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# Effect of chloroplastic overproduction of ascorbate peroxidase on photosynthesis and photoprotection in cotton leaves subjected to low temperature photoinhibition

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## Abstract

The photosynthetic performance of leaf discs of transgenic cotton with fourfold elevated activity of ascorbate peroxidase in the chloroplast stroma (APX+ plants) was compared to that of wild type (*Gossypium hirsutum* L. cv. Coker 312) during exposure to 10 °C and 500 μmol photons m<sup>-2</sup> s<sup>-1</sup>. APX+ leaves did not exhibit as large of an increase in cellular H<sub>2</sub>O<sub>2</sub> that was evident in wildtype leaves shortly after the imposition of the chilling treatment. In addition, APX+ leaves exhibited slightly, but significantly, less photosystem (PS)I and PSII photoinhibition. However, the greatest genotypic difference in H<sub>2</sub>O<sub>2</sub> scavenging did not coincide with the greatest difference in PSI inactivation, and APX+ leaves exhibited a greater quantum yield for PSII. Therefore, genotypic differences in H<sub>2</sub>O<sub>2</sub> scavenging may not have been the sole mechanism enhancing PSI protection in APX+ leaves. No evidence of competition for reduced ascorbate between ascorbate peroxidase and violaxanthin de-epoxidase was found. The dynamics of xanthophyll cycle carotenoid conversions, as well as the level of thermal dissipation, were similar for wildtype and transgenic plants throughout the chilling treatment and subsequent recovery.

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**Keywords:** Antioxidants; Ascorbate peroxidase; Chilling-induced photoinhibition; *Gossypium hirsutum*; Photoprotection; Photosynthesis

## 1. Introduction

Ascorbate peroxidase (APX, EC 1.11.1.11) scavenges H<sub>2</sub>O<sub>2</sub> and is a component of the enzymatic antioxidant defence system in chloroplasts [1]. In this system, referred to as the water–water cycle, the combined action of superoxide dismutase (EC 1.15.1.1) and APX detoxifies reactive oxygen species (ROS) resulting from the photoreduction of O<sub>2</sub> [1,2]. Documented upregulation in APX activity under adverse environmental conditions suggests that it plays an important role in plant stress tolerance [3–6].

Low temperatures combined with moderate to high photon flux density (PFD) are highly conducive to O<sub>2</sub>

photoreduction, particularly in chilling-sensitive plants [7]. H<sub>2</sub>O<sub>2</sub> formed from photogenerated superoxide may disrupt photosynthesis by deactivating certain Calvin–Benson cycle enzymes, such as the bisphosphatases [8], or via molecular damage brought about by Fenton-type hydroxyl radical generation [4,9]. Indeed, a negative correlation between antioxidant enzyme activity (including APX) and the extent of chilling-induced photoinhibition has been reported [10–13].

Photosystem (PS)I reaction centers may be the primary targets of damage mediated by photogenerated ROS. Reduced Fe–S centers on the acceptor side of P700 of PSI would be an appropriate environment for hydroxyl radical formation from H<sub>2</sub>O<sub>2</sub>, especially considering that O<sub>2</sub> reduction occurs in the vicinity [14–17]. Evidence suggests that ROS are the cause of PSI inactivation in chilling-sensitive plants during chilling in the light [14,15,17], and that H<sub>2</sub>O<sub>2</sub> accumulation is a major factor leading to the decline in PSI activity [16].

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Cotton is considered to be a chilling-sensitive species. Its photosynthetic performance and ability to rapidly recover photosynthetic activity following chilling in the light are considerably diminished with time of exposure [13,18,19]. We developed transgenic cotton plants with elevated chloroplastic APX activity (APX+ plants) to test the hypothesis that increasing chloroplastic APX activity improves the protection of photosynthesis during chilling in the light [13]. APX+ plants exhibit a substantial improvement in the initial recovery of CO<sub>2</sub>-saturated photosynthesis [13] and a lower rate constant for PSII photoinactivation (photoinhibition) following the exposure of leaf discs to 10 °C and moderate PFD for 3 h [12]. Previous studies did not examine the effect of APX overproduction on PSI photoinhibition.

Xanthophyll cycle-dependent thermal dissipation of excess absorbed light energy is an important component of photoprotection [20–22]. This process is particularly important for protecting PSII in cotton, especially during chilling [23]. In response to excess light absorption, zeaxanthin and antheraxanthin, the de-epoxidized constituents of the xanthophyll cycle are formed by violaxanthin de-epoxidase (VDE) activity, which requires ascorbate [24]. Ascorbate-deficient *Arabidopsis* mutants exhibit less thermal dissipation and de-epoxidation of violaxanthin than wildtype under excessive PFD [25]. Therefore, the possibility exists that competition for ascorbate between APX and VDE could occur in the chloroplasts of APX+ plants during chilling in the light, leading to the inhibition of xanthophyll cycle de-epoxidation and thermal energy dissipation. In our preliminary study, steady-state levels of thermal dissipation in PSII complexes estimated using chlorophyll *a* fluorescence analysis were the same for APX+ and wildtype [12]. However the direct effect of the overproduction of APX on the xanthophyll cycle was not investigated.

The present study addressed the following questions with respect to the effect of elevated, chloroplastic APX activity during chilling in the light: (1) Are H<sub>2</sub>O<sub>2</sub> levels diminished? (2) Is there improved protection of PSI against photoinhibition? (3) Are the induction and steady-state levels of thermal energy dissipation and violaxanthin de-epoxidation inhibited?

