme complex that includes PSI, SOD and APX (Asada, 1996). If such a complex exists, it could greatly minimize the potentially harmful effects of ROS generation by catalyzing superoxide detoxification in an assembly-line fashion, thus limiting ROS escape.

Three known mechanisms re-reduce monodehydroascorbate back to ascorbate. Monodehydroascorbate can be photoreduced directly, a reaction that is thought to occur at either the cytochrome b_6/f complex or at PSI (Miyake and Asada, 1992; Grace *et al.*, 1995). It can also be reduced via the activity of monodehydroascorbate reductase (MDAR), which utilizes NADH (and to a lesser extent NADPH) as a reductant (Hossain *et al.*, 1984) (Fig. 1). Additionally, two monodehydroascorbate radicals can disproportionate to form ascorbate and the two electron oxidation product of ascorbate, dehydroascorbate, which can be recycled back to ascorbate via dehydroascorbate reductase utilizing reduced glutathione (GSH) as a reductant (Hossain and Asada, 1984). Finally, oxidized glutathione (GSSG) is re-reduced enzymatically by glutathione reductase (GR; EC 1.6.4.2) utilizing NADPH as a reductant (Smith *et al.*, 1989) (Fig. 1).

In addition to the largely enzyme-driven reaction sequence described above, an ROS detoxification/ascorbate regeneration pathway that is non-enzymatic, with the exception of GSSG reduction via GR, appears to be chemically feasible (Kornyeyev *et al.*, 2003b). Ascorbate, which can be found in greater-than 10 mM concentrations in the chloroplast (Foyer, 1993), can quench superoxide non-enzymatically (Halliwell and Gutteridge, 1999) and dehydroascorbate can be reduced by GSH non-enzymatically in the alkaline conditions that one would expect to find in the stroma during illumination (Foyer and Halliwell, 1976; Winkler *et al.*, 1994). While the relative contributions of various enzymatic versus non-enzymatic reactions to oxidative metabolism are the subject of debate (see Asada, 1999; Polle, 2001), the role of non-enzymatic steps might be greatest during exposure to chilling when the demand for GSH should be high and low temperatures limit enzyme activities.

A. The Response of the Water-Water Cycle to Environmental Stress

The activities/contents of antioxidants that participate in the water-water cycle undergo large changes in response to growth conditions. Acclimation can take place over the course of days to weeks after transitions in light or temperature regimes (Logan *et al.*, 1998b, 2003). The acclimation of antioxidants

to environmental stresses can be understood in terms of the effects these stresses have upon the balance between light energy absorption by the photosystems and light energy utilization via the Calvin-Benson cycle. It appears that ROS production correlates well with the level of excess light absorption (i.e. absorbed light that exceeds the capacity of photosynthetic utilization). Exposure of a broad taxonomic array of plant species to high light intensities (Gillham and Dodge, 1987; Mishra *et al.*, 1993, 1995; Logan *et al.*, 1996; Grace and Logan, 1996) results in several-fold increases in SOD, APX and GR activities and ascorbate and glutathione contents. The linkage between excess light (and not simply light intensity) and the levels of antioxidants is exemplified by the observation that *Vinca major*, a slow-growing ornamental with low capacities for photosynthesis, possesses greater foliar antioxidant activities than pumpkin, a fast-growing crop with high photosynthetic capacities, when both are raised under full-sunlight (Logan *et al.*, 1998a) (Fig. 2A, E-F). Although overall light inputs are equivalent in this experiment, greater photosynthetic light utilization by pumpkin resulted in lower levels of excess light absorption and consequently a lesser need to maintain ROS scavenging systems. Spinach raised hydroponically at either ~400 or 800 μmol photons m^{-2} s^{-1} possess similar SOD, APX and GR activities (B. Logan, T. Rosenstiel, B. Demmig-Adams and W. Adams, unpublished data),

