

BRIEF COMMUNICATION

TRANSGENIC OVERPRODUCTION OF GLUTATHIONE REDUCTASE DOES NOT PROTECT COTTON, *Gossypium hirsutum* (MALVACEAE), FROM PHOTOINHIBITION DURING GROWTH UNDER CHILLING CONDITIONS¹

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In some studies, tissues from plants that have been genetically transformed to overproduce antioxidant enzymes sustain less damage when abruptly exposed to short-term chilling in the laboratory. However, few studies have examined the performance of transgenic plants during longer-term growth under chilling conditions. We compared growth of transgenic cotton that overproduces glutathione reductase (GR+; ~40-fold overproduction) to growth of the wild type in a controlled environment chamber as leaf temperature was lowered from 28° to 14°C over 9 d and for a subsequent 9-d period at 14°C. In wild-type and GR+ cotton, chilling temperatures resulted in decreased dark-adapted F_v/F_m (the ratio of variable to maximal fluorescence; a measure of maximum photosystem II quantum yield) and mid-light period photosystem II quantum yield, coupled with increased $1 - q_p$ (a nonlinear estimate of the reduction state of the primary quinone acceptor of photosystem II). The capacity for photosynthetic oxygen evolution decreased during the first portion of the chilling exposure, but recovered slightly during the second half. At no point during the chilling exposure did the performance of GR+ plants differ significantly from that of wild-type plants in any of the above parameters. The absence of an effect of GR overproduction under longer-term chilling may be explained, in part, by the fact that wild-type cotton acclimated to chilling by upregulating native GR activity.

Key words: acclimation; antioxidants; chilling; chlorophyll fluorescence; cotton; glutathione reductase; Malvaceae; photoinhibition.

Chilling exacerbates oxidative stress that is experienced by plants during illumination (Baker, 1994; Allen and Ort, 2001). Production of reactive oxygen species (ROS) in excess of a plant's capacity for detoxification can lead to molecular damage and sustained decreases in photosynthetic efficiency that are commonly referred to as photoinhibition (Asada, 1999; Melis, 1999; Niyogi, 1999). Chilling-sensitive plant species, such as cotton, are particularly vulnerable to chilling-induced photoinhibition (Wise, 1995).

Reduced glutathione (GSH) is a critical constituent of chloroplastic ROS detoxification pathways. Reduced glutathione is a low-molecular-weight thiol antioxidant (Hausladen and Alscher, 1993) that serves as the reductant for dehydroascorbate reductase, which reforms ascorbate from dehydroascorbate (Hossain and Asada, 1984). Reduced glutathione can also reduce dehydroascorbate nonenzymatically under the alkaline conditions found in the stroma during illumination (Foyer and Halliwell, 1976; Winkler et al., 1994). The glutathione pool is maintained largely in the reduced state by glutathione reductase (GR), which utilizes NADPH as a reductant. This reaction affords further protection against photoinhibition by forming

NADP⁺, the preferred electron acceptor for photosynthetic electron transport.

Acclimation to chilling temperatures generally leads to increased GSH contents and GR activities (Anderson et al., 1992; Logan et al., 1998b). Attempts to enhance chilling tolerance via transgenic overproduction of GR have met with some success (Foyer et al., 1995; Kornyejev et al., 2001, 2003; Payton et al., 2001). For example, 30- to 40-fold overproduction of chloroplastic GR in cotton decreased the levels of photosystem II (PSII) and photosystem I (PSI) photoinhibition by approximately 28% and 20%, respectively, during abruptly imposed, short-term exposure of leaf discs from warm-grown plants to 500 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 10°C (Kornyejev et al., 2001, 2003). The maintenance of greater rates of photochemistry, along with decreased PSII reduction states, partly explains the enhanced chilling tolerance exhibited by these transgenic plants under these conditions (Melis, 1999; Kornyejev et al., 2001, 2003).

