

Enhanced photochemical light utilization and decreased chilling-induced photoinhibition of photosystem II in cotton overexpressing genes encoding chloroplast-targeted antioxidant enzymes

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The aim of this study was to determine whether increases in stromal superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2) via transformation could reduce photosystem (PS) II photoinhibition at low temperature for cotton (*Gossypium hirsutum* L.) plants and to determine by what mechanism this protection may be realized. During 3-h exposures of lincomycin-treated leaf discs to 10°C and a photon flux density of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, all transgenic plants exhibited significantly greater PSII activity and O_2 evolution than did wild-type plants. Also, the rate constant of PSII photoinactivation was significantly lower for all transgenic plants than for wild-type plants. No significant differences existed between genotypes in non-photochemical quenching of chlorophyll *a* fluorescence and the regulated component of the thermal dissipation of excitation energy. The relationship between changes in variable to maximum chlorophyll fluores-

cence (F_v/F_m) and the time-dependent averaged excessive light exposure was similar for all genotypes. This observation excluded the possibility that differences in PSII photodamage were due to improvements in the direct protection of PSII from active oxygen by antioxidant enzyme overproduction. Similar decreases in F_v/F_m during the stress treatment for all genotypes when leaves were pre-treated with 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) suggested that the effect of overproduction involved events downstream of PSII in the electron transfer pathway. Since all transgenic plants exhibited a significantly higher photochemical quenching of chlorophyll fluorescence during the chilling treatment, we concluded that, under the conditions used in this study, the enhancement of the protection of PSII from photodamage by increasing the stromal antioxidant enzyme activity in cotton leaves was due to the maintenance of a higher rate of electron transport and, consequently, a lower reduction state of Q_A .

Introduction

Chilling temperatures limit the activity of Calvin cycle enzymes, thus reducing the utilization of absorbed light energy for CO_2 assimilation (Leegood 1995, Wise 1995). This diminished demand for absorbed light energy can render plants more susceptible to photoinhibition (Melis 1999). In

fact, even relatively moderate photosynthetic photon flux densities (PPFDs) can induce photoinhibition at chilling temperatures.

The rate of oxygen photoreduction increases during chilling, increasing the production of the reactive oxygen species

Abbreviations – APX, ascorbate peroxidase; D, fraction of light absorbed in PSII antennae that is dissipated via thermal dissipation; D_{CON} , D_{REG} and D_{PI} , components of the thermal dissipation, constitutive in the dark-adapted state before photoinhibitory treatment, regulated (reversible in the dark), and photoinhibitory (non-reversible), respectively, expressed as the fraction of the light absorbed in PSII antennae; E, fraction of excessive light absorbed in PSII antennae; F , actual level of fluorescence; F_0 , F_m and F_v , initial, maximal and variable chlorophyll fluorescence yield in dark-adapted samples, respectively; F'_0 , F'_m and F'_v , initial, maximal and variable chlorophyll fluorescence yield in illuminated samples, respectively; GR, glutathione reductase; k_{PI} , rate constant of photoinhibition; NBT, nitroblue tetrazolium; NPQ, non-photochemical fluorescence quenching ($F_m/F'_m - 1$); P, fraction of light absorbed in PSII antennae that is utilized in photosynthetic electron transport; PPFD, photosynthetic photon flux density; Q_A , primary quinone acceptor of PSII; q_p , coefficient of photochemical quenching; ROS, reactive oxygen species; SOD, superoxide dismutase.

(ROS), superoxide (O_2^-), H_2O_2 and, potentially, the hydroxyl radical (Wise and Naylor 1987, Hodgson and Raison 1991, Wise 1995, Prasad 1996). There is evidence that ROS may be produced at both PSII and PSI (Foyer and Harbinson 1994, Osmond and Grace 1995, Melis 1999). Because these highly reactive substances can damage proteins involved in photosynthetic electron transport and thylakoid membrane lipids (Halliwell and Gutteridge 1999), as well as deactivate certain Calvin cycle enzymes (Charles and Halliwell 1981), they are implicated in the mechanism of chilling-induced photoinhibition.

Chloroplasts possess a system of antioxidant enzymes that can detoxify ROS before they damage cellular constituents, although this system can be overwhelmed during chilling in the light (for reviews see Asada 1994, 1999, Foyer and Harbinson 1994, Foyer et al. 1994b, Logan et al. 1999). This antioxidant system includes superoxide dismutase (SOD) and ascorbate peroxidase (APX), whose combined activities catalyze the conversion of O_2^- to H_2O , while oxidizing ascorbate (Asada 1999). Two mechanisms to regenerate ascorbate involve reduced glutathione, either as a substrate for dehydroascorbate reductase (reviewed in Noctor and Foyer 1998) or perhaps via non-enzymatic reduction of oxidized ascorbate under the alkaline conditions present in the stroma during illumination (Winkler et al. 1994). Chloroplastic GR maintains the pool of reduced glutathione, using NADPH as a reductive substrate. The importance of antioxidant enzymes in the protection of the photosynthetic apparatus during photoinhibitory conditions, such as those that develop with chilling in the light, is indicated by the following observations: (1) the activities of these enzymes generally increase during acclimation to chilling temperatures (Schöner and Krause 1990, Prasad 1996, Hull et al. 1997, Fryer et al. 1998, Logan et al. 1998), presumably to cope with increased ROS production; (2) a correlation exists between resistance to chilling-induced photoinhibition and high antioxidant enzyme activities in comparative studies of chilling-sensitive versus chilling-resistant cultivars or species (Wise and Naylor 1987, Janke et al. 1991, Kocsy et al. 1996, Hodges et al. 1997); (3) supplementing isolated thylakoid preparations with antioxidant enzymes reduces their susceptibility to photoinhibition (reviewed in Tyystjärvi et al. 1999).

