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Elevated chloroplastic glutathione reductase activities decrease chilling-induced photoinhibition by increasing rates of photochemistry, but not thermal energy dissipation, in transgenic cotton

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Abstract. The effect of the overproduction of glutathione reductase (GR+) in cotton (*Gossypium hirsutum* L. cv. Coker 312) chloroplasts on the response of photosynthetic parameters to chilling in the light was examined. After 180 min at 10°C and 500 μmol photons m^{-2} s^{-1} in the chamber of an oxygen electrode, leaf discs of GR+ plants exhibited lower levels of sustained PSII photoinhibition than leaf discs of wild-type plants. No genotypic differences in thermal energy dissipation, leaf pigment composition, or the dynamics of xanthophyll cycle de-epoxidation were observed. The rate of induction and steady-state levels of photochemistry were greater for GR+ in comparison to wild-type plants. Enhanced photochemistry in GR+ plants could not be attributed to higher rates of CO_2 assimilation at 10°C. Although GR overproduction afforded some increased protection against PSI photoinactivation, suggesting improved scavenging of reactive oxygen species, higher PSI activities could not completely explain the greater rates of photochemistry. Pools of glutathione and ascorbate were significantly more reduced in GR+ plants. Increased demand for reducing power to maintain these constituents in the reduced state may contribute to the higher rates of photochemistry observed in GR+ plants.

Keywords: antioxidants, glutathione reductase, *Gossypium hirsutum*, photochemistry, photoprotection, reactive oxygen species.

Introduction

Chilling temperatures increase the susceptibility of chilling-sensitive plants to sustained depressions in photochemical efficiency, commonly referred to as photoinhibition. Chilling limits Calvin-Benson cycle activity, resulting in a reduction in the utilisation of absorbed light energy for CO_2 assimilation (Baker 1994; Allen and Ort 2001) and an increase in the production of reactive oxygen species (ROS) in light harvesting complexes and reaction centres (Foyer and Harbinson 1994; Osmond and Grace 1995; Melis 1999). Plants possess photoprotective mechanisms that minimise ROS-mediated cellular damage. However, these mechanisms can be overwhelmed during exposure to chilling, particularly in chilling-sensitive plants (Wise and Naylor 1987).

Thermal energy dissipation involving the xanthophyll cycle and the suite of enzymatic and non-enzymatic antioxidants are the most critical components of plant photoprotection. Thermal energy dissipation occurs in the light harvesting complexes and results in the harmless removal of excess irradiance as heat (for reviews see Demmig-Adams and Adams 1996; Demmig-Adams *et al.* 1996; Ort 2001), presumably limiting singlet oxygen production. Both a low pH in the thylakoid lumen and the presence of de-epoxidised members of the xanthophyll cycle, zeaxanthin (Z) and possibly antheraxanthin (A), are required to engage thermal energy dissipation (Gilmore 1997; Gilmore and Yamamoto 2001). The chloroplastic system of antioxidant enzymes includes superoxide dismutase (SOD, EC 1.15.1.1) and ascorbate peroxidase (APX, EC 1.11.1.11), whose combined activities catalyse the detoxification of photogenerated superoxide to water in the water–water cycle (for reviews see Asada 1999; Logan *et al.* 1999), and also glutathione reductase (GR, EC 1.6.4.2), which utilises NADPH as a reductive substrate to maintain the pool of reduced glutathione (GSH). GSH participates in ascorbate reduction as the reductive substrate for dehydroascorbate reductase (for review see Noctor and Foyer 1998) and possibly as a direct reductant under the alkaline conditions present in the stroma during illumination (Foyer and Halliwell 1976; Winkler *et al.* 1994). Levels of thermal energy dissipation, as well as contents or activities of xanthophyll cycle pigments and antioxidant systems, generally increase in response to exposure to chilling temperatures (Schöner and Krause

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1990; Anderson *et al.* 1992; Mishra *et al.* 1993; Adams and Demmig-Adams 1994, 1995; Adams *et al.* 1995; Logan *et al.* 1998). In addition, comparisons of species and cultivars report that a positive correlation exists between the capacity of antioxidant systems and resistance to chilling-induced photoinhibition (Wise and Naylor 1987; Janke *et al.* 1991; Hodges *et al.* 1997; Yu *et al.* 2002).

Increased chilling tolerance has been achieved via genetic manipulation leading to the overproduction of various antioxidant enzymes in the chloroplasts of several plant species. For example, poplar overproducing chloroplastic GR (Foyer *et al.* 1995) and tobacco overproducing either Mn-SOD (Foyer *et al.* 1994) or Cu/Zn-SOD (Sen Gupta *et al.* 1993*a*, *b*) exhibited reduced levels of photoinhibition after the abrupt imposition of chilling temperatures in a laboratory setting. However, it should be noted that there are reports wherein antioxidant overproduction had little or no effect on levels of photoinhibition (Payton *et al.* 1997; Tyystjärvi *et al.* 1999).

We reported that transgenic cotton overproducing chloroplastic GR (GR+) sustained less photoinhibition than wild-type cotton when exposed to chilling and moderate photon flux density (PFD) in the presence of the chloroplast protein synthesis inhibitor lincomycin (Kornyeyev *et al.* 2001). The aim of the present study was to examine the mechanisms underlying enhanced chilling tolerance in GR+ cotton along with the responses of energy dissipation, the xanthophyll cycle and carbon assimilation to chilling under moderate PFD in the absence of lincomycin.

Materials and methods

Plant material and growth conditions

Cotton, *Gossypium hirsutum* L. cv. Coker 312, was transformed to overproduce chloroplast-targeted glutathione reductase (GR+) using the *Arabidopsis* gene for GR as described previously in Payton *et al.* (2001). Subsequent generations exhibited a 36-fold increase in total leaf GR activity over that for the wild type, with the majority of the increase in GR activity associated with the chloroplast (Kornyeyev *et al.* 2001; Payton *et al.* 2001).

