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+/C27/+ plants) was compared to that of wild type (Gossypium hirsutum L. cv. Coker 312) during exposure to 10 °C and 500 μmol photons m−2 s−1. APX+/C27/+ leaves did not exhibit as large of an increase in cellular H2O2 that was evident in wildtype leaves shortly after the imposition of the chilling treatment. In addition, APX+/C27/+ leaves exhibited slightly, but significantly, less photosystem (PSI) and PSII photoinhibition. However, the greatest genotypic difference in H2O2 scavenging did not coincide with the greatest difference in PSI inactivation, and APX+/C27/+ leaves exhibited a greater quantum yield for PSII. Therefore, genotypic differences in H2O2 scavenging may not have been the sole mechanism enhancing PSI protection in APX+/C27/+ 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.

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

1. Introduction

Ascorbate peroxidase (APX, EC 1.11.1.11) scavenges H2O2 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 O2 [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 O2 photo reduction, particularly in chilling-sensitive plants [7]. H2O2 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 (PSI) 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 H2O2, especially considering that O2 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 H2O2 accumulation is a major factor leading to the decline in PSI activity [16].
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 CO2-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 H2O2 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 N2. 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 cm2) 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−2 s−1. Humidified air containing 5.28% CO2 (v/v) was passed through the chamber during the treatment.

For attached leaves used to measure H2O2 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 ΦPSII 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−2 s−1. The leaf temperature of 10 °C was maintained by means of a PLC temperature controller (ADC Ltd, Hoddesdon, UK).

2.4. H2O2 content

After various times of exposure to the chilling treatment, the leaf discs (10 cm2) or attached leaves were rapidly frozen in liquid N2. The frozen tissue was
ground to a powder at liquid N$_2$ temperature, and the 
H$_2$O$_2$ was extracted with ice-cold 0.2 N HClO$_4$. After 
normalization with KOH and centrifugation, the extract 
was assayed for H$_2$O$_2$ 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 glu-
thione were extracted from leaf tissue previously 
ground in liquid nitrogen in 2 ml of ice-cold 2% meta-
phosphoric acid with 2 mM EDTA. The extract was 
centrifuged at 10000 × g for 10 min at 4 °C and 
normalized 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 spectrophotometri-
cally before and after (respectively) the addition of 2-
vinylypyridine. Both assays were conducted according to 
the modified method of Rao and Ormrod [29] as 
described in Kornyeyev et al. [30].

2.6. Pigment analysis

Samples (0.25 cm$^2$) 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, Effel-
trich, 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 trans-
port by PSII was calculated as $\Phi_{\text{PSII}} = (F_m - F)/F_m$ 
[35], where $F$ and $F_m$ are the steady-state and maximal 
chlorophyll fluorescence for light-acclimated leaves, 
respectively. The level of the thermal energy dissipata-
in PSII antennae was estimated by calculating the non-
photochemical chlorophyll fluorescence quenching co-
efficient ($\text{NPQ} = F_m/F_m - 1$) and the excitation capture 
efficiency of PSII using the ratio $F_v/F_m$, where $F_v = 
F_m - F_o$ 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 $F_o$, 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 KL 1500 light source (Schott, Wiesbaden, 
Germany). Leaf discs of both genotypes exhibited 
average values of $F_v/F_m$ of 0.78 ± 0.02 (mean ± S.D.) 
without significant differences were found 
750 µM.

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$^+$ 
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)$^{-1}$ h$^{-1}$ for wildtype and APX$^+$ plants, respec-
tively, 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)$^{-1}$ h$^{-1}$ 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$_2$O$_2$ levels and the reduction states of ascorbate and glutathione during chilling treatments

For wildtype leaf discs, the total leaf H$_2$O$_2$ level increased quickly upon illumination at 10 °C, reaching 130% of the pre-illumination value in 3 min (Fig. 1A). At 180 min, H$_2$O$_2$ content was similar to the level obtained just before the light treatment. However, the H$_2$O$_2$ level in APX+ leaf discs remained close to the pre-illumination value at 3, 20 and 180 min of illumination.

Since H$_2$O$_2$ can accumulate in cotton leaves because of wounding [38], we performed additional experiments to estimate H$_2$O$_2$ accumulation in leaf discs during the dark-acclimation period. Indeed, H$_2$O$_2$ content increased dramatically during 1.5-h of dark acclimation at room temperature plus 20 min at 10 °C (from 1.9 ± 0.7 nmol/mg Chl to 257.3 ± 24.5 nmol/mg Chl). However, no significant increase in H$_2$O$_2$ levels occurred with an additional 3 min or 20 min in the dark at 10 °C. Therefore, one may suggest that the increase in H$_2$O$_2$ content at 3 and 20 min of illumination at 10 °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 °C after the 1.5 h at room temperature and 20 min at 10 °C in darkness.