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Cotton, *Gossypium hirsutum* L. cv. Coker 312, was transformed to overproduce chloroplast-targeted ascorbate peroxidase (APX+) as described previously by Payton et al. [13]. Wildtype plants (Coker 312) and plants from three independently transformed lines of the APX+ genotype were grown from seed in 8-l pots in a

greenhouse at ~30/26 °C (day/night) with a natural photoperiod. Plants were fertilized with Hoagland's solution twice a week. The first fully expanded leaf of 5–8-week-old plants was used for enzyme assays and fluorescence measurements. To check the identity of APX+ plants, extracts of fully expanded cotyledons were assayed spectrophotometrically for APX activity as described below.

### 2.2. APX activity

Leaf discs harvested to assess APX activity were rapidly removed using a cork borer and immediately frozen in liquid N<sub>2</sub>. Frozen leaf tissue was ground to a powder at liquid nitrogen temperature using a mortar and pestle and then rapidly homogenized in 1 ml of ice-cold extraction solution in a glass tissue grinder. Aliquots were taken before centrifugation for total chlorophyll determination in 80% acetone according to Lichtenthaler [26].

The extraction and assay for APX activity were performed as described by Sen Gupta et al. [27]. APX activity was measured spectrophotometrically by monitoring ascorbate oxidation at 290 nm. The assay temperature was 25 °C, and the assays were initiated within 1.5 min after commencing the extraction with the addition of 25 µl of centrifuged extract in a total volume of 1 ml.

### 2.3. Chilling treatment

Leaf discs (10 cm<sup>2</sup>) 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, UK) prior to illumination at a PFD of 500 µmol m<sup>-2</sup> s<sup>-1</sup>. Humidified air containing 5.28% CO<sub>2</sub> (v/v) was passed through the chamber during the treatment.

For attached leaves used to measure H<sub>2</sub>O<sub>2</sub> levels, plants were dark acclimated at room temperature for 1.5 h and then placed in a pre-cooled (9–10 °C) climate chamber for an additional 20 min in the dark. The light treatment used the same lamp and PFD to which leaf discs were exposed. For attached leaves used for  $\Phi$ PSII determinations, plants 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<sup>-2</sup> s<sup>-1</sup>. The leaf temperature of 10 °C was maintained by means of a PLC temperature controller (ADC Ltd, Hoddesdon, UK).

### 2.4. H<sub>2</sub>O<sub>2</sub> content

After various times of exposure to the chilling treatment, the leaf discs (10 cm<sup>2</sup>) or attached leaves were rapidly frozen in liquid N<sub>2</sub>. The frozen tissue was

ground to a powder at liquid N<sub>2</sub> temperature, and the H<sub>2</sub>O<sub>2</sub> was extracted with ice-cold 0.2 N HClO<sub>4</sub>. After neutralization with KOH and centrifugation, the extract was assayed for H<sub>2</sub>O<sub>2</sub> following the modified method of Okuda et al. [28]. The concentration of 3-methyl-2-benzothiazoline hydrazone in the reaction mixture was 750 μM.

### 2.5. Ascorbate and glutathione content

Oxidized and reduced forms of ascorbate and glutathione were extracted from leaf tissue previously ground in liquid nitrogen in 2 ml of ice-cold 2% metaphosphoric acid with 2 mM EDTA. The extract was centrifuged at 10 000 × *g* for 10 min at 4 °C and neutralized with sodium citrate as previously described [29]. The amounts of reduced and total ascorbate were determined by monitoring the decline in absorbance at 265 nm upon the addition of ascorbate oxidase from *Cucurbita* (4 units) to leaf extracts either before or after (respectively) reduction using dithiothreitol. Total and oxidized glutathione were assayed spectrophotometrically before and after (respectively) the addition of 2-vinylpyridine. Both assays were conducted according to the modified method of Rao and Ormrod [29] as described in Kornyejev et al. [30].

### 2.6. Pigment analysis

Samples (0.25 cm<sup>2</sup>) were removed from the leaf disc in the oxygen electrode chamber before, during, and after the chilling treatment. The tissue was immediately frozen in liquid nitrogen where it remained stored until processing. Pigment composition was analyzed by high performance liquid chromatography as described in Adams and Demmig-Adams [31] using the column and gradient system of Gilmore and Yamamoto [32].

### 2.7. Chlorophyll fluorescence

Chlorophyll *a* fluorescence emission was measured from leaf discs 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 chilling treatment.

The experimental protocol described by Schreiber et al. [33] and nomenclature of van Kooten and Snel [34] were used. The quantum efficiency for electron transport by PSII was calculated as  $\Phi_{\text{PSII}} = (F_{\text{m}}' - F) / F_{\text{m}}'$  [35], where *F* and *F*<sub>m</sub>' are the 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 =  $F_{\text{m}} / F_{\text{m}}' - 1$ ) and the excitation capture

efficiency of PSII using the ratio  $F_{\text{v}}' / F_{\text{m}}'$ , where  $F_{\text{v}}' = F_{\text{m}}' - F_{\text{o}}'$  is variable chlorophyll fluorescence for light-acclimated leaves. According to Harbinson et al. [36], the ratio of variable to maximal PSII fluorescence estimates the efficiency of excitation energy transfer to PSII reaction centers. Measurements of *F*<sub>o</sub>', 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 KL 1500 light source (Schott, Wiesbaden, Germany). Leaf discs of both genotypes exhibited average values of  $F_{\text{v}}' / F_{\text{m}}'$  of 0.78 ± 0.02 (mean ± S.D.) prior to the treatment in the laboratory. Post-stress values of  $F_{\text{v}}' / F_{\text{m}}'$  were determined for leaf discs following 3 h of dark acclimation at room temperature.

### 2.8. P700 activity

The relative amounts of photooxidizable P700 were measured from leaf discs by means of a PAM 101/103 modulated fluorometer (Heinz Walz GmbH) equipped with an ED-P700DW emitter-detector unit. P700<sup>+</sup> formation was induced by illumination with saturating far-red light and monitored as differential absorbance changes (810 minus 860 nm) selective for absorbance changes caused by P700 [37]. The measurements were conducted on the leaf discs previously exposed to the chilling treatment for different time periods and a subsequent 3 h period of dark acclimation at room temperature.