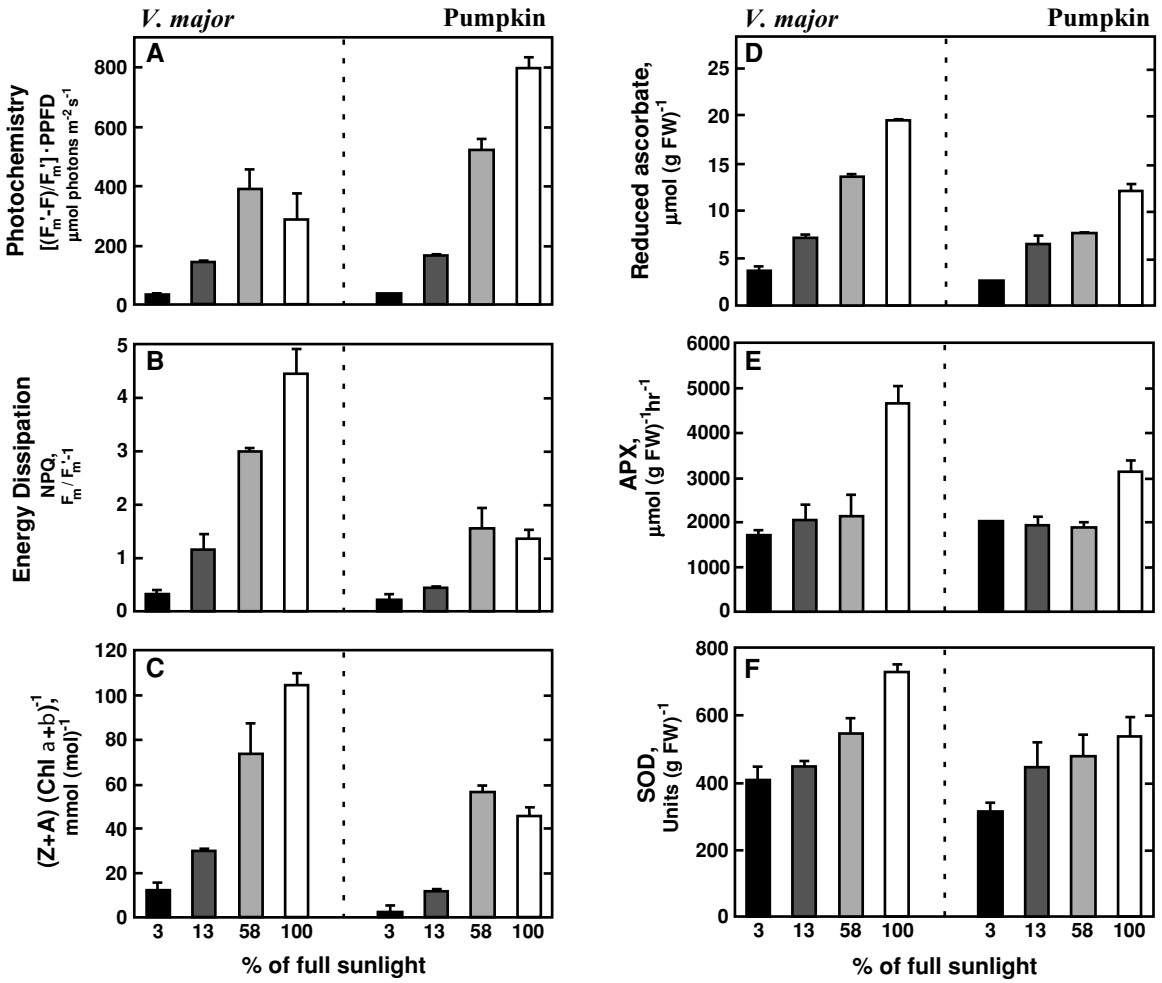

Fig. 2. Midday rate of photochemistry (a), energy dissipation (as nonphotochemical quenching) (b), the content of zeaxanthin and antheraxanthin (Z + A) per total Chl $a + b$ (c), reduced ascorbate content (d), ascorbate peroxidase (APX) activity (e) and superoxide dismutase (SOD) activity (e) for *Vinca major* and pumpkin acclimated to four different growth light intensities in the field and measured in their respective growth light environments. Growth light environments were achieved using neutral density shade cloth of various weaves. Values are means of three measurements from separate leaves. Error bars represent standard deviations. Redrawn from Logan *et al.* (1998a).

probably because the additional light absorbed at the higher intensity could be accounted for fully by photosynthetic utilization and therefore did not result in increased excess light absorption.

Chilling temperatures suppress Calvin-Benson cycle enzyme activity, but have little effect on the biophysical process of light absorption (Wise, 1995). Consequently, seasonally colder temperatures or experimentally-imposed chilling can greatly increase excess light absorption, even during exposure to moderate light intensities. Furthermore, chilling temperatures can also suppress antioxidant enzyme activities, further exacerbating the potential for oxidative damage. In many plant species, acclimation to chilling has been shown to involve profound increases in the activities of antioxidants (Schöner and Krause, 1990; Anderson *et al.*, 1992; Mishra *et al.*, 1993; Logan *et al.*, 1998c, 2003). The increase in antioxidant activity of white pine needles from summer to winter can exceed one hundred-fold (Anderson *et al.*, 1992). In addition to up-regulation in overall activity, some species have been shown to respond to chilling with preferential expression of antioxidant enzyme isoforms with lower temperature optima and other biochemical features that would favor activity at colder temperatures (Guy and Carter, 1984).

The response of antioxidants to drought varies. Experimental drought of wheat has been shown to bring about a short-term rise in SOD activity followed by a depression (Zhang and Kirkham, 1994), while GR activities were unaffected by drought in a field experiment (Gamble and Burke, 1984). In peas, SOD and APX activities increased as stomatal conductance fell after the onset of drought in a study by Mittler and Zilinskas (1994), whereas Moran *et al.* (1994) reported drought-induced decreases in APX, GR, ascorbate and glutathione. Some of the seeming contradictions in the findings above are likely to be the result of the many ways in which water stress can be imposed experimentally. It may also be that leaf wilting and drought-induced decreases in leaf Chl content may reduce overall light absorption and thus also reduce excess light absorption in some plant species.

