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Protecting cotton photosynthesis during moderate chilling at high light intensity by increasing chloroplastic antioxidant enzyme activity

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Abstract

This study examined the effect of increasing chloroplastic superoxide dismutase (SOD), ascorbate peroxidase (APX), or glutathione reductase (GR) activity via plant transformation of cotton on the initial recovery of photosynthesis following exposures to 10 °C and high photon flux density (PFD). Growing wild-type or non-expressing segregate plants (controls) and transformants at two PFDs (600 μmol m^{-2} s^{-1} and full sun) resulted in a range of total antioxidant enzyme activities. Total SOD activities above that for control leaves grown in full sun did not substantially improve the recoveries of CO_2-saturated photosynthesis, especially for stress treatments lasting more than 1 h, while elevated APX or GR activity did improve recoveries after 1–3 h of the chilling treatment. No synergistic effects were noted when the activities of more than one antioxidant enzyme were elevated in transgenic hybrids. Although these results suggest that the protection of photosynthesis can be realized by reducing either superoxide or H_2O_2 levels, thereby reducing the possibility of hydroxyl radical formation, the situation is complicated, since elevated APX or GR activity can improve recoveries even when additional SOD activity has no effect. In conclusion, to enhance the protection of photosynthesis using stroma-targeted antioxidant enzymes, enhancing metabolism associated with H_2O_2 is more effective than enhancing the capacity for superoxide scavenging. Although small, the improvement in the protection of photosynthetic capacity may be sufficient to improve cotton yield in temperate regions with large diurnal temperature fluctuations.

Key words: Oxidative stress, cotton photosynthesis, chilling-sensitive plants, antioxidant enzymes, transgenic plants.

Introduction

Photosynthesis of chilling-sensitive plants is severely inhibited after exposures to suboptimal temperatures and high photon flux densities (PFD). Exposures to severe chilling (3–5 °C) for a few hours cause dramatic reductions (63% to nearly 100%) in photosynthesis for tomato and tobacco at moderate PFD (Sassenrath *et al*., 1990; Sen Gupta *et al*., 1993*a*, *b*). Cotton photosynthesis is especially sensitive to chilling at high PFD. After 35 min at 5 °C and a PFD of 1700 μmol m^{-2} s^{-1}, photosynthesis measured in optimum conditions rapidly recovers to only 25% of the initial, prestress value (Payton *et al*., 1997). Even an exposure of cotton leaves to 20 °C and full sun for 1 d reduces photosynthesis measured at 20 °C by 40% (Königer and Winter, 1993). The sensitivity of cotton to chilling is of agronomic importance to cotton growers in temperate regions where morning temperatures on clear days early and late in the growing season can be well below 20 °C.

The generation of reactive oxygen intermediates (ROIs) may be a factor in causing this slowly-reversible reduction in photosynthesis following chilling at high PFD (Bowler *et al*., 1992; Smirnoff, 1995; Jakob and Heber, 1996; Alscher *et al*., 1997; Tyystjärvi *et al*., 1999).

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Such conditions enhance the possibility of O_2 reduction to superoxide (the Mehler reaction) at photosystem (PS) I and the subsequent formation of H_2O_2 (Schreiber and Neubauer, 1990; Osmond and Grace, 1995; Alscher *et al*., 1997), which has been shown to cause the deactivation of stromal fructose-1,6-bisphosphatase (FBPase), a key enzyme of the photosynthetic carbon reduction cycle (Charles and Halliwell, 1981). Additionally, if the levels of superoxide and H_2O_2 are not kept low, they can react in the presence of Fe^{2+} to form the highly-reactive hydroxyl radical (Smirnoff, 1995; Jakob and Heber, 1996). Hydroxyl radical-mediated inactivation of PSI and PSII has been demonstrated using isolated thylakoids (Jakob and Heber, 1996). However, PSI may be most vulnerable to conditions favouring hydroxyl radical formation, because its Fe-S centres in the vicinity of P700 are an appropriate environment for hydroxyl radical formation (Sonoike, 1996, 1998). Indeed, considerable evidence exists showing that O_2-dependent PSI photoinactivation occurs (Sonoike, 1996, 1998; Terashima *et al*., 1998; Tjus *et al*., 1999), but this photoinactivation of PSI is most evident during chilling at low to moderate PFD. The extent to which PSI photoinactivation occurs during chilling at high PFD is questionable, since those conditions cause substantial photoinactivation of PSII, which should protect PSI (Sonoike, 1998).

The importance of scavenging ROIs to photosystem inactivation is strongly suggested by reports that acclimation to chilling and high PFD or high PFD, alone, involves increases in the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and monodehydroascorbate reductase (MDHAR) (Schöner and Krause, 1990; Smirnoff, 1995; Grace and Logan, 1996; Logan *et al*., 1998*a*, *b*). Tyystjärvi *et al*. suggest that transformation of plants to enhance the antioxidant enyme activity of chloroplasts may be a method to determine the extent to which ROIs are involved in the photoinactivation of photosystems (Tyystjärvi *et al*., 1999). In several studies, increasing the activities of SOD, APX, or GR using plant transformation has improved the recovery of photosynthesis following chilling in the light. Such is the case for transgenic tobacco plants with increased chloroplastic Cu/Zn SOD activity (Sen Gupta *et al*., 1993*a*, *b*), although the elevated activity of the native APX associated with the higher SOD activity in these plants may be most critical to their recovery of photosynthesis (Sen Gupta *et al*., 1993*b*). In fact, transgenic tobacco plants with elevated APX activity, alone, either in the chloroplast stroma or in the cytosol, exhibit increased protection of photosynthesis, as well (Allen *et al*., 1997). Even increasing chloroplastic GR activities in poplar leaves to increase the capacity to regenerate ascorbate via the ascorbate–glutathione cycle results in a better initial recovery of photosynthesis after chilling at high PFD than for the wild-type poplars (Foyer *et al*., 1995).