These observations underlie attempts to increase plant resistance to chilling-induced photoinhibition via genetic manipulation of leaf antioxidant systems (for reviews see Foyer et al. 1994a, Allen 1995). Such attempts have met with mixed results. For example, various degrees of protection from chilling-induced photoinhibition at high PPFD were reported for overproduction of chloroplastic GR in poplar (Foyer et al. 1995) and MnSOD (Foyer et al. 1994a) and Cu/ZnSOD in tobacco (Sen Gupta et al. 1993a,b). However, little protective effect was observed for overproduction of chloroplastic FeSOD in poplars, cytosolic GR in tobacco (Tyystjärvi et al. 1999), or chloroplastic MnSOD in cotton (Payton et al. 1997) during exposure to severe stress conditions. In those studies where genetic manipulation did lead to significantly enhanced chilling tolerance, the actual mechanism of this enhancement was not determined.

The aim of the present study was to examine the effects of increasing the activity of SOD (MnSOD), APX and GR in

the chloroplast stroma of cotton leaves on the activity of PSII, an important site of photoinhibition, during exposure to 10°C and a moderate PPFD ($500 \mu\text{mol m}^{-2} \text{s}^{-1}$). Leaves were treated with lincomycin, an inhibitor of chloroplast protein synthesis, to evaluate the kinetics of PSII photoinactivation without the influence of repair processes. During the stress treatment, we assessed the allocation of absorbed light energy to photosynthetic electron transport and to components of thermal energy dissipation.

Materials and methods

Plant material

The development of cotton plants, *Gossypium hirsutum* L. cv. Coker 312, which overproduced chloroplast-targeted MnSOD (SOD + plants), has been described previously (Payton et al. 1997). To transform plants that overproduced chloroplast-targeted APX (APX + plants), the APX cDNA from pea (Mittler and Zilinskas 1991) was developed using polymerase chain reaction to add a Sal I site at the translation start codon (ATGGGA to GCTGAC) of the pea cytosolic APX cDNA. To target the APX to the stroma, the modified cDNA was fused with a fragment from the pea chloroplastic Cu/ZnSOD cDNA that encodes the transit peptide (Scioli and Zilinskas 1988). To generate plants that overproduced GR (GR + plants), a modified GR cDNA was amplified from total leaf RNA of *Arabidopsis thaliana*, ecotype Columbia, using primers designed from a cDNA described by Kubo et al. (1993). The mutated cDNA was developed with a Nco I site at the transcription start site (AGATGG to CCATGG) to allow for ligation into the TEV leader sequence start codon in the expression vector pRTL2. The predicted polypeptide contained an N-terminal chloroplast transit peptide sequence of 74 amino acids. In all transgenic lines, the coding sequence was under the control of an enhanced cauliflower mosaic virus 35S promoter. Transformation was accomplished by inoculating cotton hypocotyls with *Agrobacterium tumefaciens*. Plants were generated from transformed callus cells by somatic embryogenesis (Bayley et al. 1992).

The T_6 or T_7 generation plants from two independently transformed lines of SOD + and the T_4 or T_2 generation plants from three lines of APX + and GR +, respectively, were grown from seed in 8 l pots in a greenhouse at approximately 30/26°C (day/night) with a natural photoperiod from mid-February to late June. Plants were fertilized with Hoagland's solution twice per week. The first fully expanded leaf of plants 5–8 weeks old was used for the enzyme assays and fluorescence measurements. Plants of cv. Coker 312 were used as controls (wild-type genotype).

Identification of transgenic plants

To confirm that the SOD + plants that we used had high levels of active MnSOD, the seedlings were screened by subjecting extracts prepared from fully expanded cotyledons to non-denaturing polyacrylamide gel electrophoresis according to Beauchamp and Fridovich (1971) as modified by van Camp et al. (1994). Representative gels are shown in Payton et al. (1997). To screen for APX + and GR +

plants, extracts of cotyledons were assayed for APX and GR activity spectrophotometrically as described below.

Determination of SOD, APX and GR activities from whole-leaf extracts

Leaf discs were rapidly removed using a cork borer and immediately frozen in liquid N₂. Frozen leaf discs were ground to powder at liquid N₂ temperature using a mortar and pestle and then rapidly homogenized in 1 ml of the appropriate ice-cold extraction solution in a glass tissue grinder. Aliquots were taken before centrifugation for chlorophyll determination in 80% acetone according to Lichtenthaler (1987). The assays were initiated within 1.5 min after commencing the extraction with 25 µl of centrifuged extract. The assay temperature was 25°C.