Plants from three independently-transformed lines of the GR+ genotype and wild-type control (Coker 312) were grown from seed in 8-L pots in a greenhouse at ~ 30°C/26°C (day/night) with a natural photoperiod. Plants were fertilised with Hoagland's solution twice a week. The first fully-expanded leaf of 5- to 8-week-old plants was used for enzyme assays and fluorescence measurements. To verify that GR+ plants overproduced GR, extracts of cotyledons were assayed spectrophotometrically for GR activity as described below.

GR activity

Leaf discs were rapidly removed using a cork borer and immediately frozen in liquid N_2. Frozen leaf tissue was ground to a powder at liquid nitrogen temperature using a mortar and pestle and then rapidly homogenised in 1 mL of 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 extraction and assay for GR activity were performed as described by Sen Gupta *et al.* (1993*b*). GR activity was measured spectrophotometrically by monitoring the oxidation of NADPH at 340 nm.

Chilling treatment of leaf discs

Leaf discs (10 cm^2) were harvested at sunrise and allowed 1.5 h of dark acclimation at room temperature followed by 20 min at 10°C in the chamber of an oxygen electrode (Hansatech, King's Lynn, Norfolk, UK) prior to illumination. For the chilling treatment, the discs were exposed to a PFD of 500 μmol m^{-2} s^{-1} at 10°C. For fluorescence, ascorbate, and glutathione analyses, the leaf discs were exposed to humidified air containing 5.28% CO_2 (v/v). In the case of glyceraldehyde application, CO_2-free air containing 4.99% O_2 (v/v) was used.

Chlorophyll fluorescence

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 oxygen electrode chamber at various times during the 180-min exposure to 10°C and a moderate PFD of 500 μmol m^{-2} s^{-1}. This instrument was also used for fluorescence measurements conducted simultaneously with CO_2-exchange measurements through the window in the leaf chamber of the LCA-4 photosynthesis system.

The experimental protocol described by Schreiber *et al.* (1986) and nomenclature of van Kooten and Snel (1990) were used. The quantum efficiency for electron transport by PSII was calculated as $\phi_{PSII} = (F_m' - F)/F_m'$ (Genty *et al.* 1989), where F and F_m' are steady-state and maximal chlorophyll fluorescence for light-acclimated leaves, respectively. The level of the thermal energy dissipation in PSII antennae was estimated by calculating the non-photochemical chlorophyll fluorescence quenching coefficient (NPQ = $F_m/F_m' - 1$) and the excitation capture efficiency of PSII (F_v'/F_m') (Harbinson *et al.* 1989), where F_v' is variable chlorophyll fluorescence for light-acclimated leaves ($F_v' = F_m' - F_o'$). Measurements of F_o', minimal chlorophyll fluorescence for light-acclimated leaves, were performed after a 10-s application of low-intensity far-red light. Saturating light pulses, 2-s in duration, were provided by a KL1500 light source (Schott, Wiesbaden, Germany). Leaf discs of both genotypes exhibited mean values of F_v/F_m of 0.78 ± 0.02 prior to the chilling PFD treatment in the laboratory. For attached leaves, the mean values were 0.79 ± 0.01. Post-stress values of F_v/F_m were determined for leaf discs following 3-h of dark acclimation at room temperature.

P_{700} activity

The relative amounts of photo-oxidizable P_{700} were measured from leaf discs by means of a PAM 101/103 modulated fluorometer (Heinz Walz GmbH, Effeltrich, Germany) equipped with an ED-P700DW emitter-detector unit. P_{700}^+ formation was induced by illumination with saturating far-red light and monitored as differential absorbance changes (810–860 nm) selective for absorbance changes caused by P_{700} (Klughammer and Schreiber 1998). The measurements were conducted on the leaf discs previously exposed to a PFD of 500 μmol m^{-2} s^{-1} at 10°C in the oxygen electrode chamber for different periods of time and a subsequent period of 3-h dark acclimation at room temperature. During the chilling treatment, leaf discs were exposed to humidified air containing 5.28% CO_2 (v/v).

Treatment with glyceraldehyde

In one analysis, leaves were pre-treated with glyceraldehyde to inhibit Calvin-Benson cycle activity (Stokes and Walker 1972) prior to chlorophyll fluorescence measurements. These leaves were harvested with their petioles under water in the early morning. They were immediately transferred to microfuge tubes containing 60 mM DL-glyceraldehyde (Sigma, St. Louis, MO USA) and placed in dim

light (~ 3 μmol m^{-2} s^{-1}). At the end of the 3-h incubation period, the concentration of glyceraldehyde in the bulk leaf tissue (C_I) was 41–56 mM as estimated from the formula: $C_I = C_S(W_S/W_L)$, 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). No CO_2 fixation at room temperature was detected for leaves treated with glyceraldehyde (data not shown). Leaf discs removed from the inhibitor-treated leaves were considered to be dark-acclimated and were subjected to the chilling treatment in an oxygen electrode chamber as described.

Pigment analysis

Leaf discs (0.25 cm^2) were harvested before, during, and after the chilling treatment in the oxygen electrode chamber. The tissue was immediately frozen in liquid nitrogen where it remained stored until processing. Pigment composition was analysed by high performance liquid chromatography as described in Adams and Demmig-Adams (1992) using the column and gradient system of Gilmore and Yamamoto (1991).