### 2.9. Data analysis

Data for the APX+ genotype were compared with data for wildtype using a Student's *t*-test. Means were considered significantly different for  $P \leq 0.05$ .

## 3. Results

### 3.1. APX activity

Our previously published data [13] indicated that the cotton plants overexpressing the pea transgene for APX exhibited a five to sevenfold increase in total leaf APX activity over that for wildtype, with an eightfold increase in the APX activity associated with the chloroplast [13]. Plants used in the present study possessed a similar enhancement in APX activity in whole-leaf extracts of APX+ plants (619 ± 152 versus 3047 ± 721 μmol (mg Chl)<sup>-1</sup> h<sup>-1</sup> for wildtype and APX+ plants, respectively, mean ± S.D., *n* = 23–28). When APX activity was calculated separately for each of the three independently transformed lines, no significant differences were found between the lines (3121 ± 603, 3040 ± 750 and 2986 ± 889 μmol (mg Chl)<sup>-1</sup> h<sup>-1</sup> for lines, mean ± S.D., *n* = 6–11).

In fact, for all measurements described below, statistically significant differences between independently transformed lines of APX+ cotton were never observed (data not shown). Therefore, data from all lines were combined.

### 3.2. Effect of enhanced chloroplastic APX activity on the leaf $H_2O_2$ levels and the reduction states of ascorbate and glutathione during chilling treatments

For wildtype leaf discs, the total leaf  $H_2O_2$  level increased quickly upon illumination at  $10^\circ\text{C}$ , reaching 130% of the pre-illumination value in 3 min (Fig. 1A). At 180 min,  $H_2O_2$  content was similar to the level obtained just before the light treatment. However, the  $H_2O_2$  level in APX+ leaf discs remained close to the pre-illumination value at 3, 20 and 180 min of illumination.

Since  $H_2O_2$  can accumulate in cotton leaves because of wounding [38], we performed additional experiments

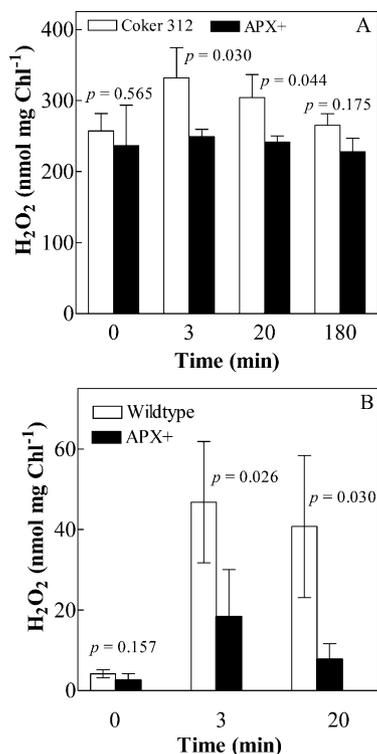


Fig. 1. The changes in the content of  $H_2O_2$  in leaf discs (A) and attached leaves (B) of wildtype cotton and transgenic cotton plants overproducing chloroplastic ascorbate peroxidase (APX+) during illumination at low temperature ( $10^\circ\text{C}$ , PFD of  $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Leaf discs were exposed to 5.28% (v/v)  $\text{CO}_2$ , while attached leaves were exposed to ambient  $\text{CO}_2$  in a climate chamber. Prior to illumination, leaf discs were acclimated to darkness at room temperature for 1.5 h and at  $10^\circ\text{C}$  for an additional 20 min. For attached leaves, plants were dark acclimated at room temperature for 1.5 h and then placed in a climate chamber at  $9\text{--}10^\circ\text{C}$  for an additional 20 min in the dark prior to illumination as used for leaf discs. Error bars represent S.D.,  $n = 4\text{--}5$ . Values above the bars are the  $P$  values from a Student's  $t$ -test comparing means at each time point for transgenic and wildtype.

to estimate  $H_2O_2$  accumulation in leaf discs during the dark-acclimation period. Indeed,  $H_2O_2$  content increased dramatically during 1.5-h of dark acclimation at room temperature plus 20 min at  $10^\circ\text{C}$  (from  $1.9 \pm 0.7$  nmol/mg Chl to  $257.3 \pm 24.5$  nmol/mg Chl). However, no significant increase in  $H_2O_2$  levels occurred with an additional 3 min or 20 min in the dark at  $10^\circ\text{C}$ . Therefore, one may suggest that the increase in  $H_2O_2$  content at 3 and 20 min of illumination at  $10^\circ\text{C}$  was the result of illumination and not wounding of the leaves. Note that the 0-min point for Fig. 1 represents the start of illumination at  $10^\circ\text{C}$  after the 1.5 h at room temperature and 20 min at  $10^\circ\text{C}$  in darkness.

In order to completely exclude the influence of wounding on  $H_2O_2$  content, we conducted similar analyses using attached leaves (Fig. 1B). As for leaf discs, attached leaves of APX+ plants maintained significantly lower levels of  $H_2O_2$  at 3 and 20 min of illumination at  $10^\circ\text{C}$  than leaves of wildtype plants.

Elevated chloroplastic APX activity in the cotton leaves had no effect on the total leaf content of ascorbate and glutathione, nor did it affect the reduction states of these antioxidants during the chilling treatment. At the end of the 3-h of illumination at  $10^\circ\text{C}$ ,  $85.7 \pm 2.5\%$  and  $82.5 \pm 2.1\%$  ( $n = 4$ ,  $P = 0.100$ ) of the ascorbate pool was reduced in the leaves of wildtype and APX+ plants, respectively. At the same time,  $88.6 \pm 3.5\%$  and  $88.3 \pm 3.4\%$  ( $n = 4$ ,  $P = 0.232$ ) of the glutathione pool was reduced in the leaves of wildtype and APX+ plants, respectively.

