Thylakoid-Bound Ascorbate Peroxidase Mutant Exhibits Impaired Electron Transport and Photosynthetic Activity

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In chloroplasts, stromal and thylakoid-bound ascorbate peroxidases (tAPX) play a major role in the removal of H₂O₂ produced during photosynthesis. Here, we report that hexaploid wheat (Triticum aestivum) expresses three homeologous tAPX genes (TaAPX-6A, TaAPX-6B, and TaAPX-6D) mapping on group-6 chromosomes. The tAPX activity of a mutant line lacking TaAPX-6B was 40% lower than that of the wild type. When grown at high-light intensity photosystem II electron transfer, photosynthetic activity and biomass accumulation were significantly reduced in this mutant, suggesting that tAPX activity is essential for photosynthesis. Despite the reduced tAPX activity, mutant plants did not exhibit oxidative damage probably due to the reduced photochemical activity. This might be the result of a compensating mechanism to prevent oxidative damage having as a consequence a decrease in growth of the tAPX mutant plants.

Plant cells are continuously exposed to reactive oxygen species (ROS) generated as by-products of fatty acid β-oxidation, photorespiration, and photosynthesis. Both biotic and abiotic stresses usually lead to an enhanced ROS production, therefore ROS-scavenging mechanisms acting in different organelles play a major role in plant survival and productivity, particularly in extreme environments. Under ROS-generating stress conditions, many antioxidant enzymes such as catalase (CAT), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione S-transferase (GST), glutathione reductase (GR), superoxide dismutase (SOD), and ascorbate peroxidase (APX) counteract the otherwise uncontrolled oxidation of cellular components (Noctor and Foyer, 1998). Among the enzymes involved in ROS-removal, APX (EC 1.11.1.11) plays a major role in H₂O₂-scavenging in plants (Asada, 1992). Three major groups of APX isoenzymes (i.e. chloroplastic, cytosolic, and glyoxysomal APXs) have been identified based on their subcellular location (Mittler and Zilinskas, 1992; Miyake and Asada, 1992; Bunkelmann and Trelease, 1996). Cytosolic APXs (cAPX) are likely to be involved in pathogen response, whereas glyoxysomal APXs remove the H₂O₂ generated by fatty acid β-oxidation and photorespiration (Bunkelmann and Trelease, 1996; Mittler et al., 1998). Chloroplastic APX (chAPX) composed of thylakoid-bound (tAPX) and stromal (sAPX) isoforms scavenge the H₂O₂ generated during photosynthesis, which is a major H₂O₂-producing metabolic process in green tissues (Nakanono and Asada, 1981).

Detoxification of ROS is required to avoid damage to the photosynthetic machinery. In chloroplasts, large amounts of O₂⁻ are produced by the transfer of electrons from the donor side of photosystem I (PSI) to O₂ (Asada, 1994). Dismutation of O₂⁻ to H₂O₂ occurs either spontaneously or by a SOD-catalyzed reaction. Subsequent reduction of H₂O₂ by chAPX produces water and the monodehydroascorbate radical, which can be regenerated to ascorbic acid (AA) by either reduced ferredoxin or NAD(P)H in a reaction catalyzed by MDHAR (Sano and Asada, 1994; Sano et al., 1995). This process, known as the water-
water cycle, dissipates the excess of excitation energy incoming to photosystems through the transfer of electrons to molecular oxygen and, in addition, reinforces the trans-thylakoid pH gradient used for ATP synthesis (Asada, 1999). SOD and APX located at the stromal side of thylakoid membranes locally remove the ROS produced at the donor side of the PSI. However, tAPXs constitute themselves a primary target for inactivation by ROS under oxidative stress, such as that produced by methylviologen (MV) and excess of excitation energy (Mano et al., 2001).

Because tAPX mutant plants have not been generated and/or identified so far, the in vivo role of chAPX has been analyzed in transgenic plants overexpressing ROS-scavenging enzymes. Studies with transgenic tobacco (Nicotiana tabacum) plants that overexpress bacterial CAT in chloroplast demonstrated that chAPXs are strongly inhibited under oxidative stress, which suggests that these enzymes could be a limiting component of the plant antioxidative defense (Shikanai et al., 1998; Miyagawa et al., 2000). Therefore, the overexpression of chAPXs might protect the plants from oxidative damage under stress. Accordingly, cotton (Gossypium hirsutum) plants that overexpress recombinant cAPX in chloroplasts showed an enhanced resistance to chilling-associated oxidative stress (Payton et al., 2001). In addition, it has been recently shown that transgenic tobacco plants that overexpress tAPX are more resistant to MV and chilling than the wild-type plants (Yabuta et al., 2002). Overall, these results showed that tAPXs are key components of the chloroplastic antioxidant defenses in vivo.