Much of our understanding of the physiological responses to chilling of transgenic plants that overproduce antioxidant enzymes derives from short-term experiments, such as those described earlier, wherein leaf discs from warm-grown plants are abruptly subjected to conditions that are more extreme than those typically encountered in the field. While these studies have yielded insight into the mechanisms of chilling tolerance and the regulation of oxidative metabolism, there is a need to examine the performance of such transgenic genotypes during growth under longer-term chilling. In the present study, we

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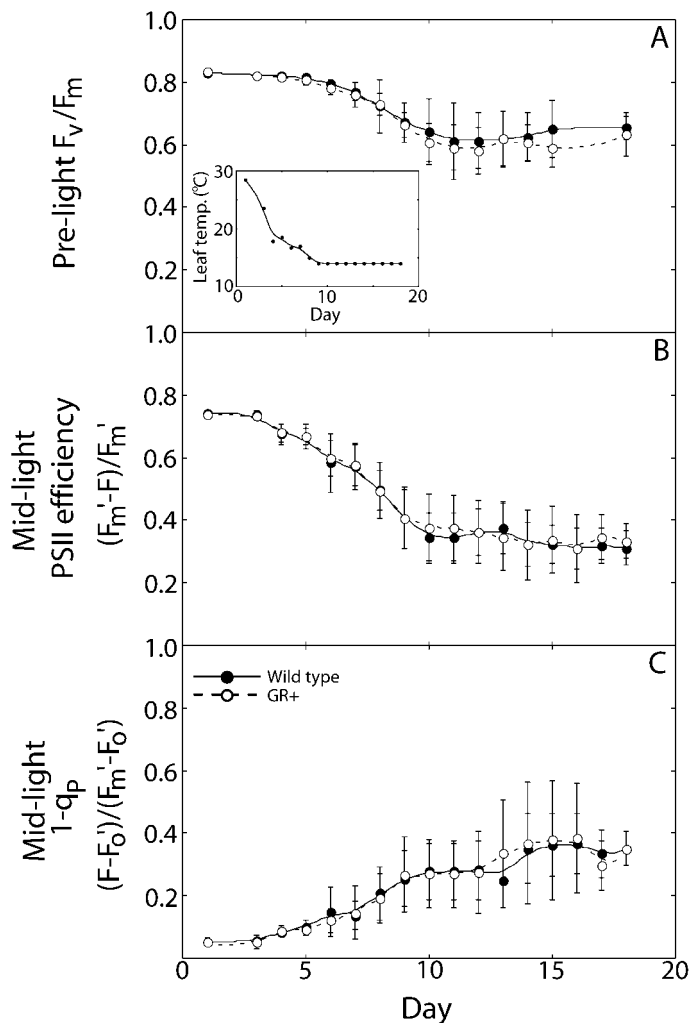


Fig. 1. Time courses for pre-light period F_v/F_m (A), mid-light period PSII efficiency (B), $1 - q_p$ (C), and leaf temperature (inset of A) from cotton that overproduces glutathione reductase (GR+; open circles) and wild-type cotton (closed circles) during exposure to gradually imposed chilling. Data are means \pm SD; $N = 8-16$.

primarily analyzed chlorophyll fluorescence to compare the performance of cotton overproducing chloroplastic GR to that of wild-type plants during growth in a controlled environment chamber as leaf temperature was lowered from 28° to 14°C over 9 d and for a subsequent 9-d period at 14°C.

MATERIALS AND METHODS

Plant material and chilling exposure—Cotton plants, *Gossypium hirsutum* L. cv. Coker 312, were transformed to overproduce glutathione reductase (GR+; EC 1.6.4.2), using a modified GR cDNA from *Arabidopsis thaliana*, ecotype Columbia, as described previously (Kornyejev et al., 2001; Payton et al., 2001). Wild-type cotton (cv. Coker 312) and two independently transformed GR+ lines of cotton were grown from seed in 8-L pots in a greenhouse with a natural photoperiod in December/January. Three weeks after planting, seedlings were transferred to a controlled environment chamber with a photon flux density of 300 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, a 14-h photoperiod, and a 28°/26°C light/dark temperature regime. After 1 wk in the chamber, the temperature of the chamber was reduced over a 9-d period such that mean leaf temperature fell gradually until it reached 14°C (day and night). The

plants were then maintained at this leaf temperature for 9 d (see inset, Fig. 1A). Plant positions within the chamber were random and changed daily. Plants were fertilized with a complete nutrient medium twice weekly. The first fully expanded leaves at the time that the chilling exposure was initiated were used for all measurements.

Glutathione reductase activities—To confirm that the GR+ plants possessed elevated enzyme activities, the GR activities of fully developed cotyledons were assayed spectrophotometrically as the rate of NADPH oxidation as described in Logan et al. (1998b). Analyses of the effect of chilling on foliar GR activities employed the same assay.