Nitrogen limitation leads to depressed photosynthetic activities, as the demand for photosynthate falls with whole-plant growth and less nitrogen is available for maintenance of the photosynthetic apparatus. The resultant decrease in photosynthetic light use may be expected to lead to a compensatory increase in excess light absorption. However, plants acclimate to limiting nitrogen availability by strongly decreasing leaf Chl contents (Verhoeven *et al.*, 1997; Logan *et al.*, 1999b), and in doing so, can effectively limit excess light absorption. Thus when the antioxidant enzyme activities (SOD, APX and GR) of nitrogen-limited spinach are compared to those of nitrogen-replete plants, they do not differ statistically when expressed on a per Chl basis and are actually significantly lower on a leaf area basis. This means of coping with excess light minimizes the nitrogen investment in Chl, antioxidant enzymes and glutathione.

Little is known about the response of antioxidants to growth at elevated CO_2. This is remarkable given the substantial attention paid to the effects of elevated CO_2 on many aspects of plant biology and the relevance of this abiotic perturbation to global change. Schwanz and Polle (2001) examined the foliar antioxidants of pendunculate oak (*Quercus robur*) and maritime pine (*Pinus pinaster*) raised at high CO_2 (either 700 or 1200 ppm). Plants of each species under elevated CO_2 possessed lower SOD activities, which is consistent with the hypothesis that elevated CO_2 increased light utilization for photosynthesis, thereby reducing excess light absorption and ROS generation.

Cross-study comparisons of the response of antioxidants to stress can be complicated by the use of multiple bases for expression for enzyme activity. The choice of a reference basis of expression for enzyme activities (e.g. per fresh weight, per protein, per leaf area) is arbitrary to a certain degree, but it can profoundly affect the nature of the trends apparent in the data. Knowledge of (and publication of) the effect of stress factors such as drought on the basis of expression, itself, is essential if one is to evaluate data relying upon it.

B. Oxygen Metabolism and the Regulation of Chloroplast Redox State

While O_2 photoreduction may have arisen as an unavoidable consequence of photosynthetic electron transport in an O_2-rich atmosphere, it now appears that superoxide production and scavenging have been co-opted into the regulatory mechanisms that minimize photoinhibition by maintaining low reduction states among electron carriers such as Q_A, the primary quinone acceptor of PSII. PSII centers with Q_A in the reduced state are more vulnerable to photoinhibitory damage because they are more likely to undergo charge recombination as electrons get "backed up" during electron transfer. Charge recombination brings about the formation of triplet-excited Chl, a long-lived excited state that can sensitize singlet O_2 formation (Melis, 1999). Singlet O_2, in turn, can irreversibly damage Chl and proteins via oxidation.

The reduction state of Q_A is determined by the balance between light energy inputs into PSII and downstream electron flow. Any process that consumes reducing equivalents and thereby increases downstream electron flow will serve to lower the reduction state of Q_A and lower its vulnerability to photoinhibition. Under conditions of stress, where the ability of Calvin-Benson cycle activity to consume reducing equivalents is compromised, electron flow through the Water-Water cycle may serve in this capacity. This effect has been demonstrated in a series of experiments examining the performance of transgenic cotton with elevated activities for chloroplastic antioxidant enzymes. In comparison to the wild type, plants that overproduce either GR or APX maintain higher rates of electron transport through PSII, lower Q_A reduction states and sustain less PSII photoinhibition during exposure to chilling in the light at 10°C and 500 μmol photons m^{-2} s^{-1} (Kornyeyev *et al.*, 2003a,b). The protective effect of APX or GR overproduction on PSII function is abolished when electron transfer from PSII is inhibited by 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) (Kornyeyev *et al.*, 2001). Thus, the protection conferred by antioxidant overexpression is not due to the direct effects of enhanced ROS scavenging, instead it is due to the effect this enhancement has upon the redox state of PSII. Antioxidant enzyme overproduction is not likely to affect the rate of O_2 photoreduction (the Mehler reaction). Rather, it increases the rate of superoxide scavenging and in doing so increases the demand for reducing equivalents to recycle ascorbate and glutathione. This is supported by the observation that during chilling, the transgenic genotypes maintain their ascorbate and glutathione pools in more highly reduced states than wild type (Kornyeyev *et al.*, 2003a,b).

The ability of antioxidant overproduction to improve the stress-tolerance of cotton is confined to moderately chilling temperatures (10 to 15°C). During exposure to 500 μmol photons m^{-2} s^{-1} at 5°C, electron flow falls to near zero as PSII is almost completely reduced in wild type and transgenic cotton alike. All genotypes are equally sensitive to photoinhibition under this extreme stress. At warm temperatures (i.e. 20 to 30°C) antioxidant overproduction also brings about no enhancement in resistance to photoinhibition. It is likely that at warm temperatures the relative contribution of the Water-Water cycle to overall electron flow declines as Calvin-Benson cycle activity consumes a greater share of reducing equivalents and as low temperature restrictions on native GR activity are lifted (Kornyeyev *et al.*, 2003b, 2005).