In the present work, three tAPX genes from wheat (Triticum aestivum; TaAPX-6A, TaAPX-6B, and TaAPX-6D) were identified. A TaAPX-6B-deleted mutant line (S-SV8) exhibits reduced weight, size, and seed production relative to the parental near isogenic line (R-SV8). The comparison of APX activities and photosynthetic parameters between the R-SV8 and the S-SV8 isolines led us to conclude that a reduced tAPX activity leads to an impaired photosynthesis.

RESULTS

The Hexaploid Wheat Genome Contains Three Homeologous Genes Encoding tAPXs

In an attempt to identify genes accountable for the phenotypic differences observed between R-SV8 and S-SV8, we isolated a cDNA for which the coding gene is absent in the mutant line. The translated sequence of this cDNA (clone TaRr16) did not show significant homology with any protein in nonredundant databases (Danna et al., 2002). However, after the 5′-RACE-mediated elongation of this sequence, we obtained a near full-length cDNA that encodes a protein highly identical to tAPX proteins from spinach (Spinacia oleracea) and pumpkin (Cucurbita pepo). Three cDNAs from wheat were identified after an extensive search based on 5′- and 3′-RACE procedures. Although their coding regions are highly similar (more than 95% identity), major differences were observed at their 3′-untranslated regions (UTRs; Fig. 1A). A comparison of their deduced amino acid sequences revealed high homology to various tAPXs from dicots (70%–80% identity). Proteins encoded by these tAPX genes from wheat show the hallmarks of prokaryotic class I peroxidases (Welinder, 1992). Their carboxy termini showed the predicted putative membrane-anchor domain characteristic of tAPXs (Ishikawa et al., 1997; Mano et al., 1997). Although predicted tAPX proteins from wheat are highly similar to other tAPXs from dicots, they have an insertion of 26 amino acid residues near the anchor domain (Fig. 1B).

Because one of the three tAPX genes (clone TaRr16) mapped on the distal region of chromosome 6BL (Danna et al., 2002), this gene was named TaAPX-6B. To determine whether the tAPXs genes are located in group-6 chromosomes, genomic DNA from Nullitetrasomic wheat lines (Chinese Spring Nuli6A-Tetra6D and Nuli6D in which 6A or 6D chromosomes are absent respectively) were analyzed by PCR. As shown in Figure 2A, these genes were located in chromosomes 6A and 6D, and consequently, they were named TaAPX-6A and TaAPX-6D. These results indicate that TaAPX-6A, TaAPX-6B, and TaAPX-6D are homeologous genes, located at chromosome 6 of the hexaploid wheat genome. High-stringency DNA hybridization analysis revealed the presence of three copies in the parental line and two in the S-SV8 mutant line (Fig. 2B). Low-stringency hybridization revealed the presence of six bands in the parental and five in the mutant (data not shown), indicating that TaAPX-6B is probably the only chAPX gene absent in the mutant line. Because chAPX genes have not been isolated from monocots so far, we used the coding region of TaAPX-6B to assess the copy number and homology in other monocot plants. A main single band was detected in maize, rice, and barley (Fig. 2C). Consistently, only one tAPX gene was identified in the complete rice genome database, which confirms the presence of a single tAPX gene in this species.

The Expression of tAPX Genes Overlaps in Most Tissues

To analyze whether the chromosomal deletion affects the expression of the tAPX genes in the mutant line, TaAPX-6A and TaAPX-6D expression was further explored in both isolines. Whereas the three genes were expressed in the R-SV8 plants, TaAPX-6A and TaAPX-6D expression was detected in the mutant (Fig. 3A). To test whether or not the three genes have a similar function, their expression was studied in different organs of R-SV8 plants. The three genes are expressed in aerial organs but not in roots. Al-
though their expression patterns essentially overlap in most tissues, there are some subtle differences in expression. Whereas TaAPX-6D and TaAPX-6B expression was detected in green tissues and reproductive organs, TaAPX-6A expression was only detected in green tissues excluding sheaths (Fig. 3B). These results indicate that tAPX genes are controlled by similar but not identical regulatory elements.