In the light of these successes in protecting photosynthesis during chilling/high PFD exposures, it appeared reasonable to expect that increasing chloroplastic SOD, APX or GR activities in cotton leaves would improve their ability to recover photosynthesis rapidly after exposures to chilling at high PFD. Although overproduction of chloroplast-targeted Mn-SOD conferred little improved protection of cotton photosynthesis after severe chilling in full sun (Payton *et al*., 1997), it was hypothesized that the presence of elevated APX and/or GR activity might be superior to Mn-SOD in protecting cotton photosynthesis. Therefore, transgenic cotton plants were developed with elevated stromal activities of APX and GR in order to compare their ability to protect photosynthetic capacity with the ability of plants expressing chloroplast-targeted Mn-SOD during conditions of moderate chilling and high PFD typical of temperate regions in which cotton is grown. Hybrids between the transgenic plants were produced with elevated activities of more than one antioxidant enzyme to determine any synergistic effects between these enzymes.

Materials and methods

Generation of transgenic plants

Cotton plants, *Gossypium hirsutum* L. cv. Coker 312, had been transformed to overproduce chloroplast-targeted Mn-SOD as described previously (Payton *et al*., 1997) (SOD+ plants). To develop transformed plants that overproduced chloroplast-targeted APX (APX+ plants), the APX cDNA from pea (Mittler and Zilinskas, 1991) was developed using the 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 mutated cDNA was fused with a fragment from the pea chloroplastic Cu/Zn-SOD cDNA that encodes the transit peptide (Scioli and Zilinskas, 1988) (Fig. 1). To generate plants that overproduced GR (GR+ plants), a mutated GR cDNA was amplified from total leaf RNA of *Arabidopsis thaliana*, ecotype Columbia, using primers designed from a cDNA described previously (Kubo *et al*., 1993). The mutated cDNA was developed with an *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 is under the control of a cauliflower mosaic virus 35S promoter.

Transformation was accomplished by inoculating cotton hypocotyls with *Agrobacterium tumefaciens*. The transformed plants were generated from transformed callus cells by somatic embryogenesis (Bayley *et al*., 1992; Horsch *et al*., 1985).

Plant material and growth conditions

Plants from at least five selected transgenic plant lines for each gene transformation were selfed, and the progeny (T_1 generation) and subsequent generations (T_2, T_3, etc.) along with

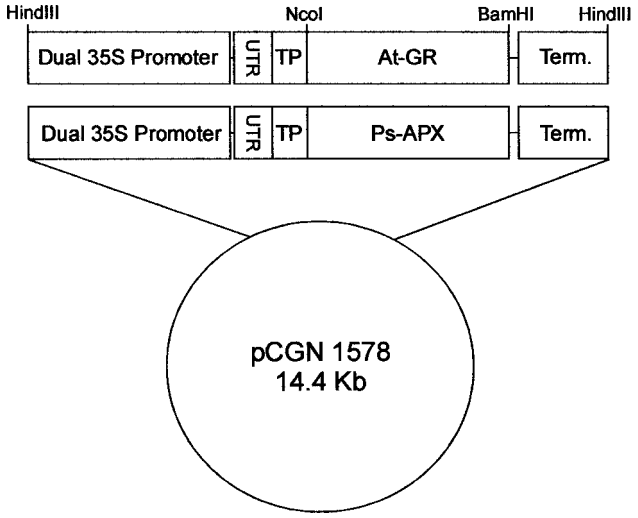

Fig. 1. A diagram of the gene constructs containing the *Arabidopsis thaliana* (At) gene for glutathione reductase (GR) or the *Pisum sativum* (Ps) gene for cytosolic ascorbate peroxidase (APX) used to transform the cotton plants. The native GR transit peptide (TP) was used to target GR to the chloroplast stroma, while the Cu/Zn superoxide dismutase TP was used to target APX to the stroma. UTR, untranslated region; Term, the 35S termination sequence.

wild-type, Coker 312, and non-expressing segregates (NX) from the transgenic lines were grown in a greenhouse with natural lighting at approximately 28/24 °C (day/night). Three lines for each transgenic genotype were chosen randomly for enzyme analyses and photosynthetic recovery was performed as described below. All analyses, except the chloroplast isolation analysis, were performed using plants grown from April to September. The chloroplast study utilized plants grown in December and January under the natural photoperiod. Using a Kruskal–Wallis, non-parametric analysis of variance, it was determined that there were no significant differences ($P > 0.05$) in mean values for lines within a genotype or between means for wild-type (Coker 312) plants and non-expressing segregates for each analysis. Therefore, data for lines within a genotype were combined for these analyses and subsequent experiments.

The plants were grown either in full sun (1700–1800 μmol m^{-2} s^{-1} at midday) or moderate PFD (approximately 600 μmol m^{-2} s^{-1} at midday), using Ball's Growing-on Mix potting soil in 10 l pots. A neutral density filter 0.6–0.7 m from the top of the plants was used to produce the moderate PFD level. All plants were fertilized with full-strength Hoagland's solution twice a week. Hybrid (F_1) plants were produced by cross-pollinating SOD+, APX+, and GR+ plants. To produce plants with elevated activities of all three enzymes, crosses between various F_1 plants or between an F_1 plant and a plant expressing a single transgene were performed. The putative hybrids were screened for the Mn-SOD transgene product using non-denaturing polyacrylamide gel electrophoresis (Payton *et al.*, 1997). Spectrophotometric assays of whole-leaf extracts were used to determine those hybrids with APX activity at least 2-fold or GR activity at least 20-fold the activity of wild-type leaves (Payton *et al.*, 1997). The fourth leaf (from the top) whose area was 90% or more of full expansion was used for photosynthesis measurements and enzyme analyses.