The SOD activity was measured by monitoring the inhibition of nitroblue tetrazolium (NBT) reduction at 560 nm. Tissue was homogenized in 50 mM KH₂PO₄ (pH 7.0). The reaction mixture contained 50 mM KH₂PO₄ (pH 7.0), 0.1 mM EDTA, 75 µM NBT, 2 µM riboflavin and 13 mM methionine. The reduction of NBT proceeded under 500 µmol photons m⁻² s⁻¹ of white light for 8 min. One unit of SOD activity was defined as the amount that inhibits the reaction by 50% (Giannopolitis and Ries 1977).

The extraction and assay solutions for APX and GR assays were as described by Sen Gupta et al. (1993b). The APX activity was determined by monitoring the H₂O₂-dependent oxidation of ascorbate at 290 nm. The GR activity was measured spectrophotometrically by monitoring the oxidation of NADPH at 340 nm.

Experimental treatments

Leaves were harvested before sunrise. Their petioles were cut under water, and they were immediately transferred to microfuge tubes containing 1 mg ml⁻¹ lincomycin (863 units mg⁻¹). At the end of the 3.5 h of dark incubation, the concentration of lincomycin in the bulk leaf tissue (C_t) was 0.8–1.5 mM as estimated from the formula: C_t = C_s(W_s/W_L), where C_s is the inhibitor concentration in the solution, W_s is the weight of the solution taken up by a leaf, and W_L is the fresh weight of the leaf (Bilger and Björkman 1994). Lincomycin inhibits chloroplast protein synthesis (Klauff and Gruijssem 1991) and, therefore, prevents the repair of photoinhibitory damage. Leaf discs treated with lincomycin and subjected to photoinhibitory conditions exhibited no increase in F_v/F_m when subsequently exposed to conditions that favored repair processes (3 h at 10 µmol photons m⁻² s⁻¹ and room temperature) (data not shown). For some experiments, lincomycin-treated leaves were wetted with 1 mM DCMU 1.5 h before the start of illumination.

Oxygen evolution and chlorophyll fluorescence measurements

After 3.5 h of dark adaptation at room temperature, leaf discs (10 cm²) were taken from lincomycin-treated leaves.

The discs were kept in the dark at 10°C for 10 min prior to treatment. Then they were exposed to a PPFD of 500 µmol m⁻² s⁻¹ and 10°C in the chamber of an oxygen electrode (Hansatech, King's Lynn, Norfolk, UK). A sponge wetted with 1 M NaHCO₃ supplied CO₂ in the chamber. This solution was exchanged every 40 min during the first 2 h and then every 30 min for the last hour. The rate of oxygen evolution was measured at the end of the third hour of exposure to the chilling/moderate PPFD treatment.

Chlorophyll fluorescence emission from leaf discs was measured with a pulse amplitude-modulated fluorometer (PAM 101/103; Heinz Walz GmbH, Effeltrich, Germany) through a port in the leaf disc electrode chamber at various times during the 180-min exposure to chilling/moderate PPFD treatment. The experimental protocol described by Schreiber et al. (1986) and the nomenclature of van Kooten and Snel (1990) were used. The level of energy dissipation in PSII was estimated from non-photochemical quenching of F'_m as F_m/F'_m - 1 (Stern-Volmer quenching or NPQ) at 20 and 40 min of treatment. Measurements of F₀ and F'₀ were performed after a 10-s application of low-intensity far-red light. The frequency of the modulated measuring light was 1.6 kHz when measurements were made in the absence of actinic light and 100 kHz in the presence of actinic light. Saturating light pulses of 2-s duration were provided by a KL 1500 light source (Schott, Wiesbaden, Germany). Leaf discs of all genotypes had F_v/F_m values of approximately 0.78 prior to the chilling/moderate PPFD treatment.

For some of the 10-cm² leaf discs, at 40, 80, 120 and 180 min of illumination at 10°C, 1-cm² discs were removed to determine F_v/F_m. These determinations were performed after 3 h of dark adaptation at 25°C. Removal of a small amount of leaf tissue from the original leaf disc rendered it impossible to assess NPQ at later time points. Instead, we used the approach developed by Demmig-Adams et al. (1996) to calculate the allocation of photons absorbed by PSII antennae to thermal energy dissipation. The level of the thermal energy dissipation (D) in PSII antennae can be expressed as the difference between maximal theoretical and actual values of PSII efficiency (D = 1 - F'_v/F'_m). F'_v/F'_m reflects the intrinsic quantum efficiency of charge separation in PSII (Harbinson et al. 1989). The rest of the light energy absorbed by PSII is presumably dissipated as heat (the share of other processes, such as fluorescence, is assumed to be relatively negligible). The fraction of light energy absorbed by PSII antennae that was utilized for photochemistry (P) was estimated as P = F'_v/F'_m × q_P = (F'_m - F)/F'_m (Genty et al. 1989), where q_P is the coefficient for photochemical quenching (q_P = (F'_m - F)/F'_v). The fraction of light not allocated to P nor D (referred to as 'excess', E) is a function of the fraction of closed PSII centers and the quantum yield of PSII centers and was estimated using the following formula from Demmig-Adams et al. (1996):

$$E = 1 - (D + P) = (F'_v/F'_m) \times (1 - q_P) \quad (1)$$

According to this formula, E is the portion of light energy absorbed by PSII antenna pigments that is trapped by

reaction centers of PSII complexes with Q_A in the reduced state ('closed' reaction centers). The result of light energy trapping by 'closed' PSII reaction centers can be the formation of triplet P680, double reduction of Q_A , and the generation of singlet oxygen, which can trigger damage of PSII reaction centers (Melis 1999, Oxborough and Baker 2000). Therefore, one might expect the existence of a relationship between the accumulation of light energy attributed to E and the extent of the decrease in PSII activity during photoinhibition. In order to verify this suggestion we introduced a new parameter, time-dependent averaged E, which is calculated as:

Time-dependent averaged E

$$= \sum_{i=2}^n (E_i + E_{i-1})/2 \times t \times \text{PPFD} \times 0.75 \times 0.5 \quad (2)$$

E_i and E_{i-1} = the levels of E measured at the current and previous time points, respectively, t = time between measurements, n = the number of data points, and 0.75 and 0.5 are the coefficients for leaf absorbance and for the sharing of absorbed photons between the two photosystems, respectively. The spectral absorbance of cotton leaves for the waveband 400–700 nm was determined by Björkman and Demmig (1987). The value of the last coefficient assumes equal distribution of excitation between PSI and PSII (Krall and Edwards 1992). We used a value for E of F_v/F_m at $t = 0$ ($E_1 = F_v/F_m$), because, at the beginning of the chilling treatment, q_P was approximately 0.

The calculation of the time-dependent averaged E includes the redox state of Q_A and the level of non-destructive thermal dissipation, both of which influence the probability of PSII photoinactivation (Demmig-Adams and Adams 1992, Öquist et al. 1992, Melis 1999). This is another reason why the time-dependent averaged E may be used as an indicator of PSII photoinactivation.

Under the conditions of our experiments, the calculation of D includes not only xanthophyll cycle-dependent thermal dissipation that is reversible in the dark, but also constitutive thermal dissipation that is responsible for lowering maximal values of F_v/F_m from 1.0 to approximately 0.8, as well as sustained thermal dissipation caused by irreversible (or very slowly reversible) inactivation of PSII (the photoinhibitory component). We suggest that D can be expressed as the sum of these components, D_{CON} (constitutive dissipation), D_{REG} (regulated dissipation) and D_{PI} (photoinhibitory dissipation), respectively:

$$D = D_{\text{CON}} + D_{\text{REG}} + D_{\text{PI}} \quad (3)$$

D_{CON} depends on the intrinsic PSII efficiency and may be estimated as the difference between the maximal theoretical level of PSII efficiency and the level observed in the dark-adapted, non-stressed samples:

$$D_{\text{CON}} = 1 - F_v/F_m \quad (4)$$

However, D_{CON} does not represent the absolute level but the relative amount of the absorbed light energy utilized as

constitutive thermal dissipation. Therefore, the changes in the level of other dissipative processes (developing regulated and photoinhibitory thermal dissipation during the light treatment) can affect the magnitude of D_{CON} . We have used the ratio $(F'_v/F'_m)/(F_v/F_m)$ to account for the influence of these processes when calculating the share of constitutive thermal dissipation under illumination:

$$D_{\text{CON}} = (1 - F_v/F_m) \times (F'_v/F'_m)/(F_v/F_m) \quad (5)$$

In the present study, in which leaf discs were exposed to stressful conditions to which they had not previously been acclimated, photoinhibitory thermal dissipation occurs. We used the following equation for the estimation of this component of D for light-adapted samples:

$$D_{\text{PI}} = (1 - (F_v/F_m)_{\text{PI}}/(F_v/F_m)) \times (F'_v/F'_m)/(F_v/F_m)_{\text{PI}} \quad (6)$$

F_v/F_m and $(F_v/F_m)_{\text{PI}}$ are the values of F_v/F_m for dark-adapted samples collected before the chilling treatment and during subsequent recovery, respectively. The influence of stressful treatments on F_v/F_m is routinely employed to estimate the level of PSII photoinhibition, as it is widely accepted that photoinhibitory inactivation to PSII lowers values of F_v/F_m that are measured during the subsequent period of dark recovery (Vavilin et al. 1995, Tyystjärvi et al. 1999). In our experiments, the recovery period was 3 h. No significant differences were observed in the magnitude of $(F_v/F_m)_{\text{PI}}$ between 3 and 6.5 h of dark recovery (data not shown).