IV. Dissipation of Excess Absorbed Energy

In addition to the antioxidants of the Water-Water cycle, which can be thought of as reactive in their protection against oxidative damage because they detoxify ROS after they are formed, chloroplasts possess an exquisitely responsive photoprotective process that minimizes damage by proactively preventing the formation ROS altogether. This process, which is referred to as "energy dissipation," safely converts absorbed light energy into heat before it can potentiate singlet O_2 formation. Energy dissipation is regulated over time-scales ranging from seconds to seasons to remove excess light without compromising light use for CO_2 assimilation.

The biochemical reactions that comprise the photosynthetic pathway generally exhibit saturation at light intensities well below full sunlight (which is ~2000 μmol photons m^{-2} s^{-1}) in C3 plants. In contrast, the biophysical process of light absorption by Chl saturates well in excess of full sunlight. Therefore, with the exception of those under deep, continuous shade, plants growing in the field commonly absorb light energy that exceeds their capacity for photosynthetic light utilization. Environmental stresses such as drought and chilling exacerbate the absorption of excess light because they further limit the photosynthetic pathway without exerting similar effects of light absorption (Wise, 1995).

Excess light absorption can have dangerous consequences. Chl, like most molecules, is singlet in the ground state, meaning that all electrons are found in pairs with opposing spins. When Chl absorbs a photon, it is converted to a singlet-excited state. If the energy of this singlet-excited molecule is not trapped by charge separation in the reaction center, Chl has a low, but significant, probability of undergoing a "spin flip" referred to as an intersystem crossing, which results in the formation of triplet-excited Chl (Foote, 1976). Triplet-excited Chl possesses two unpaired electrons and, like all triplet molecules, earns its name from the fact that it yields a three-line spectrum in electron paramagnetic resonance spectroscopy (Turro, 1978). Triplet-excited Chl possesses a spin restriction on energy decay back to the ground state and therefore is longer-lived than singlet-excited Chl. Triplet-excited Chl is also poised to react with ground state O_2 because O_2 is unusual in that it is triplet in the ground state (Halliwell and Gutteridge, 1999). Energy transfer between triplet excited Chl and ground-state triplet O_2 results in the formation of singlet O_2, a dangerous ROS (Asada, 1996; Niyogi,

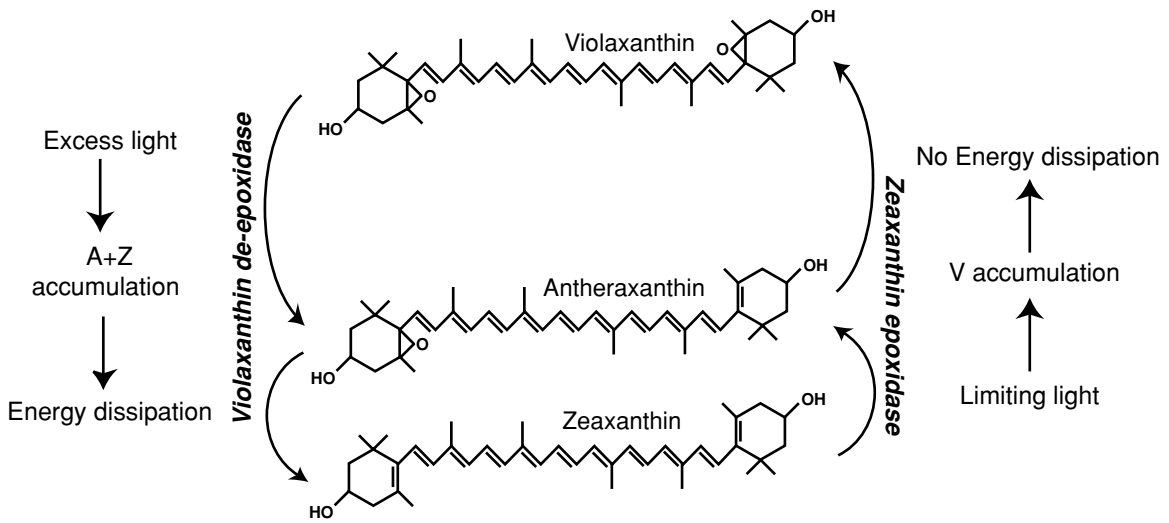

Fig. 3. The carotenoids of the xanthophyll cycle and the enzymes involved in xanthophyll cycle conversions.

1999). Under conditions that lead to excess light absorption, the lifetime of singlet-excited Chl in the light harvesting complexes lengthens since light energy trapping by reaction centers is saturated. The extended lifetime of singlet-excited Chl increases the probability that intersystem crossing leading to triplet-excited Chl will occur.