Chloroplasts of such leaves from APX+ and GR+ plants were isolated to determine whether they contained elevated activities of APX and GR. Approximately 5 g of de-ribbed leaves were homogenized gently with a mortar and pestle in small batches in 10–20 ml of 100 mM Hepes, pH 7.9, 660 mM sorbitol, 20 mM KCl, and 2 mM EDTA at 4 °C (plus 5 mM ascorbate for APX analysis). After filtration with cheesecloth, the suspension was centrifuged at 1000 *g* for 100 s. The pellet was resuspended in 30 ml of 50 mM Hepes, pH 7.9, 330 mM sorbitol, 10 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1% (w/v) bovine serum albumin (plus 5 mM ascorbate for APX analysis) and centrifuged again. After a second washing with this suspension solution, the chloroplasts were suspended in the appropriate enzyme homogenization solution and lysed by repeated freezing and thawing. Initially, aliquots of the suspended chloroplasts were loaded onto a 40% Percoll solution and centrifuged at 2500 *g* for 60 s. However, it was determined that this step could be omitted. To determine the percentage of intact chloroplasts, the activity of stromal FBPase, fully activated *in vitro*, was determined from leaf discs rapidly removed from the leaves used to isolate chloroplasts and frozen in liquid N_2 and compared to the FBPase activity in the isolated chloroplasts on a chlorophyll basis (Lichtenthaler, 1987). The extraction and assay for FBPase followed the procedure of Holaday *et al.* (Holaday *et al.*, 1992).

Enzyme assays

Leaf discs (50–100 mg fresh mass) were removed rapidly and immediately frozen and stored in liquid N_2 until homogenized for enzyme assays. The frozen discs were ground to a powder at liquid N_2 temperature and then rapidly homogenized in 1 ml of the appropriate extraction solution in a glass tissue grinder on ice. Aliquots were taken for total chlorophyll determination in 80% acetone before centrifugation (Lichtenthaler, 1987) so that activities could be compared to previously published results using this method of chlorophyll determination.

The extraction solution for SOD assays followed that described previously (Payton *et al.*, 1997). Extracts for the assay of APX, MDHAR, dehydroascorbate reductase (DHAR), and GR were prepared using a solution containing 50 mM Hepes-KOH, pH 7.0, 0.2 mM EDTA, and 1% polyvinylpolypyrrolidone. All extraction solutions contained 0.05% (v/v) Triton X-100. Following the removal of aliquots for chlorophyll determination, the extracts were centrifuged for 15 s at 16 000 *g* in a microcentrifuge.

All assays were performed at 25 °C. The SOD activity was determined by monitoring the inhibition of the reduction of *p*-nitro-blue tetrazolium chloride (NBT) at 560 nm as described earlier (Giannopolitis and Ries, 1977). The APX and GR activities were determined spectrophotometrically (Sen Gupta *et al.*, 1993*b*), while MDHAR activity was measured according to the method of Hossain *et al.* (Hossain *et al.*, 1984), and DHAR activity was measured according to the method of Nakano and Asada (Nakano and Asada, 1981).

In a separate experiment, the contents of chlorophylls *a* and *b* in leaves of wild-type and transgenic plants grown at the two PFDs were determined. The combined mass of leaf discs removed from four quadrants of each leaf was determined, and the discs were rapidly frozen in liquid N_2. To enhance the extraction of chlorophyll *a*, the chlorophylls were extracted in 100% acetone and quantified following established procedures (Lichtenthaler, 1987).

Oxidative stress analyses

The extent to which photosynthesis initially recovered after exposures to 10 ± 2 °C and a PFD of 1700 μmol m^{-2} s^{-1} was

determined in the laboratory using 10 cm^2 leaf discs. Rates of O_2 evolution at saturating CO_2 (~5% v/v) were measured at 25 °C and a PFD of 1200 μmol m^{-2} s^{-1} (a PFD that was just saturating as indicated by light response curves) using a Hansatech Leaf Disc Oxygen Electrode (Hansatech Instruments, Pentney, King's Lynn, UK) prior to and following the stress treatments of 1, 2 or 3 h. Each disc was removed from a leaf in the greenhouse and allowed to acclimate to room temperature (22 °C) for approximately 20 min at a PFD of 500 μmol m^{-2} s^{-1} (to reduce the potential for photoinhibition) on moistened filter paper in a Petri dish before the prestress O_2 evolution rates were measured. These acclimation conditions were determined by exposing leaf discs for various times to these conditions and subsequently measuring photosynthetic rates (data not shown). The acclimation period caused no photoinhibition as indicated by a lack of a change in the ratio of variable to maximum chlorophyll fluorescence (F_v/F_m) (0.780 ± 0.010 before and 0.776 ± 0.006 after the period). Following the initial photosynthetic measurement, the disc was subjected to the stress treatment on a temperature-controlled block as described previously (Payton *et al*., 1997). The post-stress rate of O_2 evolution at 25 °C required approximately 10 min to reach a steady-state and was maintained for at least 20 min, indicating that no further detecable photoinhibition that affected photosynthetic O_2 evolution was occurring during the meaurement period of 10–15 min. The post-stress rate expressed as a percentage of the initial photosynthetic rate (the percentage recovery) indicated the ability of the leaf disc to recover photosynthesis during the measurement period (Payton *et al*., 1997).

Data analysis

A Student's *t*-test was used to compare control and transgenic means, means of enzyme activity for each genotype at each PFD, and means of post-stress recovery of O_2 evolution, with differences being significant at $P \leq 0.05$. A two-way analysis of variance was conducted for the chlorophyll content of leaves, with differences between means being significant for $P \leq 0.05$.