TaAPX-6B Encodes a Protein with APX Activity

The cDNA region encoding the putative mature protein of TaAPX-6B was subcloned into plasmids for protein expression. Two versions of the recombinant protein were overexpressed in *Escherichia coli*: a long version containing the so-called membrane anchor domain (rAPX-1) and a short version in which this region was deleted (rAPX-2). Both rAPX-1 and rAPX-2 showed their predicted molecular weights in SDS-PAGE gels. The anchor-containing protein yielded an insoluble form, whereas the anchor-deleted form produced a soluble protein (data not shown). The insoluble form (rAPX-1) did not show any APX activity, but the soluble protein (rAPX-2) exhibited APX activity at 26.4 μmol AA min⁻¹ mg⁻¹ of recombinant protein in the supernatant (data not shown). The specific activity of the recombinant TaAPX-6B was similar to the activity reported for a recombinant cAPX from soybean (*Glycine max*) overexpressed in *E. coli* (Dalton et al., 1996). This result confirms that TaAPX-6B encodes an active APX protein.

The Photon Flux Density (PFD) Applied during Growth of Plants Modulates TaAPX-6B Expression

To determine whether the expression of tAPX genes is regulated by the PFD, gene expression in leaf blades of R-SV8 plants grown in the dark, in low-light condition (LL), or in high-light condition (HL) was analyzed. In darkness, TaAPX-6B expression was nearly undetectable (Fig. 4A). A 2-fold increase in the TaAPX-6B mRNA steady-state level was observed in plants grown at LL (50–100 μmol photons m⁻² s⁻¹). Plants grown at HL (700–1,000 μmol photons m⁻² s⁻¹) showed 4-fold higher TaAPX-6B expression than etiolated plants (Fig. 4A). The expression of TaAPX-6A and TaAPX-6D, roughly undetectable in the dark, increased 2-fold in LL-grown plants, but no further induction was detected in HL-grown plants (data not shown). This expression analysis indicates that tAPX

Figure 1. Sequence alignments of 3′-UTRs of tAPX cDNAs from wheat and proteins from different plant species. A, Alignment among 3′-UTRs of tAPX cDNAs from wheat. Nucleotide homology is boxed. Primers for each cDNA are indicated by bold type. The sense primer of TaAPX-6B is located over the coding region, hence, not shown (see “Materials and Methods”). Polyadenylation signals are underlined. B, Alignment among TaAPX-6B and other thylakoid-bound APX proteins from dicot species. Conserved amino acids are shown by a shadow box. Asterisks indicate essential amino acid residues for protein activity. Putative thylakoid membrane anchor domains are underlined.
genes are light-inducible but only TaAPX-6B is modulated by the PFD used for plant growth. The short-term effect of either excess excitation energy (EL) or MV-mediated oxidative stresses on tAPX gene expression was also investigated. R-SV8 plants grown at 200 to 400 μmol photons m^{-2} s^{-1} were sprayed with 20 μM MV and were exposed to EL (1,800 μmol photons m^{-2} s^{-1}). No differences in TaAPX-6B expression were detected either after EL or MV combined with EL treatments, indicating that TaAPX-6B is not regulated by a sudden oxidative stress in a 4-h temporal window (Fig. 4B). Likewise, neither MV nor EL modifies the mRNA steady-state levels of TaAPX-6A and TaAPX-6D (data not shown).

As previously reported for tAPX genes from spinach (Yoshimura et al., 2000), the mRNA level of tAPX genes from wheat is not regulated by oxidative stress.

APX Activity of Mutant Plants Is Reduced at High and Excess PFD

Because TaAPX-6B is deleted in S-SV8 plants, studies were carried out to test whether these plants have a disturbed APX activity. No significant differences in foliar APX activity were detected between R-SV8 and S-SV8 plants grown in the dark or at LL conditions. However, S-SV8 plants grown at HL, showed a 15% lower APX activity than that of the R-SV8 plants (Fig. 5A). Although these measurements cannot distinguish among different isoenzymes contributing to the foliar APX activity, the reduced activity of the mutant is likely to be due to the lack of TaAPX-6B. Because TaAPX-6B is likely to encode a tAPX enzyme, APX activity in thylakoid membranes was measured. As shown in Figure 5B, HL-grown S-SV8...
stress is compromised by the tAPX deficiency of the mutant line. The loss of tAPX activity in the mutant line.