### 3.3. Photoinactivation of PSI and PSII and photosynthetic performance during the chilling treatment

As is typical for leaves of chilling-sensitive plants, cotton leaf discs exhibited a significant inactivation of PSI during the chilling treatment (quantified after 3 h of dark acclimation as the relative amount of photooxidizable P700; Fig. 2A). For wildtype, only 48% of the initial PSI activity was detectable after 6 h of light exposure. However, at 2, 3 and 4 h of chilling, APX+ leaf discs sustained a significantly lower loss of PSI activity than wildtype. Simultaneous measurements of PSII activity (quantified as  $F_v/F_m$ ) indicated that under our experimental conditions, the extent of PSII inactivation was discernibly lower than that of PSI (73% of initial PSII activity was observed for wildtype after 6 h of exposure, see Fig. 2B). Slight, though statistically significant, differences between APX+ and wildtype plants in the level of PSII photoinhibition were observed at all time points except 20 min after the start of illumination.

As we observed for leaves treated with lincomycin to inhibit chloroplast repair processes [12], during the 3-h chilling treatment, leaf discs of APX+ plants maintained a higher efficiency of electron transport through PSII, as indicated by the chlorophyll  $a$  fluorescence

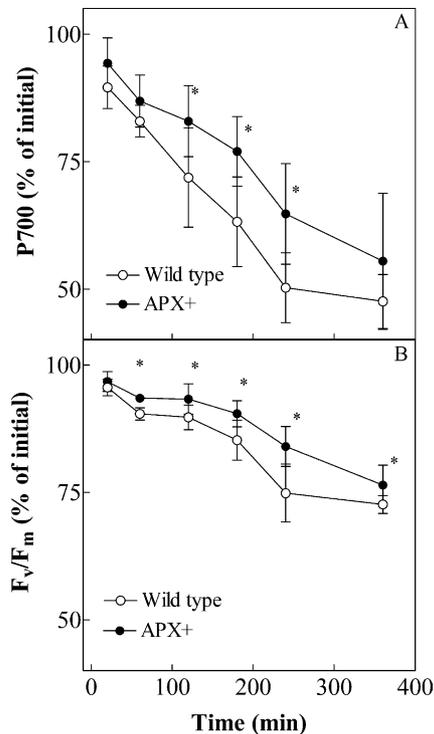


Fig. 2. Time-courses for sustained photoinactivation of PSI (A; quantified as the percent decrease in the level of photooxidizable P700 relative to pretreatment values) and PSII (B; quantified as the percent decrease in variable to maximum chlorophyll fluorescence,  $F_v/F_m$ , relative to pretreatment values) with exposure to 10 °C, a PFD of 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and 5.28% (v/v)  $\text{CO}_2$  for leaf discs from wildtype cotton and transgenic cotton overproducing chloroplastic ascorbate peroxidase (APX+). Error bars represent SD,  $n=4-21$ . Means marked with an asterisk were significantly different ( $P \leq 0.05$ ) from the corresponding means for wildtype plants using a Student's *t*-test. 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 photooxidizable P700 and  $F_v/F_m$  were conducted. Average values of  $F_v/F_m$  of  $0.78 \pm 0.02$  were observed for both genotypes prior to the light treatment.

parameter,  $\Phi\text{PSII}$  (Fig. 3A). The most pronounced differences between APX+ and wildtype cotton were observed during the induction period. Similar results were obtained for attached leaves (Fig. 3B).

#### 3.4. Non-photochemical energy dissipation in PSII complexes during exposure to chilling at moderate PFD

The level of non-photochemical energy dissipation in PSII complexes was estimated using the coefficient of non-photochemical chlorophyll *a* fluorescence quenching (NPQ) and the efficiency of excitation transfer to open PSII reaction centers ( $F_v/F_m$ ). No significant differences were observed between genotypes in the time-courses for these parameters during the 3-h exposure to 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 10 °C (Fig. 4A and B).

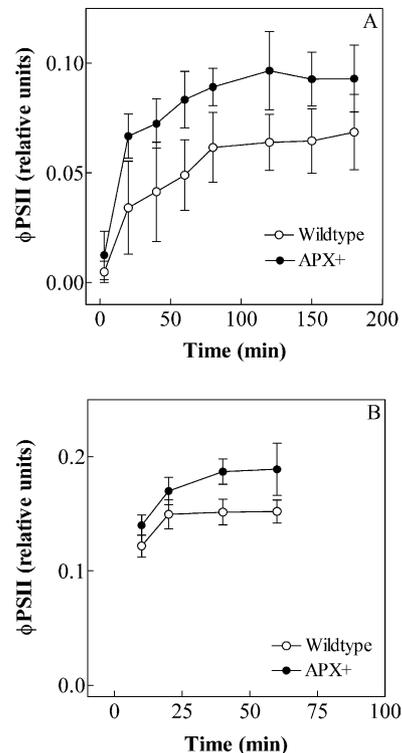


Fig. 3. Time-course for the quantum yield of PSII photochemistry ( $\Phi\text{PSII}$ ) during exposure to 10 °C at a PFD of 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for leaf discs (A) and attached leaves (B) of wildtype cotton and transgenic cotton overproducing chloroplastic ascorbate peroxidase (APX+). Leaf discs were exposed to 5.28% (v/v)  $\text{CO}_2$  (A), while attached leaves were exposed to ambient  $\text{CO}_2$  that was determined to be saturating for photosynthesis at 10 °C. 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. Attached leaves were kept in the dark for 1.5 h at 30 °C followed by an additional 0.5 h at 10 °C in the dark prior to illumination.