Results

Enzyme activity and chlorophyll content in leaves developing in moderate PFD and full sun

Previous gel electrophoresis work established that chloroplasts of SOD+ plants contained high Mn-SOD activity (Payton *et al*., 1997). Isolated chloroplasts of APX+ and GR+ plants grown in full sun during December and January had elevated activities of the transgene products, as well (Table 1). Although the whole-leaf activities of APX, especially for the wild-type (Coker 312) plants, for greenhouse plants in the winter were lower than the activities for plants grown in the spring and summer (Table 2), the activity in APX+ leaves was 7-fold that in wild-type leaves. The ratio of chloroplast activity to whole-leaf activity for APX and GR was not substantially different between wild-type and transgenic plants, suggesting that an increase in the extrachloroplastic activity of APX and GR occurred in transgenic plants.

Growth of wild-type plants (Coker 312 and non-expressing segregates) at moderate PFD (approximately 600 μmol m^{-2} s^{-1}) halved the total leaf SOD and DHAR activities and diminished MDHAR activity by 32% from the activities for leaves of plants grown in full sun (all means between PFDs for each enzyme were significantly different), but the activities of APX and GR were not significantly ($P > 0.05$) affected by the lower growth PFD (Tables 2, 3). This pattern generally held for DHAR in the transgenic plants and their hybrids (with the exception of SOD/GR plants) and for MDHAR in APX+ plants. However, with the exception of SOD/APX hybrid plants, MDHAR activities in the hybrids, SOD+, and GR+ plants were not significantly different at both PFDs. The MDHAR activity in SOD/APX hybrids was halved by growth at moderate PFD.

Growth at moderate PFD significantly ($P \leq 0.05$) reduced the enhancement of total leaf SOD, APX, and GR activities in transgenic plants over the wild-type activities. Total extractable leaf SOD activity was increased 2-fold, APX activity 3-fold, and GR activity 15-fold over the activities of wild-type plants for the respective transgenic plants at moderate PFD (Table 3), while in full sun, the enhancement was 4-fold for SOD activity, 5-fold for APX activity, and 30-fold for GR activity (Table 2). The leaves of SOD+ plants grown at moderate PFD had as much total SOD activity as

Table 1. *Mean activities ± standard deviation of ascorbate peroxidase (APX) and glutathione reductase (GR) in whole-leaf extracts and in chloroplasts isolated from those leaves for wild-type cotton plants (Coker 312) and transgenic plants overproducing chloroplast-targeted APX (APX+) and GR (GR+) grown in full sun during December and January*

The percentage of intact chloroplasts was estimated by comparing the fully activated activity of stromal fructose bisphosphatase on a chlorophyll basis in whole leaf and chloroplast extracts. The APX activity is expressed in terms of μmol of ascorbate oxidized, and the GR activity is expressed as μmol of oxidized glutathione reduced. $N = 3$ for APX and 4 for GR data. nd = not determined.

Genotype	APX			GR		
	Whole leaf (μmol mg^{-1} Chl h^{-1})	Chloroplast (μmol mg^{-1} Chl h^{-1})	% Intact	Whole leaf (μmol mg^{-1} Chl h^{-1})	Chloroplast (μmol mg^{-1} Chl h^{-1})	% Intact
Wild type	447 ± 107	259 ± 50	83 ± 10	80 ± 6	65 ± 11	78 ± 12
APX+	3237 ± 250	2091 ± 459	86 ± 13	nd	nd	nd
GR+	nd	nd	nd	2232 ± 282	1596 ± 155	84 ± 5

Table 2. *Mean activities ± standard deviation in whole-leaf extracts of superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) for leaves of wild-type cotton plants (Coker 312 and non-expressing plants), transgenic plants, and F_1 hybrids between the transgenic genotypes grown in full sun from April through September*

The APX, MDHAR, DHAR, and GR activities are expressed as μmol of ascorbate oxidized, MDHA reduced, DHA reduced, and oxidized glutathione reduced, respectively. $N = 15$ for SOD, APX, and GR. $N = 6$ for MDHAR and DHAR. The asterisk indicates that the mean is significantly different ($P \leq 0.05$) from the mean value for wild-type plants.

Genotype	SOD (units mg^{-1} Chl)	APX (μmol mg^{-1} Chl)	MDHAR (μmol mg^{-1} Chl h^{-1})	DHAR (μmol mg^{-1} Chl h^{-1})	GR (μmol mg^{-1} Chl h^{-1})
Wild type	520 ± 45	900 ± 167	79 ± 9	312 ± 130	53 ± 8
SOD+	1949 ± 75*	1145 ± 245	64 ± 11	220 ± 30	50 ± 4
APX+	566 ± 63	4495 ± 601*	75 ± 14	325 ± 80	57 ± 7
GR+	482 ± 89	1162 ± 177	75 ± 9	280 ± 2491	1612 ± 224*
SOD/APX	1254 ± 98*	3107 ± 220*	60 ± 5*	236 ± 20	72 ± 14
APX/GR	445 ± 145	2466 ± 767*	62 ± 18	220 ± 59	1011 ± 281*
SOD/GR	1717 ± 254*	1287 ± 201	58 ± 4*	139 ± 58*	1036 ± 286*
SOD/APX/GR	891 ± 161*	2011 ± 215*	70 ± 6	266 ± 45	1016 ± 104*

Table 3. *Mean activities ± standard deviation in whole-leaf extracts of superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) for leaves of wild-type cotton plants (Coker 312 and non-expressing plants), transgenic plants, and F_1 hybrids between the transgenic genotypes grown at a moderate PFD from April through September*

The APX, MDHAR, DHAR, and GR activities are expressed as μmol of ascorbate oxidized, MDHA reduced, DHA reduced, and oxidized glutathione reduced, respectively. $N = 6$. The asterisk indicates that the mean is significantly different ($P \leq 0.05$) from the mean value for wild-type plants.