Since D is a sum of its components, Eqs (5) and (6) can be used to determine D_{REG} for the light-adapted samples:

$$D_{\text{REG}} = D - D_{\text{CON}} - D_{\text{PI}} = 1 - (F'_v/F'_m)/(F_v/F_m)_{\text{PI}} \quad (7)$$

D_{CON} , D_{REG} and D_{PI} are the quantum yields for different forms of thermal dissipation. Therefore, they can be expressed as ratios between the rate constants for individual forms of thermal dissipation to the sum of the rate constants for all processes that consume light energy absorbed by PSII. Replacing the fluorescence yields by their expressions in terms of the rate constants, one obtains the following equations:

$$D_{\text{CON}} = (k_{\text{con}} + k_i)/(k_{\text{con}} + k_f + k_p + k_i + k_{\text{reg}}) \approx k_{\text{con}}/(k_{\text{con}} + k_f + k_p + k_i + k_{\text{reg}}) \quad (8)$$

$$D_{\text{PI}} = k_i/(k_{\text{con}} + k_f + k_p + k_i + k_{\text{reg}}) \quad (9)$$

$$D_{\text{REG}} = k_{\text{reg}}/(k_{\text{con}} + k_f + k_p + k_i + k_{\text{reg}}) \quad (10)$$

k_{con} , k_i , k_{reg} , k_f and k_p are the rate constants for constitutive, photoinhibitory and regulated thermal dissipation, fluorescence and photochemistry, respectively. The rate constants are related to the fluorescence parameters in the following ways: $F_0 = k_f/(k_{\text{con}} + k_f + k_p)$ for PSII with 'open' reaction centers in dark-adapted, non-stressed samples; $F_m = k_f/(k_{\text{con}} + k_p)$ for PSII with 'closed' reaction centers in dark-adapted, non-stressed samples; $(F_0)_{\text{PI}} = k_f/(k_{\text{con}} + k_i +$

Table 1. Activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) in the leaves of transgenic (SOD +, APX +, GR +) and control plants (Coker 312). Data are means $\pm$ SD (n = 6–10). ND = not determined.

Genotype	SOD units (mg Chl) ⁻¹	APX	GR
		$\mu\text{mol (mg Chl)}^{-1} \text{ h}^{-1}$	
Coker 312	695 $\pm$ 208	531 $\pm$ 118	66 $\pm$ 15
SOD +	1916 $\pm$ 593	599 $\pm$ 73	63 $\pm$ 10
APX +	ND	2581 $\pm$ 732	ND
GR +	ND	ND	2448 $\pm$ 558

$k_f + k_p$) for PSII with ‘open’ reaction centers in dark-adapted sample after photoinhibitory treatment; $(F_m)_{PI} = k_f / (k_{con} + k_i + k_f)$ for PSII with ‘closed’ reaction centers in dark-adapted sample after photoinhibitory treatment; $F'_0 = k_f / (k_{con} + k_i + k_f + k_p + k_{reg})$ for PSII with ‘open’ reaction centers in light-adapted sample during photoinhibitory treatment; $F'_m = k_f / (k_{con} + k_i + k_f + k_{reg})$ for PSII with ‘closed’ reaction centers in light-adapted sample during photoinhibitory treatment. The value of k_f is relatively small, so we assume that $k_{con} + k_f \approx k_{con}$. It is important to note that the rate constants presented here apply to the leaf pool of PSII complexes collectively, not individual PSII complexes.

Statistical analysis

Data for all analyses were obtained from plants of two independently transformed lines of SOD + and three lines of APX + and GR + genotypes. Student's *t*-test indicated that there were no significant differences between the responses of individual lines within a given genotype. Therefore, data from all lines of each particular genotype were combined. Means were considered to be significantly different when $\rho \leq 0.05$.

Results

Enzyme activities of the cotton genotypes

The SOD activity of whole-leaf extracts from SOD + cotton plants was nearly threefold greater than the activity for wild-type (Coker 312) plants (Table 1). Leaves of APX + plants possessed fivefold greater APX activity, while the leaves of GR + plants had 37-fold greater GR activity than wild-type leaves.

Table 2. The influence of a 3-h exposure to 500 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ and 10°C on photochemical quenching (q_p), PSII efficiency (F_v/F_m) after 3 h of dark adaptation, the rate constant of photoinhibition (k_{PI}), and oxygen evolution for leaf discs from control (Coker 312) cotton plants and those plants overproducing superoxide dismutase (SOD +), ascorbate peroxidase (APX +), or glutathione reductase (GR +). Leaves were pre-treated with lincomycin. The data are means $\pm$ SD (n = 7–13). The results of Student's *t*-test are presented as * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$ and NS for $P > 0.05$.

Genotype	q_p	F_v/F_m % of pre-treatment value	$k_{PI} (-\text{DCMU})$ h^{-1}	$k_{PI} (+\text{DCMU})$ h^{-1}	Oxygen evolution $\mu\text{mol m}^{-2} \text{ s}^{-1}$
Coker 312	0.176 $\pm$ 0.048	72.2 $\pm$ 3.44	0.112 $\pm$ 0.012	0.218 $\pm$ 0.055	2.46 $\pm$ 0.58
SOD +	0.288 $\pm$ 0.049***	78.0 $\pm$ 3.20***	0.087 $\pm$ 0.013***	0.187 $\pm$ 0.040 ^{NS}	3.20 $\pm$ 0.60*
APX +	0.306 $\pm$ 0.039***	78.7 $\pm$ 1.67***	0.087 $\pm$ 0.010***	0.202 $\pm$ 0.052 ^{NS}	3.26 $\pm$ 0.44***
GR +	0.325 $\pm$ 0.046***	79.9 $\pm$ 4.16***	0.079 $\pm$ 0.017***	0.215 $\pm$ 0.056 ^{NS}	3.43 $\pm$ 0.88**

Response of fluorescence parameters and oxygen evolution to chilling at moderate PPFD

No statistically significant differences were observed in the magnitude of NPQ between genotypes at 20 and 40 min of the chilling treatment (data not shown). At 3 h of chilling, steady-state values of q_p were significantly greater for all three of the transgenic genotypes (SOD +, APX + and GR +) than for Coker 312 (Table 2). PSII efficiencies (F_v/F_m) measured after 3 h of chilling followed by 3 h of recovery in the dark were significantly higher for all three of the transgenic genotypes than for wild-type plants (Table 2). Rates of CO₂-saturated oxygen evolution measured 3 h into the treatment were significantly higher in all three transgenic genotypes than in wild-type plants (Table 2).