In order to completely exclude the influence of wounding on H$_2$O$_2$ 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$_2$O$_2$ at 3 and 20 min of illumination at 10 °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 °C, 85.7 ± 2.5% and 82.5 ± 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 ± 3.5% and 88.3 ± 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
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 μmol m$^{-2}$ s$^{-1}$ and 10 °C (Fig. 4A and B).

parameter, $\Phi$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).

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-


during exposure of leaf discs to 10

during exposure of leaf discs to 10 °C at a PFD of 500 μmol m⁻² s⁻¹ and 5.28% (v/v) CO₂ 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.

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 (F̂(V/Fm*)) (B) during exposure of leaf discs to 10 °C at a PFD of 500 μmol m⁻² s⁻¹ and 5.28% (v/v) CO₂ 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.

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 μmol m⁻² s⁻¹ and 5.28% (v/v) CO₂ 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.

Table 1
Pigment composition of cotton leaf tissue from wildtype cotton (cv. Coker 312) and transgenic cotton overproducing ascorbate peroxidase (APX+)

<table>
<thead>
<tr>
<th>Pigment(s)</th>
<th>Genotype</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Wildtype</td>
<td>APX+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(μmol m⁻²)</td>
<td>(μmol m⁻²)</td>
<td></td>
</tr>
<tr>
<td>Chl a</td>
<td>464.6 ± 99.8</td>
<td>449.6 ± 94.6981</td>
<td></td>
</tr>
<tr>
<td>Chl b</td>
<td>130.0 ± 26.2</td>
<td>116.7 ± 25.89044</td>
<td></td>
</tr>
<tr>
<td>β-Carotene</td>
<td>50.4 ± 14.9</td>
<td>46.9 ± 11.79069</td>
<td></td>
</tr>
<tr>
<td>Xanthophyll cycle pigments</td>
<td>48.2 ± 13.8</td>
<td>51.7 ± 4.89061</td>
<td></td>
</tr>
<tr>
<td>Lutein</td>
<td>64.2 ± 12.0</td>
<td>62.0 ± 9.89076</td>
<td></td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>24.3 ± 3.8</td>
<td>22.8 ± 4.59059</td>
<td></td>
</tr>
</tbody>
</table>

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 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 H₂O₂ content [28] (Fig. 1). Overproduction of APX in the chloroplasts (presumably in the stroma) of cotton leaves resulted in enhanced scavenging of H₂O₂ 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 H₂O₂ that occurred once illumination commenced at 10 °C but before photochemistry (ΦPSII) reached a steady state. These genotypic differences were most striking for attached leaves. The high, illumination-independent H₂O₂ content of leaf discs may have partially masked the H₂O₂ increases dependent on illumination. This large illumination-independent H₂O₂ 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$_2$O$_2$ is thought to occur in cell walls (not in internal cell organelles like chloroplasts) [41]. Since our measurements were of the total leaf H$_2$O$_2$ content, it is possible that greater differences in H$_2$O$_2$ between wildtype and transgenic plant leaf discs occurred at the chloroplast level. Whatever the localization of the illumination-independent H$_2$O$_2$, it had little effect on the genotypic differences in photochemistry (ΦPSII), since differences in ΦPSII 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 chillingsensitive species [16,42]. PSI is vulnerable to damage by hydroxyl radicals generated via a Fenton-type reaction between photogenerated H$_2$O$_2$ and Fe$^-S$ centers in PSI. Therefore, H$_2$O$_2$ is considered critical to PSI photodamage during chilling stress [16]. One may suggest that improved H$_2$O$_2$ scavenging can positively affect PSI photoprotection. Indeed, the transgenic cotton plants with increased chloroplastic APX activity did have a lower H$_2$O$_2$ 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$_2$O$_2$ 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$_2$ photo-reduction.

This hypothesis is consistent with the finding that the APX+ leaves exhibited greater photochemistry (ΦPSII) 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 QA, the primary quinone acceptor in PSII. PSII is more vulnerable to photoinhibitory damage when photon energy is trapped by reaction centers with QA 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 PSI 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$_2$O$_2$ 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 over-production will require the creation of the transgenic plants with similar levels of isozyme overexpression.

5. Conclusions

Overproduction of APX in the chloroplast stroma of cotton leaves resulted in enhanced scavenging of \( \text{H}_2\text{O}_2 \) and a small enhancement in the resistance to photoinhibition of PSI and PSII during exposure to chilling temperatures at a moderate PFD. Factors aside from enhanced \( \text{H}_2\text{O}_2 \) 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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