Genotype	SOD (units mg^{-1} Chl)	APX (μmol mg^{-1} Chl)	MDHAR (μmol mg^{-1} Chl h^{-1})	DHAR (μmol mg^{-1} Chl h^{-1})	GR (μmol mg^{-1} Chl h^{-1})
Wild type	240 ± 77	934 ± 190	54 ± 2	155 ± 19	57 ± 11
SOD+	445 ± 103*	995 ± 197	55 ± 8	161 ± 36	47 ± 10
APX+	249 ± 89	2928 ± 195*	51 ± 1	123 ± 5*	45 ± 15
GR+	221 ± 109	749 ± 123	66 ± 11	115 ± 20*	846 ± 38*
SOD/APX	377 ± 138*	3350 ± 1205*	28 ± 6*	101 ± 35*	58 ± 12
APX/GR	168 ± 14	1564 ± 100*	53 ± 9	135 ± 28	417 ± 11*
SOD/GR	390 ± 74*	1123 ± 428	55 ± 3	143 ± 23	740 ± 99*
SOD/APX/GR	403 ± 137*	2975 ± 137*	72 ± 14*	129 ± 27	883 ± 77*

wild-type plant leaves grown in full sun. In most cases, the hybrids grown at moderate PFD exhibited leaf activities of SOD, APX, and GR that were enhanced over wild-type activities to nearly the same extent as for their respective transgenic parents grown at moderate PFD (Table 3), but when grown in full sun, the enhancement for hybrids ranged from 88–45% of that for the respective parents in full sun (Table 2). These comparisons of enzyme activities based on chlorophyll content could be made, because a two-way analysis of variance indicated that genotype and growth PFD had no significant effect on total leaf chlorophyll (Table 4).

The chilling stress treatments for periods of 1–2 h had no effect on the activities of SOD, APX, or GR of leaves developed at either PFD (data not shown). After 3 h of stress, an increase (18–30%) in SOD activity in wild-type leaf discs was observed, but no change in the activities of APX and GR was observed.

Rapid recovery of photosynthesis at saturating CO_2 following chilling at high PFD

Transgenic plants with elevated activities of SOD, APX, or GR grown at moderate PFD exhibited a significantly improved recovery of CO_2-saturated photosynthesis compared to wild-type plants following all periods of exposure to 10 °C and a PFD of 1700 μmol m^{-2} s^{-1} (Fig. 2A). Growth of wild-type plants in full sun resulted in significantly higher recoveries of photosynthesis following chilling/high PFD stress than when the plants were grown at moderate PFD (Fig. 2B). However, all transgenic lines grown in full sun exhibited significantly increased recoveries of photosynthesis compared to wild-type plants following 1 h of chilling stress. For exposures longer than 1 h, the APX+ and GR+ plants exhibited a significantly enhanced recovery of photosynthesis, but the SOD+ plants did not. Of the hybrids

Table 4. *Mean chlorophyll contents ± standard deviation for leaves of wild-type cotton plants (Coker 312 and non-expressing plants) and transgenic plants overexpressing genes for superoxide dismutase (SOD+), ascorbate peroxidase (APX+), and glutathione reductase (GR+) grown at moderate PFD (MPFD) and in full sun;* N = 4

Genotype	Chl *a* (mg Chl g^{-1} leaf)	Chl *b* (mg Chl g^{-1} leaf)	Chl *a*/Chl *b* (mg Chl g^{-1} leaf)	Total Chl (mg Chl g^{-1} leaf)
MPFD				
Wild type	1.36 ± 0.14	0.48 ± 0.06	2.83 ± 0.08	1.84 ± 0.20
SOD+	1.65 ± 0.19	0.59 ± 0.07	2.78 ± 0.04	2.25 ± 0.26
APX+	1.46 ± 0.24	0.45 ± 0.07	3.21 ± 0.01	1.91 ± 0.31
GR+	1.70 ± 0.08	0.56 ± 0.04	3.06 ± 0.13	2.26 ± 0.12
Full sun				
WildType	1.44 ± 0.18	0.45 ± 0.04	3.19 ± 0.15	1.89 ± 0.22
SOD+	1.43 ± 0.09	0.48 ± 0.04	2.97 ± 0.09	1.91 ± 0.12
APX+	1.37 ± 0.22	0.44 ± 0.07	3.11 ± 0.08	1.82 ± 0.29
GR+	1.47 ± 0.19	0.48 ± 0.08	3.11 ± 0.18	1.95 ± 0.27