FBPase activity

Fructose-1,6-bisphosphatase (FBPase) activity was measured from 10 cm^2 leaf discs that were rapidly removed from the oxygen electrode chamber under illumination and frozen in liquid N_2. The activity of FBPase was measured at 30°C for comparison with published activity data (Holaday *et al.* 1992) by monitoring the reduction of NADPH at 340 nm. Leaf tissue was homogenised in chilled 50 mM borate, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM fructose 1,6-bisphosphate, 15 mM 2-mercaptoethanol, and 0.1% (v/v) Triton X-100. Initial (not activated *in vitro*) activity was measured by adding centrifuged extract to 50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM NADPH, 0.1 mM fructose 1,6-bisphosphate, and 4 units each of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase in a final volume of 1 mL. Total activity was determined by adding centrifuged leaf extract to a similar solution but with 4 mM fructose 1,6-bisphosphate and 15 mM DTT.

Ascorbate and glutathione content

Oxidised and reduced forms of ascorbate and glutathione were extracted in 2 mL of ice-cold 2% meta-phosphoric acid with 2 mM EDTA from leaf tissue previously ground in liquid nitrogen. The extract was centrifuged at 10 000 *g* for 10 min at 4°C and neutralised with sodium citrate as previously described (Rao and Ormrod 1995). Reduced ascorbate was assayed by monitoring the total decline in absorbance at 265 nm upon the addition of ascorbate oxidase from *Cucurbita* (4 units) to an assay mixture containing 100 mM phosphate buffer (pH 5.6) and extract neutralised by 10% sodium citrate (Rao and Ormrod 1995). The total amount of ascorbate was determined after reduction of oxidised forms to ascorbate in the reaction mixture containing 20 mM DTT and HEPES buffer (pH 7.5). Total glutathione was assayed in a solution containing 0.5 M phosphate buffer (pH 7.5), 6.3 mM EDTA, 0.03 mM 5,5′-dithiobis-2-nitrobenzoic acid, 0.3 mM NADPH, and neutralised extract as described by Rao and Ormrod (1995). The reaction was initiated with 1 unit of GR from spinach and the rate of NADPH oxidation was monitored at 340 nm. The amount of oxidised glutathione (GSSG) was measured after adding 20 μL of 2-vinylpyridine to 0.5 mL of neutralised extract and incubating at 25°C for 1 h. The assay was the same as described above for total glutathione.

Chilling treatment and CO_2 assimilation of intact, attached leaves

Attached leaves were dark-acclimated at sunrise for 1.5 h at 30°C followed by an additional 0.5 h at 10°C before commencing illumination at 500 μmol m^{-2} s^{-1}. The leaf temperature of 10°C was maintained by means of a PLC temperature controller (ADC Ltd, Hoddesdon, UK). Measurements of CO_2 exchange were conducted in the greenhouse using an LCA-4 portable photosynthesis system (ADC Ltd, Hoddesdon, UK) at ambient CO_2 concentration (360–380 μmol mol^{-1}) under natural illumination. The calculation of the rate of CO_2 fixation accounted for the rate of mitochondrial respiration in the dark.

Statistical analysis

Data for transgenic genotypes were compared with data for wild-type using a Student's *t*-test. Means were considered significantly different for $P \leq 0.05$.

Results

Genotypic GR activity and leaf pigment composition

Consistent with our previously published studies (Kornyeyev *et al.* 2001; Payton *et al.* 2001), the leaves of the GR+ plants exhibited a 36-fold greater GR activity than wild-type plants (2471 ± 498 and 69 ± 17 μmol (mg Chl)$^{-1}$ h^{-1}, respectively, mean ± s.d., n = 7–15). When GR activity was calculated separately for each of the three independently-transformed lines, no significant differences were found between the lines (2695 ± 169, 2162 ± 555, and 2596 ± 491 μmol (mg Chl)$^{-1}$ h^{-1} [n = 3–7]). In all of the measurements described below, statistically significant differences between independently-transformed lines of GR+ cotton were never observed (data not shown). Therefore, data from all lines were combined. Overproduction of GR did not result in a statistically significant change in the pigment composition of GR+ in comparison to wild-type plants (Table 1).

Photochemistry and non-photochemical energy dissipation during exposure of leaf discs to chilling at moderate PFD

Leaf discs of GR+ plants exhibited significantly ($P \leq 0.01$) higher quantum yields of PSII photochemistry (ϕ_{PSII}) than those of wild-type plants throughout a 3-h exposure to chilling (10°C) at 500 μmol m^{-2} s^{-1}, except at 3 min of illumination (Fig. 1). The differences between GR+ and wild-type cotton were most pronounced during induction.

Table 1. Pigment composition (μmol m^{-2}) of cotton leaf tissue from wild-type cotton (cv. Coker 312) and transgenic cotton overproducing chloroplastic glutathione reductase (GR+)

Superscripts represent *P*-values from *t*-tests comparing GR+ and wild-type plants. Mean ± s.d., n = 5

Pigment(s)	Genotype	
	Wild-type	GR+
Total Chl *a*+*b*	594.6 ± 125.4	$553.2 \pm 88.8^{0.70}$
Chl *a*/*b*	3.6 ± 0.2	$3.6 \pm 0.1^{0.47}$
Total carotenoids	187.1 ± 44.0	$179.2 \pm 57.9^{0.81}$
β-carotene	50.4 ± 14.9	$47.6 \pm 14.7^{0.77}$
Xanthophyll cycle pigments	48.2 ± 13.8	$49.0 \pm 15.0^{0.93}$
Lutein	64.2 ± 12.0	$61.8 \pm 18.7^{0.82}$
Neoxanthin	24.3 ± 3.8	$24.2 \pm 7.2^{0.98}$

At 3-h of illumination at 10°C, GR+ plants maintained photochemical quantum yields that were 26% higher than those of control plants.

Genotypic differences in ϕ_{PSII} were temperature-dependent. Steady-state values of ϕ_{PSII} were significantly greater for GR+ only at 5 and 10°C (Fig. 2).