accumulation was carried out with plants grown in the dark (D), at 50 to 100 μmol photons m⁻² s⁻¹ (LL), or at 700 to 1,000 μmol photons m⁻² s⁻¹ (HL). B. Analysis of the short-term mRNA accumulation in plants suddenly exposed to 1,800 μmol photons m⁻² s⁻¹ (EL) combined with the application of MV for 4 h. In this case, plants were previously grown at 200 to 400 μmol photons m⁻² s⁻¹. From left to right, a base-two serial dilution of 100 and 10 ng of total cDNA was used as template for the amplification of TaAPX-6B and Actin, respectively. Asterisks indicate the last dilution at which TaAPX-6B was detected. Actin was detected up to the fifth cDNA dilution in every sample. Results are representative of two independent experiments.

plants showed a 40% lower activity in the thylakoids than HL-grown R-SV8 plants. This result is consistent with the proposed subcellular location of TaAPX-6B in thylakoid membranes and indicates that TaAPX-6A and TaAPX-6D did not fully compensate the loss of tAPX activity in the mutant line.

To determine whether the response to oxidative stress is compromised by the tAPX deficiency of the S-SV8 line, plants grown at 200 to 400 μmol photons m⁻² s⁻¹ were subjected to EL (1,800 μmol photons m⁻² s⁻¹) and MV. Foliar APX activity increased in R-SV8 while it decreased in S-SV8 plants 4 h after exposure to EL (Fig. 5C). Addition of MV under EL conditions resulted in a sharp reduction of APX activity in R-SV8 plants 4 h after treatment, whereas a drastic reduction in APX activity was observed in the mutant as soon as 1 h after this treatment. It is noteworthy that the magnitude of the APX inhibition in S-SV8 plants 4 h after EL was similar to that observed 1 h after MV. These results suggest that an elevated tAPX activity may be required to avoid foliar APX inhibition under a sudden oxidative stress.

The Ascorbate-Glutathione Cycle Is Not Altered in tAPX Mutant Plants

Transgenic plants overexpressing antioxidative enzymes usually show altered activities of ROS-scavenging endogenous enzymes (Sen Gupta et al., 1993a, 1993b). To determine whether the tAPX deficiency of S-SV8 plants causes any alteration in the activity of other antioxidative enzymes involved in the ascorbate-glutathione cycle, DHAR, MDHAR, and GR activities were assayed. No differences were detected between R-SV8 and S-SV8 plants growing at HL (non-stressing PFD), indicating that tAPX deficiency has no effect on the activity of these enzymes (Table I). Although HL-grown mutant plants had roughly 40% lower tAPX activity than R-SV8 plants, the level of AA was similar in both the isolines (Table I).

tAPX Mutant Plants Show Impaired Electron Transport and Photosynthetic Activity and Reduced Growth under Normal PFD

Because tAPXs remove H₂O₂ generated by dismutation of O₂⁻ generated at the donor side of PSI, the possible role of TaAPX-6B in the protection of the photosynthetic apparatus was investigated by comparing CO₂ assimilation and chlorophyll fluorescence parameters in both isolines. HL-grown S-SV8 plants displayed a significantly lower photosynthetic activity than that of R-SV8 plants. The quantum yield of PSII (ΦPSII) and photochemical quenching (qP) were also reduced in the mutant, but there were no significant differences in non-photochemical quenching between both isolines (Table II). The reduced ΦPSII in the mutant plants was only observed at high PFD (800 μmol photons m⁻² s⁻¹), suggesting that the mutant is more susceptible to photodamage than the parental line (Table III). To study the effect of the reduced PSII electron transfer and photosynthetic activity on growth and biomass accumulation in S-SV8 plants, leaf area and dry weight of R-SV8 and S-SV8 plants were measured. R-SV8 and S-SV8 plants grown at a PFD of 200 μmol photons m⁻² s⁻¹ showed non-significant differences in dry matter and foliar area. However, at moderate (400 μmol photons m⁻² s⁻¹) and high (800 μmol photons m⁻² s⁻¹) PFD, the mutant plants developed a smaller leaf area and accumulated less dry matter than the parental plants (Fig. 6, A and B). To determine whether the reduced accumulation of biomass in the mutant could be due to photodamage, measurements of protein carbonylation, which indicates oxidative damage, were carried out. No significant differences were detected between the parental and the mutant (data not shown), indicating that mutant plants do not experience a chronic oxidative damage at LL or HL conditions. To determine whether the tAPX deficiency of S-SV8 plants leads to an enhanced susceptibility to photodamage under stress conditions, the response to a sudden increase in PFD was investigated. HL-grown mutant and parental plants were suddenly exposed for 4 h to EL. A decrease in Fv/Fm indicating PSII photoinhibition was detected in both the isolines, but this decrease was significantly higher in the mutant than in the parental plants (Table
IV). In summary, these results suggest that a reduced tAPX activity leads to an increased susceptibility to an abrupt oxidative stress.