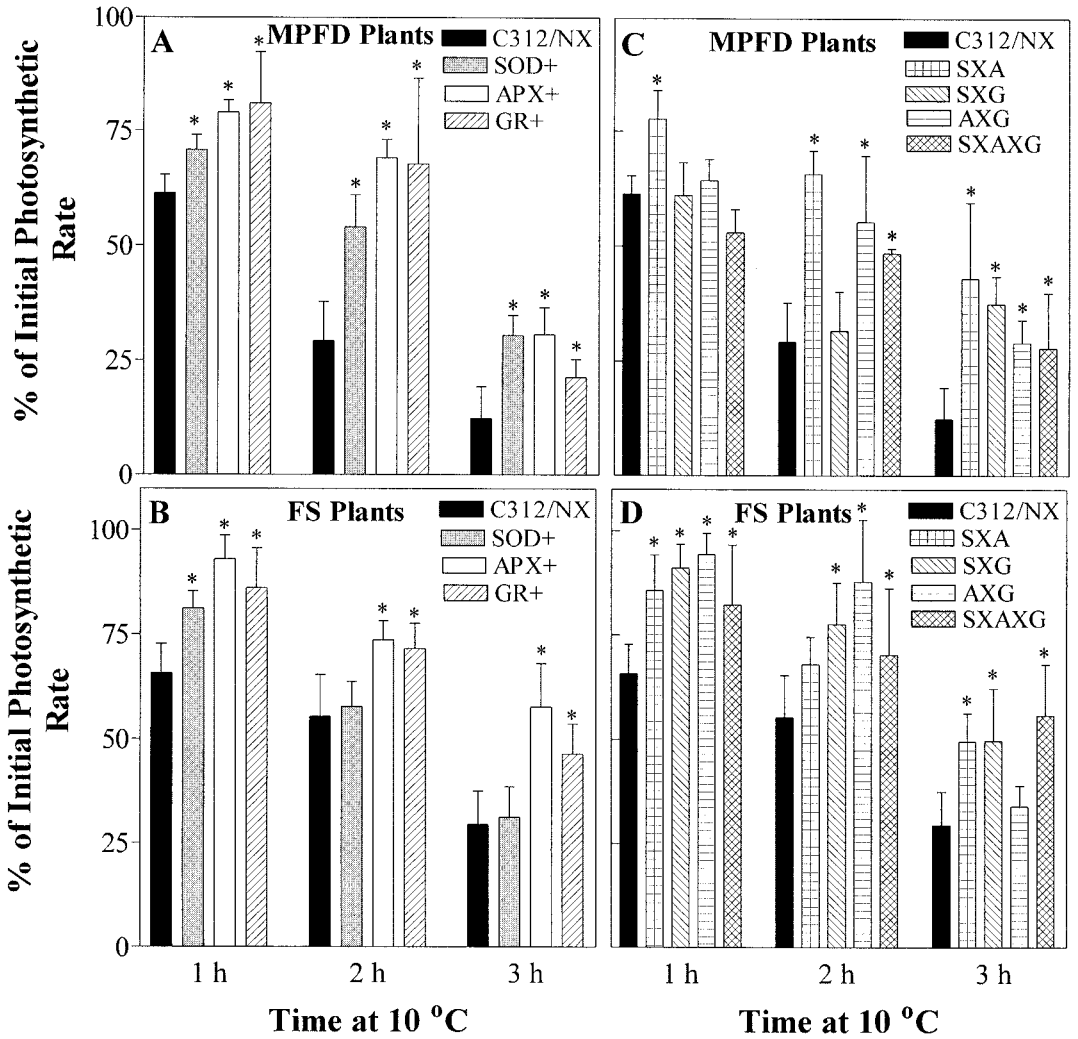

Fig. 2. The initial recovery of photosynthesis following 1, 2 or 3 h at 10 °C and a PFD of 1700 μmol m^{-2} s^{-1} expressed as a percentage of the prestress photosynthetic rate for leaf discs from wild-type cotton plants (Coker 312 and non-expressing segregates), transgenic plants overexpressing the gene for superoxide dismutase (SOD+), ascorbate peroxidase (APX+), or glutathione reductase (GR+), (A, B) and hybrid transgenic plants (C, D) grown at either moderate PFD (MPFD) or full sun (FS). Mean initial rates of photosynthesis for MPFD plants were 19.5 ± 4.4 μmol O_2 m^{-2} s^{-1} for wild-type, 20.5 ± 0.6 for SOD+, 17.3 ± 2.6 for APX+, 18.0 ± 0.8 for GR+, 15.7 ± 4.0 for SOD/APX, 16.7 ± 1.5 for APX/GR, 16.3 ± 1.5 for SOD/GR, and 20.4 ± 3.7 for SOD/APX/GR plants. $N = 4$ for wild-type and transgenic plants and $N = 5$ for the hybrids. For FS plants, mean initial rates were 27.6 ± 4.4 μmol O_2 m^{-2} s^{-1} for wild-type, 24.4 ± 4.8 for SOD+, 23.2 ± 8.1 for APX+, 26.1 ± 3.1 for GR+, 24.2 ± 4.0 for SOD/APX, 29.0 ± 2.4 for APX/GR, 31.6 ± 7.0 for SOD/GR, and 28.8 ± 4.9 for SOD/APX/GR plants. $N = 14$ for wild-type and GR+, 10 for SOD+ and APX+, and 4–6 for the hybrid plants. Bars indicate one standard deviation. An asterisk indicates that the mean is significantly different ($P \leq 0.05$) from the mean value for wild-type plants.

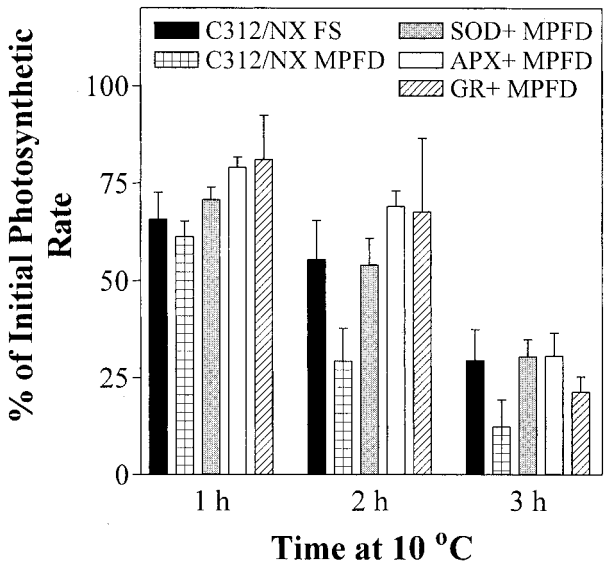

Fig. 3. A comparison of the initial recovery of photosynthesis following 1, 2 or 3 h at 10 °C and a PFD of 1700 μmol m^{-2} s^{-1} expressed as a percentage of the prestress photosynthetic rate for leaf discs from wild-type cotton plants (Coker 312 and non-expressing plants) grown at moderate PFD (MPFD) or in full sun (FS) with the recoveries for transgenic plants overexpressing the gene for superoxide dismutase (SOD+), ascorbate peroxidase (APX+), or glutathione reductase (GR+) grown at moderate PFD. Data are from Fig. 2A and B. Bars indicate one standard deviation. $N = 4$ for plants grown at moderate PFD and 14 for wild-type plants grown in full sun.

grown at moderate PFD, the SOD/APX plants performed the best during all of the stress periods (Fig. 2C). With one exception, the other hybrid genotypes had greater recoveries of photosynthesis than wild-type plants for 2 h and 3 h of stress. When the hybrid plants were grown in full sun, the combination of elevated APX and/or GR activities with elevated SOD activity resulted in significantly higher photosynthetic recoveries for 2 h and 3 h of stress than recoveries for the SOD+ plants (Fig. 2D). However, no hybrid genotype was better than APX+ or GR+ plants at each growth PFD.