Treatment of leaves with glyceraldehyde prevents CO_2 and O_2 assimilation in the Calvin-Benson cycle by inhibiting the formation of ribulose bisphosphate via phosphoribulokinase (Stokes and Walker 1972; Wu *et al.* 1991). We applied glyceraldehyde to compare ϕ_{PSII} of GR+ and wild-type cotton in the absence of any Calvin-Benson cycle activity. After 60 min of light acclimation at 500 μmol m^{-2} s^{-1} and 10°C in the absence of CO_2 but the presence of 5% O_2, ϕ_{PSII} was significantly higher for glyceraldehyde-treated GR+ in comparison to wild-type leaf discs (0.032 ± 0.005 and 0.017 ± 0.009 for GR+ and wild-type respectively, mean ± s.d., $P = 0.038$, $n = 4$) indicating enhanced electron transport in GR+ plants that was not linked to Calvin-Benson cycle activity.

In order to compare the level of the thermal energy dissipation in PSII complexes between genotypes, we determined the coefficient of non-photochemical chlorophyll fluorescence quenching (NPQ) and the efficiency of excitation transfer to open PSII reaction centres (F_v'/F_m'). No significant differences were found between genotypes in the time-courses for these parameters during exposure to 500 μmol m^{-2} s^{-1} and 10°C (Fig. 3).

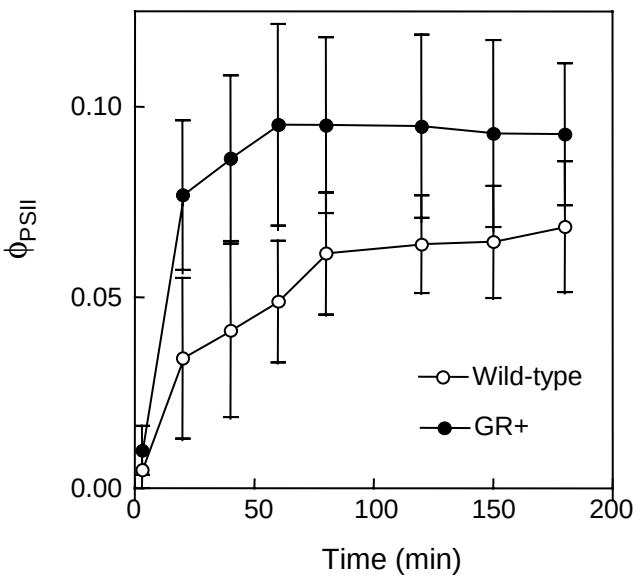

Fig. 1. Time-course for the quantum yield of PSII photochemistry (ϕ_{PSII}) during exposure to 10°C at a photon flux density of 500 μmol m^{-2} s^{-1} and 5.28% (v/v) CO_2 for wild-type cotton and transgenic cotton overproducing chloroplastic glutathione reductase (GR+). Error bars represent s.d., n = 10–13. The leaf discs were collected at dawn and kept in the dark for 1.5 h at room temperature followed by 20 min at 10°C in the dark prior to illumination.

The primary factor contributing to non-photochemical fluorescence quenching in PSII complexes under our experimental conditions was thermal energy dissipation that involves the xanthophyll cycle, since it is believed that the contribution of state 1–state 2 transitions is significant only at low light intensity (Walters and Horton 1993; Lichtenthaler and Bukart 1999). Both genotypes exhibited a similar progressive increase in the extent of xanthophyll de-epoxidation, measured as (A+Z)/(V+A+Z), from less than 0.1 to nearly 0.6 over the course of the 3-h chilling treatment (Fig. 4). A substantial fraction (~ 20%) of the total pool of xanthophyll cycle pigments was retained in the de-epoxidised forms (A+Z) after 3-h of recovery in darkness at room temperature following the 3-h chilling treatment. At no point during the chilling treatment or during recovery did the de-epoxidation state of the xanthophyll cycle differ significantly between GR+ and wild-type plants.

Chilling-induced photoinactivation of PSI and PSII

The time-courses for chilling-induced photoinactivation of PSI (quantified as the relative amount of photo-oxidizable P_{700}) and PSII (quantified as the non-reversible decrease in F_v/F_m) indicated that PSI exhibited greater rates of photoinactivation than PSII in both genotypes (Fig. 5). Only

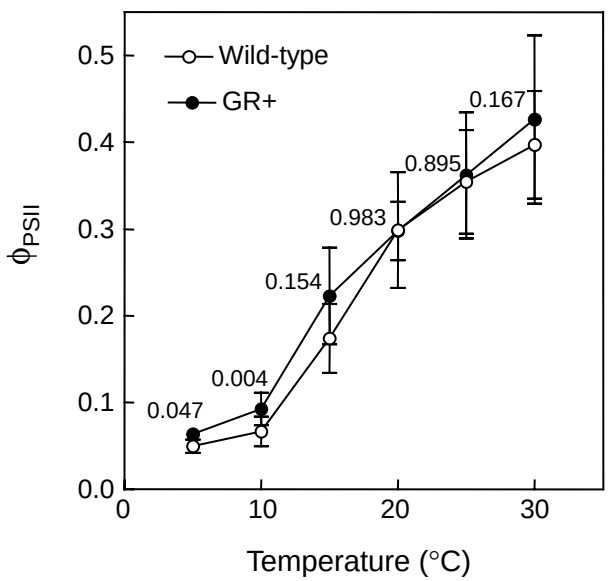

Fig. 2. Temperature dependence of the quantum yield of PSII photochemistry (ϕ_{PSII}) for wild-type cotton and transgenic cotton overproducing chloroplastic glutathione reductase (GR+) at 5.28% (v/v) CO_2 and a photon flux density of 500 μmol m^{-2} s^{-1}. Error bars represent s.d., n = 3–13. The measurements were conducted after 90 min of illumination at temperatures of 15–30°C and after 180 min of illumination at 5 and 10°C. The leaf discs were collected at dawn and kept in the dark for 1.5 h at room temperature followed by 20 min at each temperature in the dark prior to illumination. Numbers at symbols indicate P-values obtained from a t-test comparing levels of ϕ_{PSII} for wild-type and GR+ plants at each temperature.