Energy dissipation interrupts the formation of singlet O_2 by converting the energy of singlet-excited Chl to heat, which is readily exchanged with the surroundings across the leaf lamina. Energy dissipation requires the presence of either zeaxanthin (Z) or antheraxanthin (A) (Gilmore and Yamamoto, 1993a, 1993b; Demmig-Adams and Adams, 1996) and a low pH in the thylakoid lumen (Fig. 3). When energy dissipation is invoked in leaves exposed to excess light, A and Z are created from the de-epoxidation of violaxanthin (V) in a reaction catalyzed by violaxanthin de-epoxidase (VDE), an enzyme localized to the thylakoid lumen. Upon return to less stressful conditions, plants reverse this reaction and reform V from Z and A via the activity of zeaxanthin epoxidase, which is localized to the chloroplast stroma. The three carotenoids and the enzymes that catalyze their interconversions are referred to as the "xanthophyll cycle." The precise role that xanthophylls play in energy dissipation remains the subject of debate and active research; however, evidence is building in favor of a direct role involving energy or electron transfer from Chl to Z (Frank *et al.*, 1994; Owens *et al.*, 1997; Ma *et al.*, 2003; Holt *et al.*, 2005). Z appears to possess an S_1 excited state that is lower in energy than Chl, thus making energy transfer from Chl to Z thermodynamically feasible. Alternatively, absorption of light by chlorophyll has been shown to lead to the formation of a carotenoid radical under conditions that bring about high rates of energy dissipation (Holt *et al.*, 2005). This suggests that the molecular mechanism of energy dissipation involves energy transfer to a chlorophyll-zeaxanthin dimer, which undergoes charge separation followed by recombination (Holt *et al.*, 2005).

Violaxanthin de-epoxidation is a reductive process and VDE utilizes ascorbic acid, and not the ascorbate anion, as a source of electrons (Bratt *et al.*, 1995). As a result, V de-epoxidation has a relatively low pH optimum. This biochemical feature of Z and A formation can be thought of as part of the regulation of energy dissipation, since VDE is localized to the thylakoid lumen, a compartment that one would expect to become more acidic under conditions of excess light absorption, as electron transport augments the proton gradient faster than it is utilized for ATP formation.

Energy dissipation occurs on a protein subunit of PSII known as PsbS (Li *et al.*, 2000), which has been referred to also as CP22 (Funk *et al.*, 1994). This was revealed by analyses of npq4-1, a null mutant of Arabidopsis PsbS, which is deficient in energy dissipation but possesses a fully functional xanthophyll cycle (Li *et al.*, 2000). Subsequently, transgenic plants that overproduce PsbS have been shown to exhibit higher than wild type levels of energy dissipation after abrupt transfer from darkness to intense light (Li *et al.*, 2002a). PsbS belongs to the Light Harvesting Complex protein superfamily, and has four (rather than the more

common three) trans-membrane helices (Li *et al.*, 2000). Loops of PsbS that connect trans-membrane helices and project into the thylakoid lumen possess conserved glutamic acid residues that are required for energy dissipation, as demonstrated by site-directed mutagenesis (Li *et al.*, 2002b). A compelling model is emerging from this research in which acidification of the thylakoid lumen under conditions of excess light absorption brings about the protonation of critical glutamic acid residues of PsbS, which alters the conformational structure of the protein in a way that leads to Z binding or brings previously-bound Z into an alignment with Chl to permit energy dissipation. This model is consistent with the requirement for a low lumenal pH not only for VDE activity, but also for energy dissipation itself.

Plants alter the de-epoxidation state of their xanthophyll cycle on a minute-to-minute basis throughout the day to ensure adequate protection against the damaging effects of excess light, while at the same time leaving productive use of absorbed light energy for carbon assimilation uncompromised (Adams and Demmig-Adams, 1992; Demmig-Adams and Adams, 1992a, 1996). Thus, this proactive photoprotective mechanism is exquisitely responsive and efficient, requiring only relatively rapid and energetically inexpensive catalytic conversions among pigments instead of far slower and more costly de novo synthesis. Plants acclimate on longer time scales (weeks to seasons) to prevailing conditions and the potential for excess light absorption by adjusting the size of their xanthophyll cycle pool and also the expression of PsbS. In numerous field, greenhouse and growth chamber studies, plants acclimated to more intense light environments possess larger xanthophyll cycle pools (Fig. 4) and convert a greater fraction of their xanthophyll cycle pool to Z and A at midday (e.g. Demmig-Adams and Adams, 1992b; Grace and Logan, 1996; Logan *et al.*, 1998a,b,c). When raised under similar light environments, plants with a greater capacity to utilize light for photosynthetic carbon assimilation possess smaller xanthophyll cycle pools (Logan *et al.*, 1998a) (Fig. 2A-C). Environmental stresses such as wintertime cold temperatures or low soil nitrogen availability lead to lower photosynthetic capacities. Plants have been shown to adjust Chl contents downwards in response to these environmental stresses and also to increase their xanthophyll cycle pool size (when expressed per unit Chl) (Adams and Demmig-Adams, 1994; Verhoeven *et al.*, 1996, 1997; Logan *et al.*, 1998c; Burkle and Logan, 2003). Studies of the acclimation of PsbS expression to environmental stress have just begun, however increased