Increasing the activity of chloroplastic SOD, APX, or GR via transformation conditioned the plants grown at moderate PFD to perform in the stress tests at the level of wild-type plants grown in full sun (Fig. 3). Transgenic SOD+, APX+, or GR+ plants grown at moderate PFD had recoveries of photosynthesis similar to, or slightly greater than, the recoveries for wild-type plants grown in full sun.

Discussion

This study shows that some improvement in the protection of cotton photosynthetic capacity during chilling in the light can be achieved by increasing chloroplastic activities of SOD, APX, and GR. This finding suggests that ROIs are involved, to some extent, in the photoinactivation of photosynthesis for cotton. However, at this time, more information on possible mechanisms that result in improved photosynthetic recovery is needed in order to assess critically whether ROI-mediated inactivation of photosynthetic processes is actually reduced by elevated SOD, APX, and GR activities in cotton leaves.

Antioxidant enzyme activity and the protection of photosynthesis

It is known that plants grown under low PFD have decreased capacities for protection from ROIs due to a lower capacity to dissipate excess excitation energy harmlessly (Demmig-Adams and Adams, 1996) and due to a lower enzyme capacity to scavenge ROIs relative to plants grown in full sun (Gillham and Dodge, 1987; Schöner and Krause, 1990; Grace and Logan, 1996; Logan *et al.*, 1998*a*). Growth of wild-type cotton leaves at 600 μmol m^{-2} s^{-1} (moderate PFD) reduces the total leaf activities of SOD, MDHAR, and DHAR as opposed to growth at 1700 μmol m^{-2} s^{-1} (full sun in the greenhouse) and reduces the protection of photosynthesis during a chilling/high PFD exposure, while total chlorophyll content is not different at the two growth PFDs. However, increases in the activities of chloroplastic SOD, APX or GR by plant transformation are sufficient to condition cotton leaves grown at moderate PFD to perform at the level of wild-type leaves grown in full sun despite the lower capacity for ascorbate regeneration via DHAR in all plants and via MDHAR in APX+ plants. These results suggest that SOD activity, and possibly the activities of APX and GR in wild-type cotton plants grown at modertate PFD are factors causing the reduced protection of photosynthesis during chilling/high PFD stress. A plot of post-stress photosynthesis as a percentage of the initial photosynthetic rate versus total leaf SOD activities for all genotypes at the two growth PFDs (Fig. 4A) supports this idea for SOD. Since wild-type plants grown in moderate PFD or full sun have similar APX (Fig. 4B) and GR (Fig. 4C) activities, but exhibit different recoveries of photosynthesis after chilling, other factors (e.g. SOD activity) must be involved in determining the extent of the photosynthetic recovery for cotton plants grown at moderate PFD. However, elevating the chloroplastic activity of APX or GR above that of wild-type plants by transformation does confer a greater protection of photosynthesis during chilling in the light for plants grown at either PFD. For plants grown at moderate PFD, high activities of APX or GR can compensate for low total leaf SOD activity despite the lower MDHAR and DHAR activities. Therefore, the results of this study suggest that improvements in the protection of photosynthetic capacity during short-term chilling at high PFD can be realized by reducing either superoxide or H_2O_2 levels, thereby reducing the possibility of hydroxyl radical formation. This idea is supported by