48–57% of the initial PSI activity was detectable after 6-h of light exposure, while 73–77% of initial PSII activity was detectable. Photosystem activity was measured after 3-h dark acclimation of leaf discs previously subjected to the chilling treatment (10°C, 500 μmol m^{-2} s^{-1}). When examined after 2, 3 and 4 h of the treatment, GR+ leaf discs sustained significantly less loss of PSI activity than wild-type leaf discs. The extent of PSII photoinactivation was significantly less for GR+ than for wild-type from 0.2 to 4 h of light exposure.

Effect of chilling treatment on ascorbate and glutathione pool sizes and reduction states

After 40 and 180 min of illumination (500 μmol m^{-2} s^{-1}) at 10°C, the reduction state of the ascorbate pool decreased in leaf discs of both genotypes (Fig. 6*A*). However, leaf discs of GR+ plants maintained higher levels of ascorbate reduction in comparison to wild-type. Similarly, GR+ leaf discs maintained a greater percentage of glutathione in the reduced state at 40 and 180 min than wild-type leaves (Fig. 6*B*).

Fructose bisphosphatase activity and activation state for leaf discs

Stromal fructose-1,6-bisphosphatase (FBPase) is sensitive to H_2O_2 *in vitro* and the stromal redox state (Charles and Halliwell 1981; Harbinson *et al.* 1990). Therefore, increasing the capacity to scavenge H_2O_2 by increasing GR activity might lead to a more rapid activation and higher *in vivo* activity of FBPase during dark-to-light transitions at chilling temperature and potentially to greater Calvin-Benson cycle activity. However, the activation kinetics for FBPase during the 180 min of illumination were similar for both genotypes (Table 2). In addition, the 3-h chilling treatment (500 μmol m^{-2} s^{-1}, 10°C) did not affect the total activity of FBPase in either genotype. At the end of the treatment, the activities were 364 ± 24 and 355 ± 15 μmol (mg Chl)$^{-1}$ h^{-1} for GR+ and wild-type leaf discs, respectively (mean ± s.d., $P = 0.546$, $n = 4–5$).

Quantum yield of PSII photochemistry and the rate of CO_2 assimilation for attached leaves under ambient CO_2 concentrations

In addition to those measurements described above that were performed on leaf discs, we also measured chlorophyll fluorescence emission and CO_2 assimilation simultaneously from intact, attached leaves at 500 μmol photons m^{-2} s^{-1} and

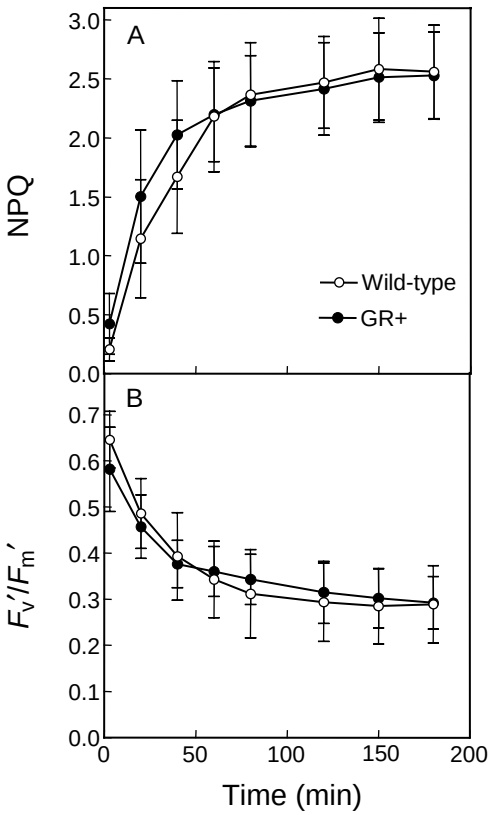

Fig. 3. Time-course for non-photochemical chlorophyll fluorescence quenching (NPQ) (*A*) and the maximal quantum yield of photochemistry for PSII complexes with 'open' reaction centres (F_v'/F_m') (*B*) during exposure to 10°C at a photon flux density of 500 μmol m^{-2} s^{-1} and 5.28% (v/v) CO_2 for wild-type cotton and transgenic cotton overproducing chloroplastic glutathione reductase (GR+). Error bars represent s.d., n = 10–13. The leaf discs were collected at dawn and kept in the dark for 1.5 h at room temperature followed by 20 min at 10°C in the dark prior to illumination.

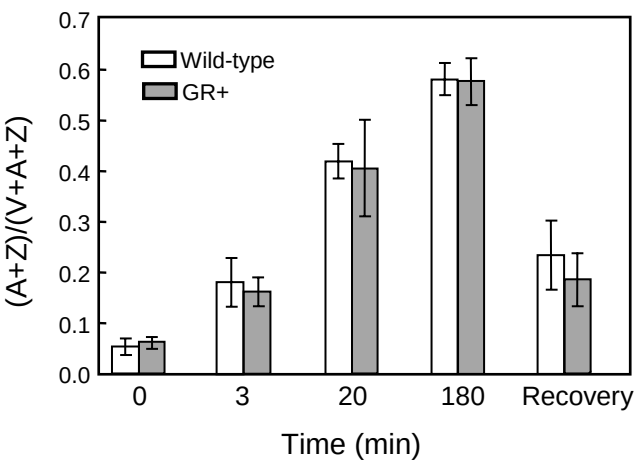

Fig. 4. Time-courses for the xanthophyll cycle de-epoxidation state, (A+Z)/(V+A+Z), during exposure to 10°C at a photon flux density of 500 μmol m^{-2} s^{-1} and 5.28% (v/v) CO_2 and subsequent recovery for 180 min in darkness at room temperature for wild-type cotton and transgenic cotton overproducing chloroplastic glutathione reductase (GR+). Error bars represent s.d., n = 5. Transgenic means were not significantly different (P>0.05) from wild-type means. The leaf discs were collected at dawn and kept in the dark for 1.5 h at room temperature followed by 20 min at 10°C in the dark prior to illumination.