The main factor contributing to non-photochemical fluorescence quenching in PSII complexes under conditions such as those experienced by leaf discs in our experiments has been suggested to be thermal energy dissipation that involves the xanthophyll cycle, since it is believed that the contribution due to state 1–state 2 transitions is significant only at low light intensity [39,40]. However, the dark relaxation kinetics of NPQ for the cotton leaves indicated that the share of medium and slow components associated with state 1–state 2 transitions and PSII photodamage, respectively, could be significant, leaving the possibility that genotypic differences in xanthophyll cycle pigments occurred during chilling.

Overproduction of chloroplastic APX did not result in a statistically significant change in the pigment composition of non-stressed APX+ leaves in comparison to wildtype leaves (Table 1). Over the course of the 3-h chilling treatment, leaf discs of both genotypes exhibited a similar increase in the extent of ascorbate-

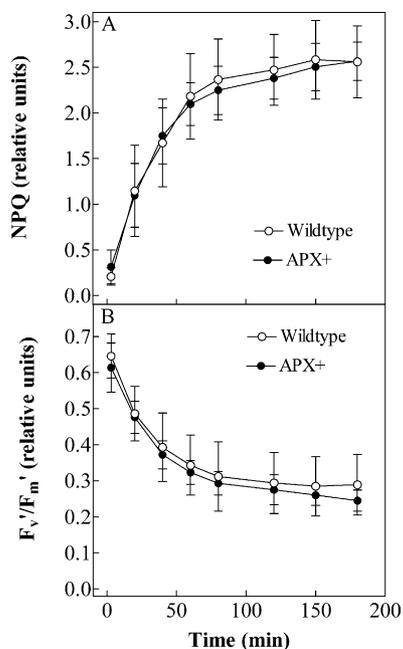


Fig. 4. Time-course for non-photochemical chlorophyll fluorescence quenching (NPQ) (A) and the maximal quantum yield of photochemistry for PSII complexes with 'open' reaction centers ( $F_v/F_m'$ ) (B) during exposure of leaf discs to 10 °C at a PFD of 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 5.28% (v/v)  $\text{CO}_2$  for wildtype cotton and transgenic cotton overproducing ascorbate peroxidase (APX+). 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.

Table 1

Pigment composition of cotton leaf tissue from wildtype cotton (cv. Coker 312) and transgenic cotton overproducing ascorbate peroxidase (APX+)

Pigment(s)	Genotype	
	Wildtype ( $\mu\text{mol m}^{-2}$ )	APX+ ( $\mu\text{mol m}^{-2}$ )
Chl <i>a</i>	464.6 ± 99.8	449.6 ± 94.9 <sup>0.81</sup>
Chl <i>b</i>	130.0 ± 26.2	116.7 ± 25.9 <sup>0.44</sup>
$\beta$ -Carotene	50.4 ± 14.9	46.9 ± 11.7 <sup>0.69</sup>
Xanthophyll cycle pigments	48.2 ± 13.8	51.7 ± 4.8 <sup>0.61</sup>
Lutein	64.2 ± 12.0	62.0 ± 9.8 <sup>0.76</sup>
Neoxanthin	24.3 ± 3.8	22.8 ± 4.5 <sup>0.59</sup>

Superscripts represent *P* values from *t*-tests comparing APX+ and wildtype plants. Mean ± S.D.,  $n = 5$ .

dependent violaxanthin (V) de-epoxidation, measured as  $(A+Z)/(V+A+Z)$  (Fig. 5). Even after 3 h of recovery in the dark at room temperature after the chilling exposure, approximately 20% of the xanthophyll cycle pool was retained as antheraxanthin (A) and zeaxanthin (Z). The dynamics of the xanthophyll cycle did not differ between leaf discs of APX+ and wildtype plants at any

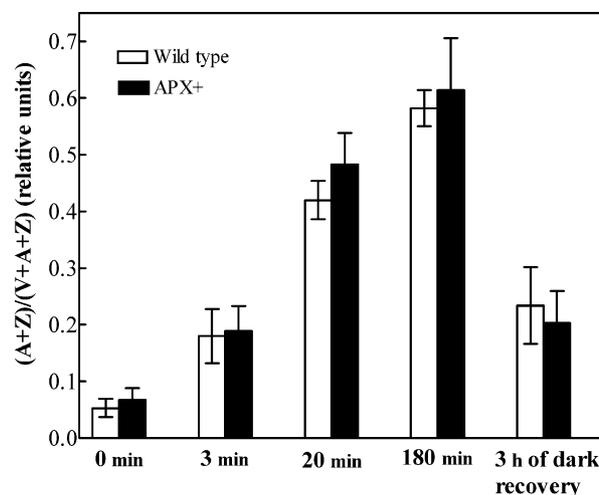


Fig. 5. Time-courses for the xanthophyll cycle de-epoxidation state,  $(A+Z)/(V+A+Z)$ , for leaf discs during exposure to 10 °C at a PFD of 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 5.28% (v/v)  $\text{CO}_2$  and subsequent recovery in darkness at room temperature for wildtype cotton and transgenic cotton overproducing ascorbate peroxidase (APX+). Error bars represent S.D.,  $n = 5$ . Transgenic means were not significantly different ( $P > 0.05$ ) from wildtype 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.

point during the chilling treatment and recovery, consistent with the NPQ data.

#### 4. Discussion

Our previous analyses indicated that wildtype and nonexpressing segregate transgenic plants were statistically indistinguishable in terms of antioxidant enzyme activities and chilling tolerance [13]. In the present study, no significant differences were observed between independently transformed APX+ lines. These observations strongly suggested that the differences in performance between wildtype and APX+ plants were due to the presence of the transgene and its specific effect on APX activity.