Chlorophyll fluorescence—Chlorophyll fluorescence emission was measured from attached leaves in their growth environment with a pulse amplitude-modulated fluorometer (FMS2, Hansatech, King's Lynn, Norfolk, UK). Measurements were taken immediately before and in the middle of the light period. The experimental protocol described by Schreiber et al. (1986) and nomenclature of van Kooten and Snel (1990) were used. The ratio of variable to maximal fluorescence emission (F_v/F_m) was calculated as $(F_m - F_o)/F_m$, where F_o and F_m are the pre-light period minimal and maximal levels of fluorescence, respectively. The fraction of light energy absorbed by PSII antennae that was utilized for photochemistry (PSII efficiency) was estimated as $(F_m' - F)/F_m'$, where F_m' is maximal fluorescence during illumination and F is steady state fluorescence during illumination (Genty et al., 1989). The value $1 - q_p$ was estimated as $(F - F_o')/(F_m' - F_o')$, where F_o' is minimal fluorescence of leaves in the light-acclimated state. Minimal fluorescence was measured after a brief application of low-intensity far-red light. Saturating light pulses of 1-s duration were provided by a white light source embedded in the fluorometer.

Oxygen evolution—The capacity for oxygen evolution was measured at the beginning, middle (day 7), and end (day 18) of the chilling exposure. Measurements were performed on leaf discs (1.1 cm^2) exposed to 1700 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 25°C in an atmosphere of humidified 5% CO_2 , 21% O_2 , and the balance N_2 in the chamber of a gas-phase oxygen electrode (Model LD-2, equipped with an LS-2 light source, Hansatech). Steady-state rates of oxygen evolution were determined, followed by measurement of respiration upon return to darkness.

RESULTS AND DISCUSSION

Prior to the initiation of the chilling exposure, mean GR activities of leaves of wild-type plants were $12 \pm 1.5 \mu\text{mol NADPH} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ ($N = 4$). Mean GR activities of the two independently transformed lines of GR+ plants used for this study were 416 ± 80.7 and $622 \pm 31.5 \mu\text{mol NADPH} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ ($N = 3$ for each line). Therefore, GR+ plants possessed ~40-fold higher foliar GR activities than wild-type plants. No significant differences were observed between the physiological performances of the two independently transformed lines of GR+ cotton (data not shown); therefore, data from each were combined. We reported previously that transgenic overproduction of GR has no effect on the size of the foliar glutathione pool in cotton (Kornyejev et al., 2003).

In both wild-type and GR+ cotton, the onset of chilling temperatures led to decreased pre-light period F_v/F_m and mid-light period PSII efficiency along with increased mid-light period values for $1 - q_p$ (Fig. 1; the results of one chilling exposure are reported; the entire experiment was repeated and yielded similar results). All three of these effects of decreasing leaf temperature were statistically significant for both wild-type and GR+ cotton (analyses of covariance, $P < 0.0001$). These responses to the chilling exposure are hallmarks of chilling stress on chilling-sensitive plant species such as cotton (Melis, 1999; Allen and Ort, 2001). Decreased pre-light period

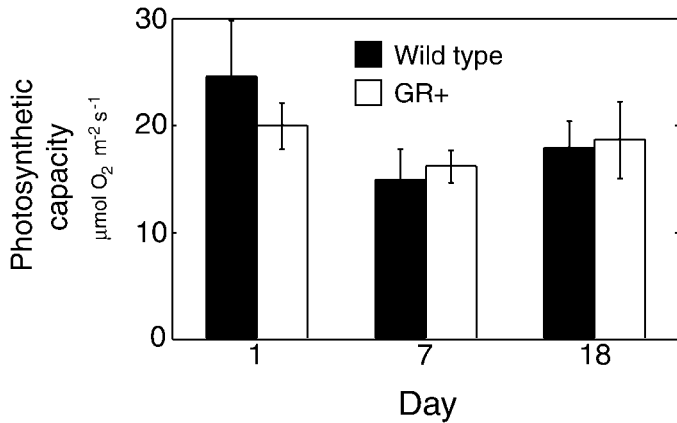


Fig. 2. Capacities for photosynthetic oxygen evolution of leaves of cotton that overproduces glutathione reductase (GR+; open bars) and wild-type cotton (closed bars) measured at three times during the chilling exposure. Data are means \pm SD; $N = 4-8$.