10°C at ambient CO_2 concentrations (360–380 μmol mol^{-1}). Ambient CO_2 concentrations were determined to be saturating for photosynthesis at 10°C (data not shown). GR+ plants exhibited significantly higher ϕ_{PSII} than wild-type plants from 20 to 180 min exposure to chilling (Fig. 7*A*). The time courses of ϕ_{PSII} were similar to those observed in leaf discs (see Fig. 1). No significant differences were observed in the rates of CO_2 assimilation determined simultaneously with chlorophyll fluorescence (Fig. 7*B*).

Discussion

Our previous analyses indicated that wild-type and non-expressing segregate transgenic plants were statistically indistinguishable in terms of antioxidant enzyme activities and the response of photosynthesis to chilling (Payton *et al.* 2001). In the present study, independently-transformed GR+ lines exhibited no significant differences in any measured parameter at any time-point. These observations strongly suggest that the differences in performance between wild-type and GR+ plants were due to the presence of the transgene and its specific effect on GR activity.

In an earlier study (Kornyeyev *et al.* 2001), we determined that GR+ plants exhibited a lower rate of PSII photoinactivation at 10°C and 500 μmol m^{-2} s^{-1} than wild-type in the presence of lincomycin, an inhibitor of chloroplast repair processes. In the present study, we showed that, even without the inhibition of repair processes, PSII photoinactivation at 10°C was reduced in GR+ plants. The enhanced chilling tolerance of GR+ plants may be the result of elevated rates of photochemistry. Elevated rates of photochemistry are protective because they bring about a decrease in the reduction state of the primary quinone acceptor of PSII, Q_A. Photon energy trapped by PSII centres

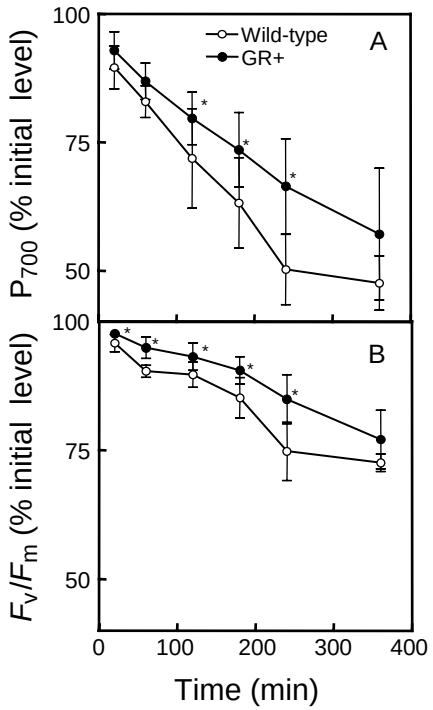

Fig. 5. Time-courses for sustained photoinactivation of PSI (*A*; quantified as the percent decrease in the level of photo-oxidizable P_{700} relative to pretreatment values) and PSII (*B*; quantified as the percent decrease in F_v/F_m relative to pretreatment values) with exposure to 10°C at a photon flux density of 500 μmol m^{-2} s^{-1} and 5.28% (v/v) CO_2 for wild-type cotton and transgenic cotton overproducing chloroplastic glutathione reductase (GR+). Error bars represent s.d., n = 4–21. The leaf discs were collected at dawn and kept in the dark for 1.5 h at room temperature followed by 20 min at 10°C in the dark prior to illumination. After the stress treatment, leaf discs were allowed 3-h of dark acclimation at room temperature before the measurements of photo-oxidizable P_{700} and F_v/F_m were conducted.

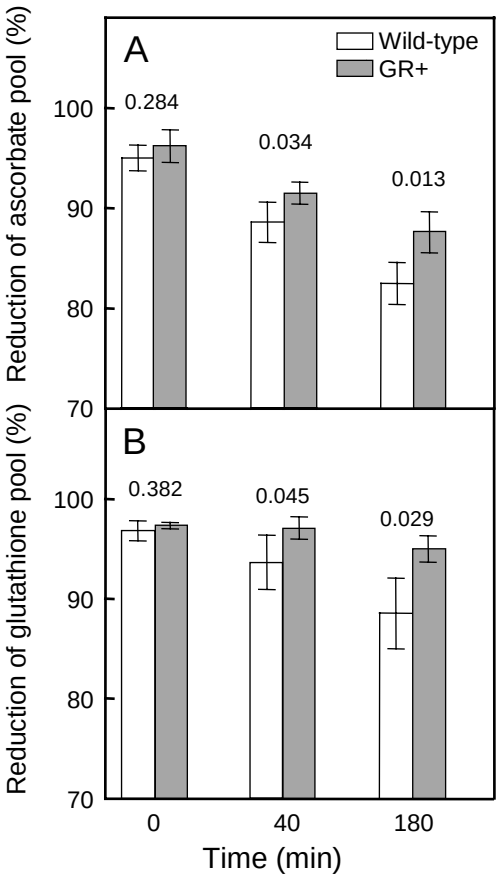

Fig. 6. The reduction states of the ascorbate (*A*) and glutathione (*B*) pools in leaf discs of wild-type cotton and transgenic cotton overproducing chloroplastic glutathione reductase (GR+) after 0, 40 and 180 min at 10°C, 5.28% (v/v) CO_2, and a photon flux density of 500 μmol m^{-2} s^{-1}. Prior to illumination, leaf discs were acclimated to darkness at room temperature for 1.5 h and at 10°C for an additional 20 min The mean total ascorbate content prior to illumination was 6475 ± 545 and 6479 ± 261 nmol g^{-1} fresh mass for wild-type and GR+ plants, respectively. The mean total glutathione content prior to illumination averaged 442 ± 17 and 405 ± 32 nmol g^{-1} fresh mass for wild-type and GR+ plants respectively. No significant change in the total ascorbate or glutathione pool size occurred over the 180 min of treatment. Error bars represent s.d., n = 4.