Illumination at low temperature causes a rapid, temporary increase in  $\text{H}_2\text{O}_2$  content [28] (Fig. 1). Overproduction of APX in the chloroplasts (presumably in the stroma) of cotton leaves resulted in enhanced scavenging of  $\text{H}_2\text{O}_2$  during illumination at 10 °C, especially during the induction phase of photosynthesis. Both leaf discs and attached leaves of the APX+ plants were able to dampen the rise in leaf  $\text{H}_2\text{O}_2$  that occurred once illumination commenced at 10 °C but before photochemistry ( $\Phi\text{PSII}$ ) reached a steady state. These genotypic differences were most striking for attached leaves. The high, illumination-independent  $\text{H}_2\text{O}_2$  content of leaf discs may have partially masked the  $\text{H}_2\text{O}_2$  increases dependent on illumination. This large illumination-independent  $\text{H}_2\text{O}_2$  content was likely a reaction

to wounding when the leaf discs were removed from the leaf [38]. It should be noted that wounding-induced accumulation of  $H_2O_2$  is thought to occur in cell walls (not in internal cell organelles like chloroplasts) [41]. Since our measurements were of the total leaf  $H_2O_2$  content, it is possible that greater differences in  $H_2O_2$  between wildtype and transgenic plant leaf discs occurred at the chloroplast level. Whatever the localization of the illumination-independent  $H_2O_2$ , it had little effect on the genotypic differences in photochemistry ( $\Phi$ PSII), since differences in  $\Phi$ PSII were similar for attached leaves and leaf discs.

Our data on the inactivation of PSI in cotton leaves support previous reports that PSI is more sensitive to chilling-induced photoinactivation than PSII in chilling-sensitive species [16,42]. PSI is vulnerable to damage by hydroxyl radicals generated via a Fenton-type reaction between photogenerated  $H_2O_2$  and Fe–S centers in PSI. Therefore,  $H_2O_2$  is considered critical to PSI photo-damage during chilling stress [16]. One may suggest that improved  $H_2O_2$  scavenging can positively affect PSI photoprotection. Indeed, the transgenic cotton plants with increased chloroplastic APX activity did have a lower  $H_2O_2$  content and exhibited less PSI photoinhibition during the chilling treatment. However, the fact that no genotypic differences in PSI inactivation occurred during the induction period when genotypic differences in  $H_2O_2$  were greatest suggests that other factors were likely to have been involved in enhancing protection of PSI, as well. For example, it is possible that a greater demand for reducing power to regenerate ascorbate occurred during chilling in APX+ leaves than in wildtype leaves. If this situation were the case, acceptor-side, non-enzymatic reduction of monodehydroascorbate to ascorbate or enzymatic ascorbate regeneration dependent on NADPH may have competed with  $O_2$  for reducing power and reduced the rate of  $O_2$  photoreduction.

This hypothesis is consistent with the finding that the APX+ leaves exhibited greater photochemistry ( $\Phi$ PSII) than wildtype leaves during chilling. The enhanced electron transport may have also been a factor contributing to improved protection of PSII against photoinhibition by increasing electron flux and thereby decreasing the reduction state of  $Q_A$ , the primary quinone acceptor in PSII. PSII is more vulnerable to photoinhibitory damage when photon energy is trapped by reaction centers with  $Q_A$  in the reduced state, due to charge recombination leading to triplet-P680 formation [43]. We previously observed a similar effect of APX overproduction on PSII photoinhibition and linear electron flow for lincomycin-treated leaves [12].

By 360 min of chilling in the light, the amounts of photooxidizable P700 were not significantly different for both genotypes (Fig. 2A). The slowing of PSI inactivation for wildtype leaves may have been the result of a

change in electron transport rate through PSII. It has been suggested that a decline in PSII activity might result in additional PSI photoprotection [15]. The development of strong NPQ and the increased accumulation of damaged PSII centers would likely have slowed electron flow from PSII with time at 10 °C, reducing the rate of inactivation of PSI.

We expected that elevated APX activity would lead to a decrease in the reduction state of the ascorbate pool, particularly during exposure to chilling temperatures, as the demand for  $H_2O_2$  scavenging, and hence the consumption of reduced ascorbate, increased. Interestingly, the reduction state of the ascorbate pool for APX+ plants was only slightly lower and did not differ significantly from that for wildtype plants during the chilling exposure. Apparently, through various enzymatic and non-enzymatic mechanisms, APX+ plants possess adequate ascorbate regeneration capacity to meet the demand of elevated APX activity in the chloroplast at 10 °C. Regeneration of ascorbate from dehydroascorbate, either enzymatically or non-enzymatically, utilizes GSH as a reductant [44,45]. Yet, the reduction state of the glutathione pool was also unaffected by transgenic APX over-production.

Thermal energy dissipation is considered a critical photoprotective mechanism [for a review see 9 and 46]. Thermal energy dissipation safely converts excess absorbed light energy into heat and is modulated by interconversions among the pigments of the xanthophyll cycle and also the strength of the trans-thylakoid membrane pH gradient. Thermal energy dissipation and the water–water cycle (of which APX is a constituent) are linked by complex and poorly understood interactions. For example, electron flow into the water–water cycle can augment the trans-thylakoid membrane proton gradient, which can, in turn, enhance levels of thermal energy dissipation. However, APX activity (particularly in APX+ plants) may effectively out-compete VDE for ascorbate and thereby limit the formation of zeaxanthin (and antheraxanthin), thus inhibiting thermal energy dissipation. In fact, we found no evidence that thermal energy dissipation or xanthophyll cycle interconversions were influenced by transgenic APX over-production. The finding was less surprising in light of the fact that APX+ plants were able to maintain ascorbate reduction states similar to those of wildtype plants. Since VDE is associated with the thylakoid membrane [46], while the overproduced APX was targeted to the stroma, this difference in localization could be a reason why competition between the enzymes for reduced ascorbate was not observed. In addition, the activity of both enzymes would be slow at low temperature, thereby lowering the demand for reduced ascorbate. It remains possible that VDE inhibition by APX over-production would be manifested at warmer temperatures.