DISCUSSION

Early studies postulated that thylakoid-bound APX enzymes play a key role in the scavenging of H$_2$O$_2$ in chloroplasts, helping plants to dissipate excess excitation energy through electron transfer from PSI to O$_2$ (Asada and Takahashi, 1987). The rate of electron flow from PSI to O$_2$ increases dramatically in plants exposed to various stresses, generating large quantities of ROS, which in turn lead to oxidative stress (Foyer et al., 1994). The relevance of tAPX activity in H$_2$O$_2$ removal under non-stressing and stressing conditions has been previously analyzed in transgenic plants (Shikanai et al., 1998; Miyagawa et al., 2000; Payton et al., 2001). Here, we report the identification of a tAPX mutant line of wheat that displays reduced tAPX activity and impaired photosynthesis.

tAPX Redundancy Allows Wheat Mutant Plants to Survive

Several tAPX cDNAs have been already isolated, and their encoded proteins have been characterized. Most of these studies have been carried out using pumpkin and spinach as models, which contain a single chAPX gene encoding both the tAPX and the sAPX by means of mRNA alternative splicing (Ishikawa et al., 1997; Mano et al., 1997). chAPX mutants have not been isolated in a nonredundant background genome so far, which suggests a conditional lethal nature for such a mutation. In fact, as reported recently, transgenic tobacco plants expressing tAPX transgene in the antisense orientation were not obtained most probably due to the lethal nature of the tAPX suppression (Yabuta et al., 2002). In Arabidopsis, tAPX and sAPX are encoded by two different loci (At1g77490 and At4g08390), and accordingly, T-DNA interrupted lines for each one of these genes were obtained (http://signal.salk.edu/cgi-bin/tdnaexpress). The hexaploid genome of wheat may facilitate the survival of a tAPX mutant and thereby allows determining the in vivo role of tAPX enzymes. DNA gel-blot hybridization analysis indicates that monocots like rice, maize, and barley contain a single tAPX gene (Fig. 2C). Unlike those species, three tAPX genes were detected in bread wheat mapping on the group-6 chromosomes. Because no additional copies of tAPX map on the deleted region of the mutant (Fig. 2B), its reduced tAPX activity could be attributed to the lack of TaAPX-6B (Fig. 5B). Moreover, in vitro activity assays of the recombinant protein confirmed that TaAPX-6B encodes a functional APX enzyme, providing additional evidence for the latter hypothesis. Because only TaAPX-6B expression is regulated by the PFD at

![Figure 5](https://www.plantphysiol.org/figure/5/)
non-stressing conditions (Fig. 4A), it seems possible that the lack of either TaAPX-6A or TaAPX-6D could have a minor effect on the phenotype of wheat plants. In this regard, it should be emphasized that no compensatory tAPX activity seems to be provided by TaAPX-6A and TaAPX-6D in the mutant (Fig. 5B). Although reproductive organs and sheaths do not express TaAPX-6A, the three tAPX genes are expressed in most aerial tissues (Fig. 3B). This overlapped pattern of gene expression could lead to functional redundancy, and such a redundancy could explain the remainder tAPX activity in S-SV8 plants (Fig. 5B) and probably the viability of the mutant.