The rate constant of photoinhibition (k_{PI}) during the chilling treatment was calculated from the light-induced decrease in F_v/F_m (Tyystjärvi et al. 1999) measured at 40, 80, 120 and 180 min. In the absence of DCMU, k_{PI} calculated over the 3-h treatment was lower for all three transgenic genotypes in comparison with wild-type plants (Table 2). In the presence of DCMU, k_{PI} for the different genotypes was not significantly different.

The actual level of PSII photochemistry (P) for all transgenic plants was significantly higher than that for wild-type plants throughout the 3-h chilling treatment (not shown). At 3 h, not only was P significantly greater for all transgenic genotypes than the wild-type P, but also the photoinhibitory component of thermal dissipation (D_{PI}) was significantly lower for all transgenic genotypes (Table 3). As was observed for NPQ at earlier time points, there were no significant differences in the level of D_{REG} between genotypes after 3 h of chilling.

The relationship between the levels of sustained decreases in F_v/F_m and the time-dependent averaged E determined after different periods of exposure to the chilling treatment (in the absence of DCMU) was described by lines with similar slopes for all genotypes (Fig. 1). Therefore, there were no significant differences between genotypes for this relationship.

Discussion

In this report, we introduce parameters that estimate the components that comprise overall energy dissipation (D_{CON} , D_{REG} , D_{PI}). Deconvoluting D in this manner is analogous to separating non-photochemical quenching of chlorophyll *a* fluorescence into its principal components (q_{Ne} , q_{Nt} , q_{Ni})

Table 3. Allocation of the light energy absorbed in PSII antennae at the end of the 3-h exposure to 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 10°C for leaf discs from control cotton plants (Coker 312) and plants overproducing superoxide dismutase (SOD+), ascorbate peroxidase (APX) and glutathione reductase (GR+). Leaves were pre-treated with lincomycin. Data are means $\pm$ SD ($n=9-13$) of different plants. The results of Student's *t*-test are presented as * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$ and NS for $P > 0.05$.

Genotype	P	D_{REG}	D_{PI}	E
Coker 312	0.041 ± 0.014	0.592 ± 0.078	0.114 ± 0.025	0.190 ± 0.038
SOD+	$0.066 \pm 0.013^{***}$	$0.626 \pm 0.035^{\text{NS}}$	$0.082 \pm 0.015^{**}$	$0.163 \pm 0.017^*$
APX+	$0.072 \pm 0.018^{***}$	$0.634 \pm 0.056^{\text{NS}}$	$0.077 \pm 0.014^{***}$	$0.159 \pm 0.022^*$
GR+	$0.074 \pm 0.016^{***}$	$0.633 \pm 0.055^{\text{NS}}$	$0.075 \pm 0.022^{***}$	$0.152 \pm 0.022^{**}$

(Walters and Horton 1993). Components of D, as well as those of non-photochemical chlorophyll *a* fluorescence quenching, are distinguished in part on the basis of their relaxation in the dark. In addition, many of the physical processes that influence D also influence non-photochemical chlorophyll *a* fluorescence quenching.

Under photoinhibitory conditions, incomplete relaxation of D and non-photochemical chlorophyll *a* fluorescence quenching is observed. The extents of sustained D and fluorescence quenching are expressed by means of parameters D_{PI} and q_{Ni} , respectively. In the case of D_{PI} , changes in minimal (F_0) and maximal (F_m) chlorophyll *a* fluorescence yield are taken into account (see Eq. 6; F_0 is used to calculate F_v), whereas only changes in F_m are involved in the determination of q_{Ni} .

The parameters, q_{Ne} and q_{Nt} , are the forms of non-photochemical fluorescence quenching that are influenced by the strength of the trans-thylakoid membrane proton gradient and state II–state I transitions, respectively (Walters and Horton 1993). These processes are employed by plants in order to regulate the delivery of excitation energy to PSII reaction centers and combine to influence D_{REG} . Therefore, D_{REG} can be used to estimate so-called ‘down-regulation’ of PSII, allowing for the quantitative comparison of the allocation of absorbed photon energy to regulated dissipation versus photochemistry (P) (see Demmig-Adams et al. 1996).

D_{CON} represents the difference between the maximal theoretical level of PSII efficiency (unity) and the level observed in dark-adapted, non-stressed leaves. This thermal dissipation cannot be assessed by F_m alone, requiring determination of the fluorescence level from PSII complexes with reaction centers in the ‘open’ state, as well, in order to account for photochemical light energy utilization (see Eq. 4).