It is possible that the overproduction of thylakoid-bound APX could lead to more pronounced effects on the xanthophyll cycle as well as on electron transport and photodamage than did the stromal-targeted enzyme used in this study [47]. A direct comparison of the effect of stromal and thylakoid membrane-bound APX overproduction will require the creation of the transgenic plants with similar levels of isoenzyme overexpression.

## 5. Conclusions

Overproduction of APX in the chloroplast stroma of cotton leaves resulted in enhanced scavenging of H<sub>2</sub>O<sub>2</sub> and a small enhancement in the resistance to photo-inhibition of PSI and PSII during exposure to chilling temperatures at a moderate PFD. Factors aside from enhanced H<sub>2</sub>O<sub>2</sub> scavenging appear to contribute to improved PSI protection, as well. An increase in APX associated with the thylakoid membrane might potentially have more significant influence on PSI sensitivity to low-temperature photoinhibition.

Overproduction of APX resulted in no inhibition of xanthophyll cycle de-epoxidation or thermal energy dissipation. Since thermal dissipation was substantial at 10 °C in cotton leaves, this finding suggests that the mechanisms that maintain the pool of reduced ascorbate are able to compensate for some putative increase in ascorbate utilization at low temperature.

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## References

- [1] K. Asada, The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50 (1999) 601–639.
- [2] B.A. Logan, B. Demmig Adams, W.W. Adams, III, Acclimation of photosynthesis to the environment, in: G.S. Singhal, G. Renger, S.K. Sopory, K.-D. Irrgang, Govindjee (Eds.), *Concepts in Photobiology: Photosynthesis and Photomorphogenesis*, Narosa Publishing House, 1999, pp. 477–512.
- [3] G. Ievinsh, A. Valcina, D. Ozola, Induction of ascorbate peroxidase activity in stressed pine (*Pinus sylvestris* L.) needles: a putative role for ethylene, *Plant Sci.* 112 (1995) 167–173.
- [4] R.G. Alscher, J.L. Donahue, C.L. Cramer, Reactive oxygen species and antioxidants: relationships in green cells, *Physiol. Plant.* 100 (1997) 224–233.
- [5] B.A. Logan, B. Demmig-Adams, W.W. Adams, III, S.C. Grace, Antioxidants and xanthophyll cycle-dependent energy dissipation in *Cucurbita pepo* L. and *Vinca major* L. acclimated to four growth PFDs in the field, *J. Exp. Bot.* 49 (1998) 1869–1879.
- [6] D.H. Lee, C.B. Lee, Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays, *Plant Sci.* 159 (2000) 75–85.
- [7] R.R. Wise, A.W. Naylor, Chilling-enhanced photooxidation. The peroxidative destruction of lipids during chilling injury to photosynthesis and ultrastructure, *Plant Physiol.* 83 (1987) 272–277.
- [8] S.A. Charles, B. Halliwell, Light activation of fructose biphosphatase in isolated spinach chloroplasts and deactivation by hydrogen peroxide, *Planta* 151 (1981) 242–246.
- [9] K.K. Niyogi, Photoprotection revisited: genetic and molecular approaches, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50 (1999) 333–359.
- [10] L.S. Janke, M.R. Hull, S.P. Long, Chilling stress and oxygen metabolizing enzymes in *Zea mays* and *Zea diploperennis*, *Plant Cell Environ.* 14 (1991) 97–104.
- [11] D. Hodges, C. Andrews, D. Johnson, R. Hamilton, Antioxidant responses to chilling stress in differentially sensitive inbred maize lines, *J. Exp. Bot.* 48 (1997) 1105–1113.
- [12] D. Kornyejev, B.A. Logan, P. Payton, R.D. Allen, A.S. Holaday, Enhanced photochemical light utilization and decreased chilling-induced photoinhibition of photosystem II in cotton overexpressing genes encoding chloroplast-targeted antioxidant enzymes, *Physiol. Plant.* 113 (2001) 323–331.
- [13] P. Payton, R. Webb, D. Kornyejev, R. Allen, A.S. Holaday, Protecting cotton photosynthesis during moderate chilling at high light intensity by increasing chloroplastic antioxidant enzyme activity, *J. Exp. Bot.* 52 (2001) 2345–2354.
- [14] B. Jakob, U. Heber, Photoproduction and detoxification of hydroxyl radicals in chloroplasts and leaves and relation to photoinactivation of photosystems I and II, *Plant Cell Physiol.* 37 (1996) 629–635.
- [15] K. Sonoike, Photoinhibition of photosystem I: its physiological significance in the chilling sensitivity of plants, *Plant Cell Physiol.* 37 (1996) 239–247.
- [16] I. Terashima, K. Noguchi, T. Itoh-Nemoto, Y.-M. Park, A. Kubo, K. Tanaka, The cause of PSI photoinhibition at low temperatures in leaves of *Cucumis sativus*, a chilling sensitive plant, *Physiol. Plant.* 103 (1998) 295–303.
- [17] S.E. Tjus, H.V. Scheller, B. Andersson, B.L. Moller, Active oxygen produced during selective excitation of photosystem I is damaging not only to photosystem I but also to photosystem II, *Plant Physiol.* 125 (2001) 2007–2015.
- [18] M. Königer, K. Winter, Reduction of photosynthesis in sun leaves of *Gossypium hirsutum* L. under conditions of high light intensities and suboptimal leaf temperatures, *Agronomie* 13 (1993) 659–669.
- [19] P. Payton, R.D. Allen, N. Trolinder, A.S. Holaday, Overexpression of chloroplast-targeted Mn superoxide dismutase in cotton (*Gossypium hirsutum* L., cv. Coker 312) does not alter the reduction of photosynthesis after short exposures to low temperature and high light intensity, *Photosynth. Res.* 52 (1997) 233–244.
- [20] B. Demmig-Adams, W.W. Adams, III, Photoprotection and other responses of plants to high light stress, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43 (1992) 599–626.
- [21] B. Demmig-Adams, W.W. Adams, III, D.H. Baker, B.A. Logan, D.R. Bowling, A.S. Verhoeven, Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of excess excitation, *Physiol. Plant.* 98 (1996) 253–264.
- [22] D.R. Ort, When there is too much light, *Plant Physiol.* 125 (2001) 29–32.