Because S-SV8 plants bear a deletion in chromosome 6BL, we cannot rule out that the lack of other genes (not known so far) could be accountable for some of the physiological differences detected between these two near-isogenic lines. However, despite the deletion, previous analysis indicated that R-SV8 and S-SV8 lines are nearly identical at a genetic level. No RAPDs were detected among 2,400 genomic loci analyzed (F. Sacco, unpublished data) and only TaAPX-6B out of 6,000 genes analyzed was differentially expressed between these two isolines (Danna et al., 2002). Moreover, no disturbances in the ascorbate-glutathione cycle were detected in the mutant (Yabuta et al., 2002). Accordingly, our observations indicate that an impaired tAPX activity leads to photodamage at the time that plants are subjected to EL. In this scenario, the rather limited tAPX activity in the mutant could be easily overwhelmed by the increased H₂O₂ production at high PFD, as previously suggested by Mano et al. (2001). Besides, upon transfer to EL, mutant plants showed a pronounced decrease of F_v/F_m, indicating photodamage to PSII, which might be the consequence of the increased H₂O₂ production at the donor side of PSII (Maxwell and Johnson, 2000; Tjus et al., 2001). A recent work reported that transgenic plants that overexpress TaAPX in chloroplasts had improved resistance to oxidative stress (Yabuta et al., 2002). Accordingly, our observations indicate that an impaired tAPX activity leads to photodamage at the time that plants are exposed to a sudden oxidative stress. On the contrary, HL-grown mutant plants, which have an impaired tAPX activity at HL steady-state condition, do not display a chronic oxidative damage (see below).

**Impairing the PSII Electron Transfer Would Impede Massive Oxidative Damage**

The decreased photosynthetic activity of mutant plants is associated with both a low \( \Phi_{\text{PSII}} \) and a low \( q_P \). Because \( q_P \) estimates the redox state of the primary acceptor of PSII, \( Q_A \), we hypothesize that the impaired electron transfer of S-SV8 plants may be due to inefficient re-oxidation of \( Q_A \). It has been reported that the amount of \( \text{psaA} \) protein (a PSI sub-
Table III. Dependency of ϕPSII on irradiance

Quantum yield measurements were performed in leaves of 2-week-old plants grown at 200, 400, or 800 μmol photons m⁻² s⁻¹. Mean values of eight measurements (plants) from two independent experiments. se is shown in parentheses.

<table>
<thead>
<tr>
<th>Plant</th>
<th>μmol Photons m⁻² s⁻¹</th>
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<tbody>
<tr>
<td></td>
<td>200</td>
</tr>
<tr>
<td>R-SV8</td>
<td>0.65 (0.01)</td>
</tr>
<tr>
<td>S-SV8</td>
<td>0.64 (0.03)</td>
</tr>
</tbody>
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* Significant differences (t test, P < 0.05).

Table IV. Photoinhibition of PSII measured as Fv/Fm

Measurements of Fv/Fm in 2-week-old plants grown at 700 to 1,000 μmol photons m⁻² s⁻¹ (HL) were already transferred to 1,800 μmol photons m⁻² s⁻¹ (EL) for 4 h. Mean values of 10 measurements (plants) from two independent experiments. se is shown in parentheses.

<table>
<thead>
<tr>
<th>Plant</th>
<th>HL</th>
<th>EL</th>
</tr>
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<tbody>
<tr>
<td>R-SV8</td>
<td>0.816 (0.004)</td>
<td>0.540a (0.030)</td>
</tr>
<tr>
<td>S-SV8</td>
<td>0.819 (0.004)</td>
<td>0.652 (0.018)</td>
</tr>
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* Significant differences (t test, P < 0.05).
Two recombinant TaAPX-6B proteins were produced. The large recombinant TaAPX-6B contained the mature coding region of TaAPX-6B and was obtained by digesting TaAPX-6B with EcoRI and by subcloning the digestion product into pTrec-His-C (BD Biosciences Clontech, Palo Alto, CA) protein expression vector. The short version, lacking the putative membrane-anchor region, was obtained by PCR using the larger version as template and the 5'-gtagctgtacagggatatggtg-3' (RamHI and EcoRI containing primer) and 5'-catcttgcatgccgaccaat-3' (EcoRI-containing) as upper and lower primers, respectively. This PCR product was digested with RamHI and EcoRI and was subcloned into pTrec-His-C vector digested with the same enzymes. Escherichia coli XL-1-Blue cultures were used for the expression of recombinant proteins. Induction of protein expression was carried out overnight for 4 h in 25 mL of Luria-Bertani medium supplemented with 200 μg mL⁻¹ ampicillin and 1 mM isopropyl-β-D-galactoside at 37°C and 300 rpm. Culture replicas were grown and isopropyl-β-D-galactoside-induced in the presence of 50 μM hemin (Sigma-Aldrich, St. Louis). Bacterial pellets were resuspended in 50 mM PO₄ buffer (pH 7.2) and lyzed by sonication at 4°C. E. coli lysates were centrifuged at 20,000g for 30 min. Total protein (30 μg) from the lysate was separated by electrophoresis in 12% (w/v) SDS-PAGE gels and was stained with Coomassie Blue.