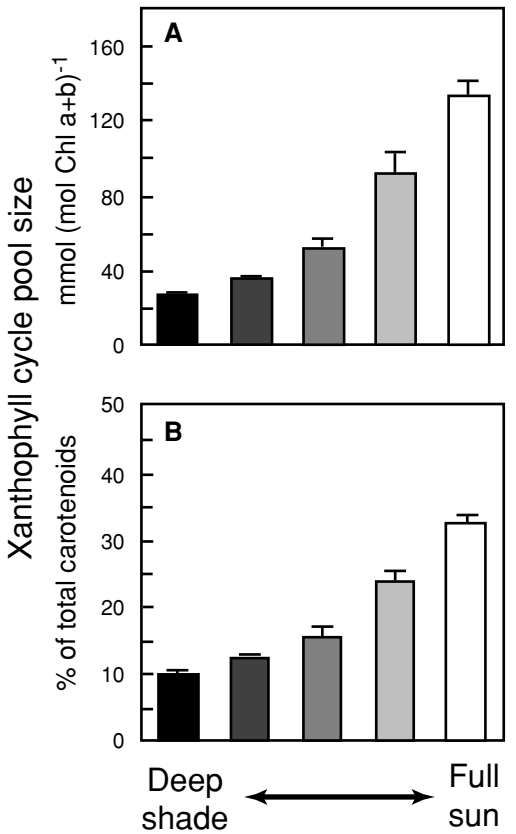

Fig. 4. Xanthophyll cycle pool sizes expressed per total Chl $a + b$ (a) and as a percentage of the total carotenoid pool (b) for plants collected from 5 sites representing a continuum from deeply shaded to full sun environments. Sites were located in Dorrigo National Park, a subtropical rainforest in New South Wales, Australia. Error bars represent standard error of the mean. n = 10, 12, 7, 4, and 8 different plant species for sites from deeply shaded to full sun, respectively. Redrawn from Logan *et al.* (1996).

expression (relative to the expression of PSII core proteins) has already been demonstrated in response to seasonally colder temperatures in pine (Ottander *et al.*, 1995; Ebbert *et al.*, 2005) and high light in Arabidopsis (B. Logan, K. Niyogi, unpublished data) and *Monstera deliciosa* (V. Ebbert, B. Demmig-Adams and W. Adams, unpublished data).

Highly dynamic light environments such as the forest understory present unique challenges to plants because they experience rapid, at-times large, and unpredictable fluctuations in light intensity. Sunflecks nearing the intensity of full sunlight can penetrate the overstory canopy and abruptly strike understory leaves that would otherwise be deeply shaded. Changes in light intensity can occur more quickly than plants can respond to via enzyme-catalyzed interconversions among

xanthophyll cycle pigments. Instead, plants have been shown to de-epoxidize V in response to the first sunflecks experienced each day and retain Z between sunflecks (Logan *et al.*, 1997; Adams *et al.*, 1999). The level of energy dissipation is then presumably rapidly modulated via the strength of the trans-thylakoid proton gradient and its effects on lumenal pH.

V. Transgenic Manipulations of Photoprotection

The observation that the response to many environmental stresses, particularly chilling, includes upregulation of antioxidant systems has led plant geneticists to attempt to improve the stress tolerance of some crop species by transforming plants with genes for chloroplast-targeted antioxidant enzymes (for reviews see Foyer *et al.*, 1994; Allen, 1995). Such attempts have met with mixed results. Various degrees of protection from chilling-induced photoinhibition at high PPFD were reported for poplar overproducing chloroplastic GR (Foyer *et al.*, 1995) and MnSOD (Foyer *et al.*, 1994) and tobacco overproducing Cu/ZnSOD (Sen Gupta *et al.*, 1993a,b). In contrast to these studies, little protection was conferred by overproduction of chloroplastic FeSOD in poplars, cytosolic GR in tobacco exposed to high light (Tyystjärvi *et al.,* 1999), or chloroplastic MnSOD in cotton exposed to high light at cold temperatures (Payton *et al.* 1997).

The studies mentioned above and most others seeking to examine the stress tolerance of transgenic plants employ abruptly imposed, severe stress exposures. Typically, leaf tissues are detached from plants raised under relatively benign conditions and placed under stresses that far exceed those that the species under study might encounter in the field. Experiments of this sort have yielded important insights into the mechanisms of chilling tolerance and the regulation of oxidative metabolism, however the enhanced stress tolerance that is occasionally observed under such artificial conditions may not be predictive of enhanced stress tolerance under field conditions. For example, when leaf discs of warm-grown transgenic cotton that possess 30- to 40-fold higher chloroplastic GR activities were abruptly exposed to 10°C at 500 μmol photons m^{-2} s^{-1}, they sustained approximately 28 and 20% lower levels of PSII and PSI photoinhibition, respectively, in comparison to wild type (Kornyeyev *et al.*, 2001, 2003b) (Fig. 5 left panel). However, chilling tolerance was not enhanced when this same cotton genotype was raised in a growth chamber in which temperatures were lowered from 28 to 14°C over 9 days and held at for a subsequent 9-day period at 14°C (Logan *et al.*, 2003) (Fig. 5 right panel). The absence of an effect of GR overproduction under longer-term, gradually imposed chilling may be