F_v/F_m is a classical manifestation of chilling-induced photo-inhibition. Inhibition of Calvin cycle activity and the resultant decrease in the demand for photochemically generated reductant is thought to underlie chilling-induced increases in $1 - q_p$, which is a nonlinear estimate of the reduction state of the primary quinone acceptor of PSII. An increase in the reduction state renders PSII more vulnerable to photoinactivation by increasing the probability of charge recombination in the reaction center that can lead to triplet chlorophyll and, ultimately, singlet oxygen formation (Melis, 1999).

For all three chlorophyll fluorescence parameters depicted in Fig. 1, there was no significant difference between the response of wild-type and GR+ cotton. The absence of differences in the performance of GR+ vs. wild-type cotton contrasts with previous studies of short-term (e.g., 3-h) exposure of leaf discs from warm-grown cotton to either 10° or 15°C at 500 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ wherein GR+ cotton maintained elevated rates of photochemistry and sustained 28% less PSII photoinhibition (Kornyejev et al., 2001, 2003).

In both wild-type and GR+ cotton, the capacity for photosynthetic oxygen evolution decreased during the first half of the chilling exposure (analysis of variance, $P < 0.0001$) and exhibited a small but statistically significant increase over the second half of the chilling exposure ($P = 0.04$; Fig. 2). However, the response of photosynthetic oxygen evolution did not differ between GR+ and wild-type cotton ($P = 0.24$).

Plants have the opportunity to acclimate to environmental stress when that stress is imposed gradually, such as in the present study. Plants have been shown to acclimate to a wide range of environmental stresses, including chilling temperatures, by increasing their activities for antioxidant enzymes and their contents of antioxidant metabolites such as GSH and ascorbate (Anderson et al., 1992; Logan et al., 1999). In chilling-tolerant evergreen species such as *Pinus strobus* (Anderson et al., 1992) and *Mahonia repens* (Logan et al., 1998b), acclimation to winter involves profound increases in the capacity to detoxify ROS. Acclimation to environmental stress can occur over a period of days and has been shown to exhibit faster kinetics in crop species (Logan et al., 1998a). In the present study, the GR activities of wild-type cotton doubled over the course of the chilling exposure (Fig. 3). The chilling exposure had no statistically significant effect on the GR ac-

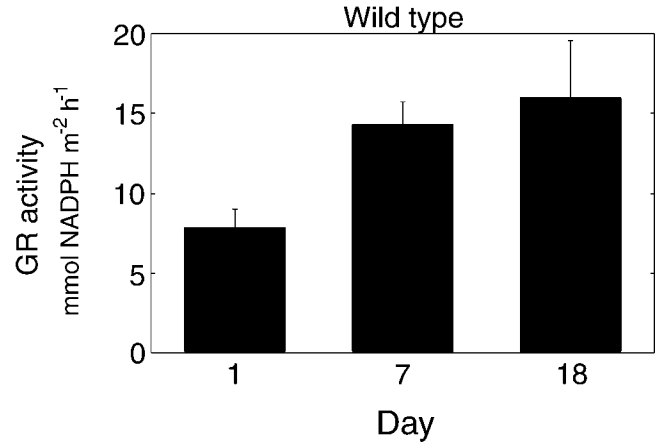


Fig. 3. Glutathione reductase activities of leaves of wild-type cotton measured at three times during the chilling exposure. Data are means \pm SD; $N = 4$.

tivities of GR+ plants (data not shown). While a large difference in GR activity between wild-type and GR+ genotypes remained even after acclimation to chilling, the twofold increase in wild-type GR activity may have been sufficient to meet the demand for enzymatic capacity to reduce oxidized glutathione under the growth conditions.

Our findings clearly indicate that the nature of the chilling exposure (i.e., short- vs. long-term) has profound effects on the response of GR+ genotypes relative to that of wild type. This should be taken into account when assessing the utility of overproducing antioxidant enzymes as a strategy for developing more stress-tolerant crop varieties for agricultural use. Furthermore, this study underscores the need to design experiments that examine the performance of transgenic genotypes under realistic conditions of chilling.