with Q_A in the reduced state is more likely to result in charge recombination that can produce triplet-P_{680}, which in turn, sensitises singlet oxygen formation (Melis 1999). Therefore, lower Q_A reduction states brought about by enhanced electron flow render leaves less vulnerable to PSII photoinactivation.

Several lines of evidence indicated that elevated rates of photochemistry observed in GR+ plants were due to increased non-assimilatory electron flow and not due to assimilatory electron flow resulting from enhanced Calvin-Benson cycle activity. Under the conditions of the chilling treatment, there were no differences between GR+ and wild-type plants in the induction kinetics and total activity of FBPase, a redox-sensitive Calvin-Benson cycle enzyme (Table 2) and no genotypic differences in the rates of CO_2 assimilation, while simultaneous measurements of chlorophyll fluorescence indicated a genotypic difference in ϕ_{PSII} (Fig. 7*B*). Furthermore, GR+ plants maintained higher ϕ_{PSII} than wild-type plants even in the presence of glyceraldehyde, an inhibitor of the Calvin-Benson cycle.

Lower rates of PSI photoinactivation in transgenic plants may have contributed to higher ϕ_{PSII}. However, genotype-specific differences in ϕ_{PSII} were observed throughout the chilling treatment, even during the early phases of induction before appreciable loss of PSI activity had occurred. Therefore, protection against PSI photoinactivation cannot fully explain the protective effect of GR overproduction on PSII. Nonetheless, our present observations support the conclusion that PSI is more sensitive to chilling-induced photoinactivation than PSII (Terashima *et al.* 1994, 1998). For cotton, the rate of PSI photoinactivation was nearly two-fold greater than that of PSII. PSI photoinactivation is thought to occur through hydroxyl radical production via a Fenton-type reaction between photogenerated H_2O_2 and Fe-S centres in PSI (Terashima *et al.* 1998). Thus, our data suggest that the GR+ cotton exhibited greater H_2O_2 scavenging than wild-type during the chilling treatment.

We propose that the elevated rates of photochemistry observed in GR+ cotton during chilling exposure were due primarily to an elevated demand for reducing power to maintain the glutathione and ascorbate pools in the reduced state. This idea is consistent with the observation that the reduction states of the glutathione and ascorbate pools were higher during chilling in GR+ plants in comparison to wild-type. The explanation suggested above for elevated rates of photochemistry in GR+ plants is compatible with the absence of significant genotypic differences in ϕ_{PSII} at temperatures greater than 10°C. As temperatures approach the optimum for CO_2 assimilation, the relative contribution of GR-dependent metabolism to the overall demand for

Table 2. Activation state (%) of FBPase in leaf discs of wild-type cotton (cv. Coker 312) and transgenic cotton overproducing chloroplastic glutathione reductase (GR+) during a dark-to-light transition at saturating CO_2, 500 µmol photons m^{-2} s^{-1}, and 10°C
Superscripts represent *P*-values from *t*-tests comparing GR+ and wild-type plants. Mean ± s.d., $n = 5$

Time of light exposure (min)	Genotype	
	Wild-type	GR+
0	26.1 ± 2.0	$25.1 \pm 1.9^{0.477}$
2	38.3 ± 3.6	$36.5 \pm 2.3^{0.392}$
5	44.2 ± 5.0	$46.7 \pm 3.2^{0.401}$
20	56.8 ± 4.4	$57.0 \pm 2.7^{0.930}$
180	67.3 ± 9.3	$69.6 \pm 7.1^{0.693}$