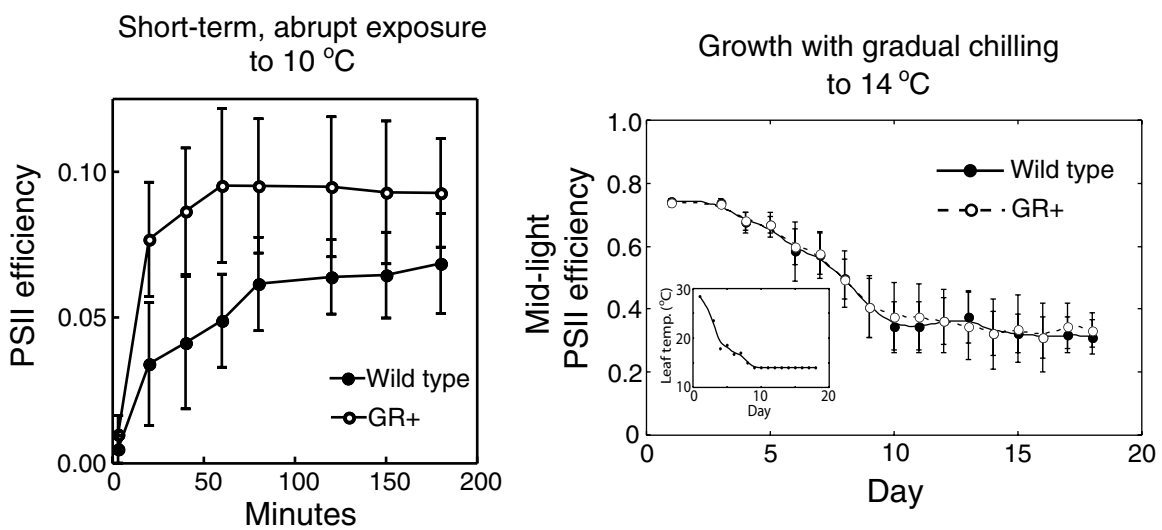

Fig. 5. The effect of two different chilling regimes, short-term abrupt exposure to 10°C (left panel) and growth with gradual chilling to 14°C (right panel), on the photosystem II efficiency ($[F_m'-F]/F_m'$) of wild-type cotton (closed circles) and transgenic cotton exhibiting a 30 to 40-fold overproduction of chloroplastic glutathione reductase (GR+; open circles). Error bars represent standard deviation, n = 10 − 13 for the short-term exposure and 8–16 for the gradual chilling. Redrawn from Kornyeyev *et al.* (2003) and Logan *et al.* (2003).

explained, in part, by the fact that wild-type cotton acclimated to this chilling regime by upregulating native GR activity two-fold (Logan *et al.*, 2003).

The recent discovery that PsbS is required for energy dissipation has yielded a means of transgenically enhancing capacities for energy dissipation. Arabidopsis plants that have been transformed to overproduce PsbS possess correspondingly higher capacities for energy dissipation (Li *et al.*, 2002a). In comparison to wild-type plants, PsbS overproducers sustained slightly, but significantly, less photoinhibition when raised in a greenhouse (B. Logan and K. Niyogi, unpublished data). Interestingly, however, no differences in photoinhibition were observed when these same genotypes were raised under continuous light of 1500 μmol photons m^{-2} s^{-1} in a growth chamber. Although the growth chamber provided much higher total daily photon fluxes than the greenhouse, the greenhouse light environment included rapid and large fluctuations in light intensity. Taken together, these results suggest that energy dissipation may be most effective at managing light stress under fluctuating light intensities.

Clearly, the nature and timing of the stress profoundly influences the response of transgenic plants with increased ROS scavenging capacity or levels of energy dissipation. This should be taken into account when assessing the utility of manipulating photoprotective processes as a strategy for developing more stress-tolerant crop varieties for agricultural use and underscores the need to design experiments that examine the performance of transgenic genotypes under realistic conditions of stress.

VI. Extra-Chloroplastic Photoprotection

Photoprotection is not confined to chloroplast biochemistry; plants have evolved biochemical, ultrastructural and anatomical means of reducing chloroplastic light stress that reside beyond the bounds of the chloroplast envelope. These include ROS scavenging in other cellular compartments, chloroplast movements and a host of leaf surface features and morphological adjustments aimed at reducing excess light absorption.