LITERATURE CITED

- ALLEN, D. J., AND D. R. ORT. 2001. Impacts of chilling temperatures on photosynthesis in warm-climate plants. *Trends in Plant Science* 6: 36-42.
- ANDERSON, J. V., B. I. CHEVONE, AND J. L. HESS. 1992. Seasonal variation in the antioxidant system of eastern white pine needles. *Plant Physiology* 98: 501-508.
- ASADA, K. 1999. The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annual Review of Plant Physiology and Plant Molecular Biology* 50: 601-639.
- BAKER, N. R. 1994. Chilling stress and photosynthesis. In C. Foyer and P. Mullineaux [eds.], *Causes of photooxidative stress and amelioration of defense systems in plants*, 127-154. CRC Press, Boca Raton, Florida, USA.
- FOYER, C. H., AND B. HALLIWELL. 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta* 133: 21-25.
- FOYER, C. H., N. SOURIAU, S. PERRET, M. LELANDAIS, K.-J. KUNERT, C. PRUVOST, AND L. JOUANIN. 1995. Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees. *Plant Physiology* 109: 1047-1057.
- GENTY, B., J. M. BRIANTAIS, AND N. R. BAKER. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* 990: 87-90.
- HAUSLADEN, A., AND R. G. ALSCHER. 1993. Glutathione. In R. Alscher and J. Hess [eds.], *Antioxidants in higher plants*, 31-58. CRC Press, Boca Raton, Florida, USA.
- HOSSAIN, H. A., AND K. ASADA. 1984. Purification of dehydroascorbate re-

- ductase from spinach and its characterisation as a thiol enzyme. *Plant and Cell Physiology* 25: 85–95.
- KORNYEYEV, D., B. A. LOGAN, P. PAYTON, R. D. ALLEN, AND A. S. HOLADAY. 2001. Enhanced photochemical light utilization and decreased chilling-induced photoinhibition of photosystem II in cotton overexpressing genes encoding chloroplast-targeted antioxidant enzymes. *Physiologia Plantarum* 113: 323–331.
- KORNYEYEV, D., B. A. LOGAN, P. PAYTON, R. D. ALLEN, AND A. S. HOLADAY. 2003. Elevated chloroplastic glutathione reductase activities decrease chilling-induced photoinhibition by increasing rates of photochemistry, but not thermal energy dissipation, in transgenic cotton. *Functional Plant Biology* 30: 101–110.
- LOGAN, B. A., B. DEMMIG-ADAMS, AND W. W. ADAMS III. 1999. Acclimation of photosynthesis to the environment. In G. Singhal, G. Renger, S. Sopory, K. Irrgang, and Govindjee [eds.], Concepts in photobiology: photosynthesis and photomorphogenesis, 477–512. Narosa Publishing House, New Dehli, India.
- LOGAN, B. A., B. DEMMIG-ADAMS, W. W. ADAMS III, AND S. C. GRACE. 1998a. Antioxidants and xanthophyll cycle-dependent energy dissipation in *Cucurbita pepo* L. and *Vinca major* L. acclimated to four growth PPFs in the field. *Journal of Experimental Botany* 49: 1869–1879.
- LOGAN, B. A., S. C. GRACE, W. W. ADAMS III, AND B. DEMMIG-ADAMS. 1998b. Seasonal differences in xanthophyll cycle characteristics and antioxidants in *Mahonia repens* growing in different light environments. *Oecologia* 116: 9–17.
- MELIS, A. 1999. Photosystem-II damage and repair cycle in chloroplasts: what modulates the rate of photodamage in vivo? *Trends in Plant Science* 4: 130–135.
- NIYOGI, K. K. 1999. Photoprotection revisited: genetic and molecular approaches. *Annual Review of Plant Physiology and Plant Molecular Biology* 50: 333–359.
- PAYTON, P., R. WEBB, D. KORNYEYEV, R. ALLEN, AND A. S. HOLADAY. 2001. Protecting cotton photosynthesis during moderate chilling at high light intensity by increasing chloroplastic anti-oxidant enzyme activity. *Journal of Experimental Botany* 52: 2345–2354.
- SCHREIBER, U., U. SCHLIWA, AND W. BILGER. 1986. Continuous recording of photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynthesis Research* 10: 51–62.
- VAN KOOTEN, O., AND J. F. H. SNEL. 1990. The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynthesis Research* 25: 147–150.
- WINKLER, B. S., S. M. ORSELLI, AND T. S. REX. 1994. The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. *Free Radical Biology and Medicine* 17: 333–349.
- WISE, R. R. 1995. Chilling-enhanced photooxidation: the production, action, and study of reactive oxygen species produced during chilling in the light. *Photosynthesis Research* 45: 79–97.