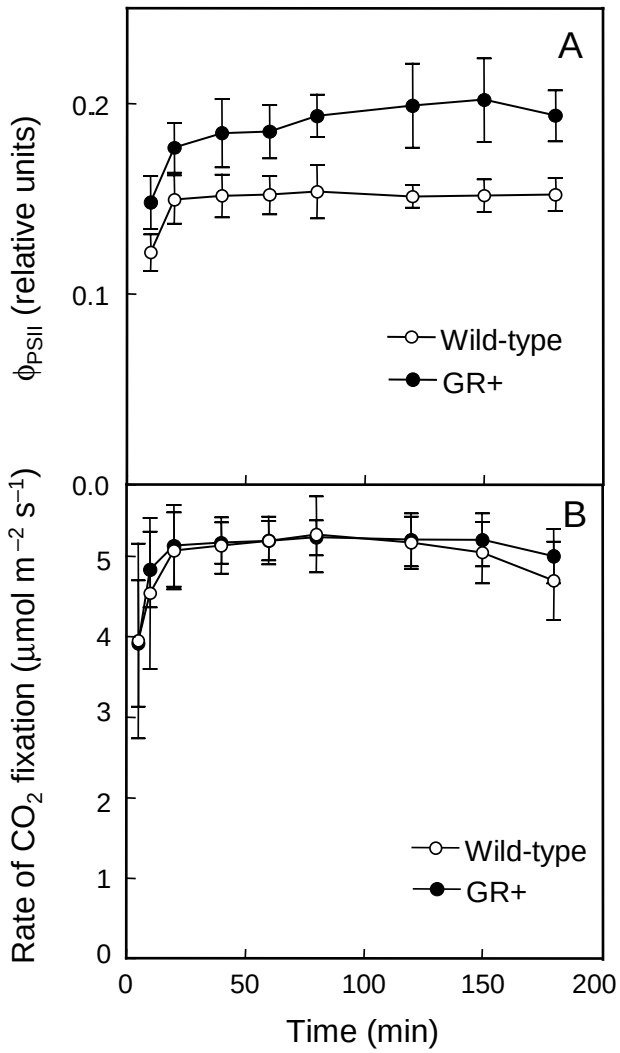

Fig. 7. Time-course for the quantum yield of PSII photochemistry (ϕ_{PSII}) (*A*) and the rates of CO_2 assimilation (*B*) at 10°C, ambient CO_2 concentration (360–380 µmol mol^{-1}), and a photon flux density of 500 µmol m^{-2} s^{-1} for attached leaves of wild-type cotton and transgenic cotton overproducing chloroplastic glutathione reductase (GR+). At dawn, the leaves were kept in the dark for 1.5 h at 30°C followed by 30 min at 10°C in the dark prior to illumination. For ϕ_{PSII}, genotypic means were significantly different ($P \leq 0.05$) at each time point, while the means were not significantly different ($P > 0.05$) for CO_2 assimilation. Error bars represent s.d., $n = 3$–9.

reducing power could decline for the following reasons: (a) increased Calvin-Benson cycle activity may increase the proportion of reducing power required to maintain CO_2 assimilation; (b) the rate of O_2 photoreduction is likely to decrease, thereby decreasing the demand for GSH; (c) as the low temperature restrictions on GR activity *in vivo* are relieved with increasing temperature, the rate of GSH formation via the native cotton GR may be sufficient to scavenge the ROS formed.

It has been previously suggested that the chloroplastic ROS scavenging system may act as an alternative electron sink (Asada 2000). However, flux analyses through the water–water cycle are only now emerging (Polle 2001) and very little is known concerning the limiting steps in this complex pathway that involves both some redundancy as well as a combination of enzymatic and non-enzymatic reactions (Asada 1999). As the water–water cycle is typically depicted, GR maintains a pool of GSH that serves as a substrate for the reduction of dehydroascorbate (DHA) that is catalysed by the enzyme DHA reductase. Considering this role alone renders GR somewhat peripheral in the water–water cycle, given that two additional mechanisms of monodehydroascorbate (MDHA) reduction have also been described, direct photoreduction and reduction via MDHA reductase (Asada 1999). Our data obtained on GR-overproducing cotton plants confirm the important role of GR in chilling tolerance. We suggest that an ascorbate regeneration and ROS detoxification pathway that is non-enzymatic, with the exception of GSH reduction via GR, may contribute to this response. Although the relative importance of enzymatic versus non-enzymatic reduction of DHA remains the subject of debate (Asada 1999; Polle 2001), GSH can reduce DHA non-enzymatically under the alkaline conditions that would be expected in the stroma during illumination (Foyer and Halliwell 1976; Winkler *et al.* 1994). Non-enzymatic reduction of DHA, coupled with non-enzymatic detoxification of superoxide by ascorbate (Halliwell and Gutteridge 1999), would allow for the safe removal of ROS that is dependent on GR only. We can offer no direct experimental evidence that such a pathway operates *in vivo*, however the mid-point reduction potentials of the participating constituents (Buettner 1993) and the high concentrations of ascorbate and glutathione found in the chloroplast (Noctor and Foyer 1998) render it worthy of consideration. This pathway may be particularly relevant during exposure to chilling when the demand for GSH should be high and enzyme activities are limited by low temperatures.

In addition to the water–water cycle, thermal dissipation of excess irradiance in the light harvesting complexes protects against ROS damage by mitigating the production of singlet oxygen. Levels of thermal energy dissipation can be modulated by the magnitude of the trans-thylakoid membrane proton gradient, which, in turn, can be augmented by the activity of the water–water cycle. Therefore, there was reason to suspect that transgenic manipulation of the water–water cycle may affect levels of thermal energy dissipation. However, we observed no such effects; neither levels of non-photochemical fluorescence quenching nor xanthophyll cycle conversion state differed between GR+ and wild-type cotton during chilling and subsequent recovery.

Despite the many-fold overproduction of GR, the observed effects on photochemistry and the protection of photosynthetic capacity (Payton *et al.* 2001) in cotton were small. This observation could be the result of a relatively low level of control exerted by glutathione metabolism mediated by GR on ROS scavenging. Alternatively, regulated thermal dissipation (downregulation of the PSII quantum efficiency) may be the most important photoprotective process involved in minimizing PSII photoinhibition under our experimental conditions, where electron transport rates are low. Because so little absorbed light was utilised for photochemistry during chilling, the 1.3 fold increase in the magnitude of ϕ_{PSII} in GR+ plants led to only a 2–3% increase in the overall non-destructive utilization of light energy absorbed by PSII complexes. Nevertheless, this small increase in light energy utilization did enhance the protection of PSII.

Conclusions

Overproduction of GR in the chloroplasts of cotton leaves resulted in small, but significant, enhancements in the resistance to photoinhibition of PSII and PSI during exposure to chilling temperatures at moderate PFDs. Thermal energy dissipation was not influenced by the transgenic manipulation. The effect on PSII photoinhibition may be attributed to an elevated rate of electron transport, which is not associated with carbon metabolism. This elevated photochemistry can be partly explained by an increased consumption of reducing power in enzymatic GSSG reduction in chloroplasts of GR+ plants at low temperature.

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