The primary aim of this study was to determine the extent to which elevated activities of three chloroplastic, antioxidant enzymes in cotton could improve the protection of PSII from photoinhibition. Previous analyses indicated that wild-type and non-expressing segregate plants were statistically indistinguishable with respect to antioxidant enzyme activities and photosynthetic recovery following chilling in the light (our unpublished results). This information and the lack of significant differences between independently transformed lines of a given transgenic genotype strongly suggest that the differences in performance between transgenic genotypes and wild type were due to the presence of the transgenes and the resultant increase in antioxidant enzyme activity (Table 1).

To our knowledge, the development of these transgenic cotton plants represents the first attempt to determine the effectiveness of increasing three different antioxidant enzymes in the same species. It is interesting to note that differing degrees of overproduction of the three different chloroplastic antioxidant enzymes yielded somewhat similar enhancements in the resistance of cotton to chilling-induced PSII photoinhibition under the moderately stressful treatment used in this study. However, these transgenic genotypes did not perform equally under severe stress (3 h at 10°C and a PPFD of 1 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$; our unpublished results). We cannot offer a certain explanation for our observations presented here and do not wish to draw quantitative comparisons between the performance of the three

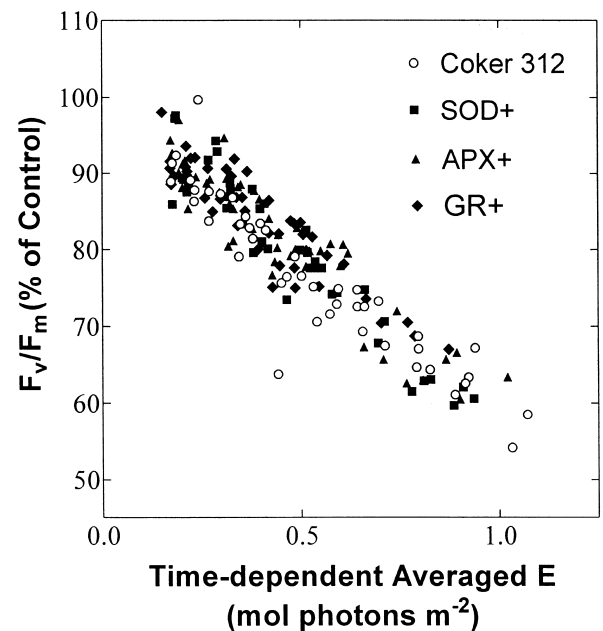


Fig. 1. Values of F_v/F_m expressed as the percent of the initial, dark-adapted values versus time-dependent averaged E (excessive light) for leaf discs from control cotton plants (Coker 312) and plants overproducing superoxide dismutase (SOD+), ascorbate peroxidase (APX), or glutathione reductase (GR+). The leaves were treated with lincomycin prior to the exposure to 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and 10°C. At 20, 40, 60, 80, 120, 150, 180, 240, 300 and 360 min after the beginning of the light treatment, E values were determined, and then small leaf discs were immediately removed for determining F_v/F_m after 3 h of dark adaptation at room temperature. Linear regression analysis yields lines that can be described by the following equations: for Coker 312, $y = 100 - 43.4x$ ($r^2 = 0.84$, $n = 44$); for SOD+, $y = 100 - 42.7x$ ($r^2 = 0.90$, $n = 44$); for APX+, $y = 100 - 41.1x$ ($r^2 = 0.86$, $n = 52$); for GR+, $y = 100 - 39.9x$ ($r^2 = 0.84$, $n = 40$).

transgenic genotypes. The enzymes, SOD, APX and GR, are all associated with the water-water cycle (Asada 1999). This cycle and associated ascorbate regeneration mechanisms are complex and include some redundant and non-enzymatic reactions (Asada 1999). It is possible that no single enzyme in the water-water cycle or ascorbate regeneration limits the overall activity of ROS scavenging under all conditions. Although the GR + plants of the present study possessed many-fold higher levels of overexpression in comparison to the SOD + and APX + genotypes, it is possible that the protective effect of this genetic manipulation saturated at lower activities. A 100–500-fold overexpression of GR in poplar seedlings was reported to confer only a small improvement in resistance to photoinhibition during chilling (Foyer et al. 1995). A comparison of GR + genotypes exhibiting differing degrees of overexpression would reveal the level of enzyme overexpression that is required for maximal effect on PSII photoinhibition.

Conducting fluorescence experiments in the presence of lincomycin allows one to examine PSII photoinactivation without the complicating effects of competing synthetic reactions. This approach does not allow one to determine any possible protective effects of enhanced antioxidant enzyme activity on chloroplastic repair processes. Effects on such processes may be important for the recovery of photosynthetic activity after stress.

The lower values of k_{PI} during the chilling treatment, i.e. the maintenance of higher PSII efficiencies (F_v/F_m), for all three transgenic cotton genotypes versus wild-type cotton indicate that elevated chloroplastic (presumably stromal) SOD, APX or GR activities improved PSII photoprotection (Table 2). One could speculate that elevated activities of antioxidant enzymes could have provided protection against PSII photoinhibition via the following mechanisms: (1) scavenging ROS that would otherwise directly inactivate PSII; (2) enhancing levels of thermal energy dissipation by increasing electron flow that augments the trans-thylakoid membrane proton gradient; (3) enhancing levels of photochemical light energy consumption (as well as decreasing Q_A reduction) by enabling chloroplasts to maintain greater rates of electron flow. Below, we consider our findings in light of these three possibilities.