Superoxide does not readily cross membranes and is sufficiently unstable that it is likely to react very near to its source. Hydrogen peroxide, on the other hand, is less reactive and capable of passing through membranes. This introduces the possibility that photogenerated H_2O_2 might diffuse out of the chloroplast and render its effects in other cellular compartments. Light stress has been shown to lead to the up-regulation of cytosolic isoforms of APX (Karpinski *et al.*, 1997; Yoshimura *et al.*, 2000). In addition, light stress very often induces the accumulation of phenolic compounds (Grace *et al.*, 1998; Grace and Logan, 2000). Certain phenolics act as efficient reductants for vacuolar guaiacol peroxidase (Yamasaki *et al.*, 1997; Yamasaki and Grace, 1998). Since phenolics tend to be concentrated in the vacuole and the vacuole occupies most of the cell volume, phenolic-assisted reduction of H_2O_2 to H_2O might be a significant pathway for ROS detoxification.

Chloroplasts are closely associated with actin filaments of the cytoskeleton, which can control organelle position within the cell. When exposed to low light intensities, chloroplasts organize along the upper and lower planes of the cell in order to maximize light interception. However, exposure to high light intensities leads chloroplasts to migrate to the lateral walls of cells to maximize self-shading and thereby minimize light interception (Haupt and Scheuerlein, 1990; Brugnoli and Björkman, 1992). Such movements probably serve to optimize light absorption under low irradiance conditions (Williams *et al.*, 2003) and may also reduce photodamage at high irradiance (Park *et al.*, 1996; Kasahara *et al.*, 2002).

Many plants acclimated to full sunlight develop leaves with steep angles (relative to horizontal orientation) in order to reduce sunlight exposure (e.g. Mooney *et al.*, 1977). Some plant species are capable of leaf movements that influence light interception (Koller, 1990). *Oxalis oregana*, an understory herb in redwood forests of the northwestern United States, folds it leaflets downwards within ~five minutes of exposure to bright sunflecks (Powles and Björkman, 1989). Leaflets that were experimentally restrained in the horizontal position suffered almost twice as much sunfleck-induced photoinhibition (Powles and Björkman, 1989). During drought, the legume *Macroptilium atropurpureum* orients its leaves parallel to the sun's rays in order to minimize light stress and photodamage, a response referred to as paraheliotropism (Ludlow and Björkman, 1984). At the other end of the spectrum, some desert plants exhibit diaheliotropism, leaf movements that maintain leaf lamina perpendicular to the sun's rays, after periods of rainfall in order to maximize their potential for photosynthetic carbon gain during the transient period when adequate water supplies permit increased rates of evapotranspiration (Ehleringer and Forseth, 1980). Under low nitrogen availability, soybean employs a combination of midday paraheliotropism and afternoon diaheliotropism to orient leaves such that they are experiencing the light intensity where electron transport and the

Calvin-Benson cycle co-limit photosynthetic activity, thereby maximizing their return on investment of nitrogen into the photosynthetic machinery and reducing light stress (Kao and Forseth, 1992).

Leaves of various species possess highly reflective surface waxes (Barker *et al.*, 1997) or pubescence (Ehleringer and Björkman, 1978) to minimize light absorption. *Mahonia repens*, a broad-leafed evergreen native to the western United States, accumulates high concentrations of anthocyanins (which are red in color) in its upper epidermis in winter, only to "regreen" during the following growing season (Grace *et al.*, 1998). Anthocyanins may serve as a sunscreen in winter when photosynthetic light use is greatly suppressed by low temperatures. *Atriplex hymenelytra*, an evergreen desert shrub, possesses bladder-like trichomes on leaf surfaces, which are filled with salty water (Mooney *et al.*, 1977). During the moist season, when photosynthetic light use is greatest, these bladders remain hydrated and are optically transparent. In the dry season, when photosynthetic light use falls with intensifying drought, these bladders collapse, leaking their contents onto the leaf surface, where the water quickly evaporates and leaves behind a highly reflective layer of crystalline salt that reduces photodamage to mesophyll cells below (Mooney *et al.*, 1977).

VII. Concluding Remarks

Chloroplasts are uniquely vulnerable to oxidative damage because they are the site of both O_2 production and of energy and electron transfer reactions that can potentiate ROS formation. Research over the last two decades has established the importance of photoprotection in maintaining a functional photosynthetic apparatus. In fact, as the elegance and intricacies of photoprotective mechanisms become more apparent, we are growing to appreciate that photosynthesis and photoprotection are tightly interwoven. Evolution has established a role for O_2 photoreduction and subsequent ROS scavenging in the regulation of photosynthetic electron transfer and chloroplast redox state. Antioxidants can no longer be viewed simply as "mop-up" agents that eradicate toxic ROS. ROS themselves have been shown to influence cellular signal transduction pathways that alter gene expression. Future research into the subtle interplay between the potentially harmful and the regulatory aspects of chloroplast oxidative metabolism may lead to novel strategies for employing transgenic technologies to improve the stress tolerance of crops. Or, perhaps, it will lead us simply to marvel further at the balancing act between autotrophic light use and protection against oxidative damage that plants accomplish.

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