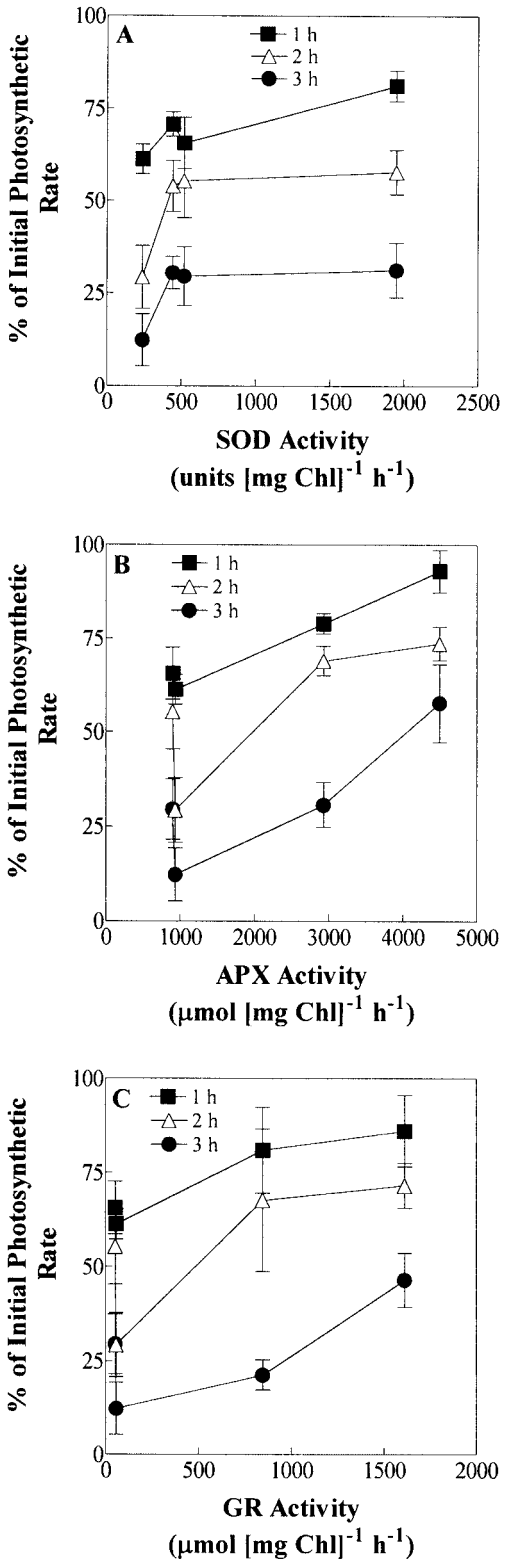

Fig. 4. The initial recovery of photosynthesis for leaf discs following 1, 2 or 3 h at 10 °C and a PFD of 1700 μmol m^{-2} s^{-1} expressed as a percentage of the prestress photosynthetic rate (from Fig. 2A, B) versus the total leaf activities of superoxide dismutase (SOD) (A), ascorbate peroxidase (APX) (B), or glutathione reductase (GR) (C) in wild-type plants and transgenic plants grown at full sun and moderate PFD (from Tables 2 and 3). Bars indicate one standard deviation.

the finding that there are no detectable synergistic effects associated with combining elevated SOD activity with elevated APX or GR activity by hybridization. However, the situation appears to be more complicated, at least for cotton plants grown in full sun, since total SOD activity above that of wild-type plants in full sun (520 units mg^{-1} Chl h^{-1}) does not improve photosynthetic recovery after stress periods of more than 1 h, but elevated APX and GR activities do improve photosynthetic recovery after longer stress periods. An explanation of these results will require further investigation into the mechanism by which additional APX and GR activity improve photosynthetic protection during chilling.

The lack of a response of GR and APX activity in wild-type plants to increased growth PFD is surprising given that overproduction of these enzymes by transformation produces an enhancement of tolerance to short-term chilling in the light similar to that for wild-type plants grown in full sun. However, it must be remembered that acclimation to high PFD may not be the same as acclimation to high PFD and low temperature, which, in combination, cause a greater imbalance between light energy absorption and utilization via CO_2 assimilation than high PFD alone.

The limitations to using increased activity of stromal anitoxidant enzymes to protect cotton photosynthesis

For cotton, the extra protection of photosynthesis afforded by having high activities of antioxidant enzymes is considerably less than it is for tobacco with elevated chloroplastic Cu/Zn-SOD along with increased native APX activity (Sen Gupta *et al.*, 1993*a*, *b*) or with increased activity of stromal APX, alone (Allen *et al.*, 1997). In addition, increasing the activity of more than one antioxidant enzyme in cotton leaves by hybridization does not improve photosynthetic protection in an additive manner and does not improve the protection beyond that afforded by increased APX or GR activity, alone. Thus, it is clear that other factors are limiting the effectiveness of this approach to improve the response of cotton photosynthesis to chilling. For example, it is possible that some mechanism of photosystem inactivation other than that mediated by the ROIs targeted in this study may operate in cotton chloroplasts to a greater degree than in tobacco chloroplasts to reduce photosynthetic capacity during chilling in the light. Alternative mechanisms leading to the inactivation of PSII have been proposed based on considerable supporting evidence (Anderson *et al.*, 1998; Tyystjärvi *et al.*, 1999), and once PSII activity is substantially decreased, the resulting reduction in electron flow to PSI reduces the chance of further ROI formation and PSI photoinactivation. It is possible, as well, that targeting the enzymes produced from transgene expression to the stroma in the

cotton plants may not be as effective as targeting them to thylakoid membranes (Van Camp *et al*., 1996; Payton *et al*., 1997; Tyystjärvi *et al*., 1999). Although it is considered that native chloroplastic GR is localized in the stroma, Cu/Zn-SOD, Fe-SOD, and the majority of the chloroplastic APX are associated with thylakoid membranes (Allen *et al*., 1997). However, it is interesting to note that elevating cytosolic APX activity in tobacco provides protection of photosynthesis to the same degree as does elevating stromal APX activity (Allen *et al*., 1997), suggesting that association of APX with thylakoid membranes in tobacco is not absolutely necessary for improved protection of photosynthesis. Transformation of cotton with genes for APX and SOD that target the enzyme to thylakoid membranes will be required to evaluate the potential problem of enzyme localization.

Another potential limitation to the effectiveness of elevated stromal APX activity is the ability to regenerate ascorbate, either via MDHAR, or via the ascorbate–glutathione cycle. This possibility is most likely for cotton plants grown at moderate PFD, which have diminished MDHAR and DHAR activities relative to the activities for plants grown in full sun. But even for plants grown in full sun, it was reported that the ratio of MDHA plus DHA to ascorbate increases in SOD+ plants under severe stress to a greater extent than in wild-type plants (Payton *et al*., 1997). Adding elevated GR activity to plants with elevated APX activity using hybridization only marginally improves the protection of photosynthesis, possibly because either DHAR or the pool of glutathione exerts considerable control over the rate of ascorbate cycling during chilling and high PFD exposures.

Concluding remarks

The results of this study suggest that ROIs are involved, to some degree, in the reduction of photosynthetic capacity of cotton during chilling/high light exposures. This hypothesis could be tested by determining precisely how elevated SOD, APX, and GR activities in the chloroplast improve the protection of photosynthesis. Although stromal SOD activity above the native activity in cotton leaves that develop at moderate to low PFDs may provide some protection of photosynthesis for sudden exposures at high PFD, increasing enzyme activity in the stroma directly or indirectly associated with H_2O_2 metabolism provides equal or greater protection for cotton leaves that develop at low or high PFD during periods of excess energy absorption. The small improvement in the protection of photosynthetic capacity as a result of raising the activity of stromal antioxidant enzymes may be sufficient to increase yield over a stressful growing season for cotton grown in temperate, semi-arid regions.

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