- [23] W. Bilger, O. Björkman, Temperature dependence of violaxanthin de-epoxidation and non-photochemical fluorescence quenching in intact leaves of *Gossypium hirsutum* L. and *Malva parviflora* L., *Planta* 184 (1991) 226–234.
- [24] C.E. Bratt, P.-O. Arvidsson, M. Carlsson, H.E. Åkerlund, Regulation of violaxanthin de-epoxidase activity by pH and ascorbate concentration, *Photosynth. Res.* 45 (1995) 169–175.
- [25] P. Müller-Moulé, P.L. Conklin, K.K. Niyogi, Ascorbate deficiency can limit violaxanthin de-epoxidase activity in vivo, *Plant Physiol.* 128 (2002) 970–977.
- [26] H.K. Lichtenthaler, Chlorophylls and carotenoids: pigments of photosynthetic membranes, *Methods Enzymol.* 148 (1987) 350–382.
- [27] A. Sen Gupta, P.R. Webb, A.S. Holaday, R.D. Allen, Overexpression of superoxide dismutase protects plants from oxidative stress: induction of ascorbate peroxidase in superoxide dismutase-overproducing plants, *Plant Physiol.* 103 (1993) 1067–1073.
- [28] T. Okuda, Y. Matsuda, A. Yamanaka, S. Sagisaka, Abrupt increase in the level of hydrogen peroxide in leaves of winter wheat is caused by cold treatment, *Plant Physiol.* 97 (1991) 1265–1267.
- [29] M.V. Rao, D.P. Ormrod, Ozone exposure decreases UVB sensitivity in a UVB-sensitive flavonoid mutant of *Arabidopsis*, *Photochem. Photobiol.* 61 (1995) 71–78.
- [30] D. Kornyeyev, B.A. Logan, P. Payton, R.D. Allen, A.S. Holaday, Elevated chloroplastic glutathione reductase activities decrease chilling-induced photoinhibition by increasing rates of photochemistry, but not thermal energy dissipation, in transgenic cotton, *Funct. Plant Biol.* 30 (2003) 101–110.
- [31] W.W. Adams, III, B. Demmig-Adams, Operation of the xanthophyll cycle in higher plants in response to diurnal changes in incident sunlight, *Planta* 186 (1992) 390–398.
- [32] A.M. Gilmore, H.Y. Yamamoto, Resolution of lutein and zeaxanthin using a nonendcapped, lightly carbon-loaded C-18 high performance liquid chromatographic column, *J. Chromatogr.* 543 (1991) 137–145.
- [33] U. Schreiber, U. Schliwa, W. Bilger, Continuous recording of photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer, *Photosynth. Res.* 10 (1986) 51–62.
- [34] O. Van Kooten, J.F.H. Snel, The use of chlorophyll fluorescence nomenclature in plant stress physiology, *Photosynth. Res.* 25 (1990) 147–150.
- [35] B. Genty, J.M. Briantais, N.R. Baker, The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence, *Biochim. Biophys. Acta* 990 (1989) 87–90.
- [36] J. Harbinson, B. Genty, N.R. Baker, Relationship between the quantum efficiencies of Photosystem I and II in pea leaves, *Plant Physiol.* 90 (1989) 1029–1034.
- [37] C. Klughammer, U. Schreiber, Measuring P700 absorbance changes in the near infrared spectral region with a dual wavelength pulse modulation system, in: G. Garab (Ed.), *Photosynthesis: Mechanisms and Effects*, vol. 5, Kluwer Academic Publishers, 1998, pp. 4357–4360.
- [38] M. Orozco-Cardenas, C.A. Ryan, Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway, *Proc. Natl. Acad. Sci. USA* 96 (1999) 6553–6557.
- [39] R.G. Walters, P. Horton, Resolution of components of non-photochemical chlorophyll fluorescence quenching in barley leaves, *Photosynth. Res.* 27 (1991) 121–133.
- [40] H.K. Lichtenthaler, S. Burkart, Photosynthesis and light stress, *Bulg. J. Plant Physiol.* 25 (1999) 3–16.
- [41] M.L. Orozco-Cardenas, J. Narvaez-Vasquez, C.A. Rayan, Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate, *Plant Cell* 13 (2001) 179–181.
- [42] I. Terashima, S. Funayama, K. Sonoike, The site of photoinhibition in leaves of *Cucumis sativus* L. at low temperatures is photosystem I, not photosystem II, *Planta* 193 (1994) 300–306.
- [43] A. Melis, Photosystem-II damage and repair cycle in chloroplasts: what modulates the rate of photodamage in vivo, *Trends Plant Sci.* 4 (1999) 130–135.
- [44] C.H. Foyer, B. Halliwell, The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism, *Planta* 133 (1976) 21–25.
- [45] G. Noctor, C.H. Foyer, Ascorbate and glutathione: keeping active oxygen under control, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49 (1998) 249–279.
- [46] B. Demmig-Adams, W.W. Adams, III, The role of xanthophyll cycle carotenoids in the protection of photosynthesis, *Trends Plant Sci.* 1 (1996) 21–26.
- [47] Y. Yabuta, T. Motoki, K. Yoshimura, T. Takeda, T. Ishikawa, S. Shigeoka, Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress, *Plant J.* 32 (2002) 915–925.