Comparisons of time-dependent averaged E and sustained decreases in F_v/F_m (Fig. 1) reveal that diminished PSII photoinhibition for all three transgenic genotypes is not due to improved scavenging of ROS that directly inactivate PSII. If antioxidant enzyme overproduction interfered directly with ROS-mediated PSII inactivation, then the relationship between the time-dependent averaged E and F_v/F_m would differ between the transgenic genotypes and wild type, with the slope of the relationship being greater in wild-type plants. However, linear regression analysis indicates that the relationship between F_v/F_m and the time-dependent averaged E was similar for all cotton genotypes.

Our analyses indicate that none of the transgenic genotypes differed from wild type in NPQ early in the chilling treatment or in D_{REG} at 3 h of chilling (Table 3). Therefore, we conclude that the enhanced protection against PSII photoinhibition provided by elevated antioxidant enzyme activities in the stroma was not due to enhanced levels of

regulated, xanthophyll cycle-dependent thermal energy dissipation.

The inability of antioxidant enzyme overproduction to affect the level of PSII inactivation in the presence of DCMU (Table 2) suggests that the effect of overproduction involves events downstream of PSII in the electron transfer pathway. Higher values of q_P , P and rates of oxygen evolution at 3 h into the chilling treatment for all three transgenic genotypes in comparison to wild type (Table 2), along with the results discussed above, show that reduced PSII photoinhibition associated with enhanced stromal activities of SOD, APX or GR in cotton leaves is due primarily to the maintenance of a higher rate of electron transport and, consequently, a more oxidized Q_A pool during the chilling treatment for transgenic plants in comparison to wild-type plants. This explanation necessitates that elevated values of q_P were observed before the onset of appreciable photoinhibition, as was the case. The transgenic plants maintained elevated levels of q_P and P throughout the entire chilling treatment (data not shown), even early in the treatment when the extent of photoinhibition was low.

The correlation between higher q_P values and less sustained decreases in PSII efficiency (F_v/F_m) is consistent with the proposal that the reduction state of Q_A modulates the susceptibility of PSII to photoinhibition at low temperature (Öquist et al. 1992, 1993, Melis 1999). Our results are also consistent with a recent report that the level of resistance to photoinhibition during exposure to chilling correlates with the level of electron transport activity among rice cultivars (Xu et al. 1999). The crucial role of q_P in the enhancement of PSII photoprotection in transgenic cotton means that the protective effect of elevated stromal SOD, APX or GR activity may be overwhelmed at PPFDs that are high enough to cause full reduction of the Q_A pool. This possibility may explain why studies that used high PPFD did not find that chloroplastic SOD overproduction conferred substantially enhanced protection against photoinactivation (e.g., Payton et al. 1997, Tyystjärvi et al. 1999).

The mechanism by which chloroplastic SOD, APX or GR overproduction increased photosynthetic electron transport activity in cotton leaves during exposure to chilling is not known. High activities of these enzymes could increase the rate of H_2O_2 metabolism, increasing the demand for reducing power required for ascorbate regeneration from oxidized ascorbate produced in the water-water cycle (Asada 1999). Previous studies have shown that exogenous H_2O_2 supplied to isolated chloroplasts can serve as a Hill oxidant and decrease the Q_A reduction state (Neubauer and Schreiber 1989, Foyer et al. 1994a, Osmond and Grace 1995). Alternatively, the enhancement of antioxidant enzyme activity could improve the scavenging of ROS that would otherwise inactivate PSI (Sonoike 1996, Terashima et al. 1998, Tjus et al. 1998) and reduce electron transport activity. Infiltration of barley leaves with KCN, an inhibitor of CuZn SOD and APX, has been reported to increase chilling-induced PSI photoinhibition (Tjus et al. 1998). In either case, increased rates of electron flow to ferredoxin may improve the activation state of Calvin–Benson cycle enzymes that depend on reduced ferredoxin for activation, potentially leading to higher rates of CO_2 assimilation at 10°C. Also, at least for

plants overproducing APX or GR, the potentially lower concentrations of H₂O₂ in the stroma may have led to higher activation states of certain Calvin–Benson cycle enzymes sensitive to H₂O₂, as has been demonstrated in vitro (Charles and Halliwell 1981). Increasing Calvin–Benson cycle activity by increasing the activation states and amounts of key enzymes is an acclimation response of chilling-resistant species to growth at low temperature (Holaday et al. 1992, Gray et al. 1996) and is thought to maintain greater electron flow and a more oxidized pool of Q_A (Gray et al. 1996).

In conclusion, enhanced protection of PSII from photo-damage at moderate PPFD and 10°C can be achieved for cotton leaves by increasing the stromal antioxidant enzyme activity via plant transformation. It is the maintenance of a higher rate of electron transport and a lower reduction state of Q_A by transgenic plants compared to wild-type plants that appear to be the principal mechanism by which the protection of PSII is enhanced.

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