**APX, MDHAR, DHAR, and GR Activities**

Fresh leaves (100–200 mg fresh weight) were frozen in liquid N₂, ground, and mixed with 2 mL of a 50 mM MES/KOH buffer (pH 7.0) containing 40 mM KCl, 2 mM CaCl₂, 1% (w/v) polyvinyl-pyrrolidone, and 1 mM AA. After thawing, 0.1% (v/v) Triton X-100 was added to the mixture, and tubes were gently mixed for 15 min at 4°C. Homogenates were then centrifuged at 4,500g for 2 min, and the supernatants were used to measure foliar APX activity. For thylakoid isolation, leaf homogenates were centrifuged at 3,000g for 10 min, the pellet was washed and centrifuged again at 3,000g for 10 min, and APX activity was measured in the pellet without addition of Triton X-100 (Guanzet et al., 2002). Recombinant APX activity was measured in 1 mL of buffer containing 20, 40, and 80 μg of total proteins from bacterial lysates (quantified spectrophotometrically by Bradford assay) as described below. Mass of rAPX-2 for specific in vitro activity was estimated by comparison with quantified bovine serum albumin in Coomassie-stained gels. APX was measured spectrophotometrically by the method of Nakano and Asada (1987). The reaction mixture (950 μL) contained 50 mM KH₂PO₄/K₂HPO₄ buffer (pH 7.0), 500 μM AA, and 0.1 mM H₂O₂. Foliar homogenates (50 μL) were added to the reaction mixture and were gently mixed. Oxidation of AA was followed by a decrease in absorbance at 260 nm in a spectrophotometer (DU-650, Beckman Coulter, Fullerton, CA) at 30°C. The reaction rates measured were linear for at least 3 min and were corrected for AA auto-oxidation in the presence of 0.1 mM H₂O₂. APX activity was calculated using an extinction coefficient of 2.8 μM⁻¹ cm⁻¹ for AA. For measurements of MDHAR (EC 1.6.5.4), DHAR (EC 1.8.5.1), and GR (EC 1.6.4.2) activities, leaves were ground in a medium containing 0.1 M Bicine (pH 7.5), 1 mM EDTA, 10% (v/v) glycerol, 4 mM Cys, and protease inhibitors (25 mM phenylmethylsulfonyl fluoride and 2 mM leupeptin). Homogenates were filtered through a 20-μm mesh and were centrifuged at 10,000g for 10 min. The supernatants were used for the determinations of enzyme activities. MDHAR and DHAR were measured essentially as by De Gara et al. (2000), and GR was measured as described previously (Bartoli et al., 1999). AA content was determined by HPLC as described by Iwase (1992).

**Photosynthetic Parameters**

Photosynthesis was measured with an infrared gas analyzer (LI-6250, LI-COR, Lincoln, NE) fitted in a 1-L assimilation chamber, at 750 μmol photons m⁻² s⁻¹, 25°C, and 340 to 360 ppm of CO₂. Chlorophyll a fluorescence was measured with an FMS2 Fluorescence Monitoring System (Hanatech, King's Lynn, UK), and fluorescence parameters were calculated as by Maxwell and Johnson (2000). Fluorescence parameters in the light-adapted state (i.e., Fₛₑ, F₀, and Fₚ) were measured in leaves exposed to the PFDs applied during growth, and then leaves were dark-adapted for 30 min before measuring Fₛₑ and F₀.
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REFERENCES


Kingston-Smith AH, Foyer CH (2000) Bundle sheath proteins are more sensitive to oxidative damage than those of the mesophyll in maize leaves exposed to paraquat or low temperatures. J Exp Bot 51: 123–130


Tjus SE, Scheller HV, Andersson B, Mollier BL (2001) Active oxygen production during selective excitation of photosystem I is damaging not only to photosystem I, but also to photosystem II. Plant Physiol 125: 2007–2015


Sano S, Asada K (1994) cDNA cloning of monodehydroascorbate radical reductase from cucumber: a high degree of homology in terms of amino acid sequence between this enzyme and bacterial flavoenzymes. Plant Cell Physiol 35: 425–437


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