Arabidopsis Chloroplastic Glutathione Peroxidases Play a Role in Cross Talk between Photooxidative Stress and Immune Responses$^{1[W][OA]}$

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Glutathione peroxidases (GPXs; EC 1.11.1.9) are key enzymes of the antioxidant network in plants and animals. In order to investigate the role of antioxidant systems in plant chloroplasts, we generated Arabidopsis (Arabidopsis thaliana) transgenic lines that are depleted specifically in chloroplastic (cp) forms of GPX1 and GPX7. We show that reduced cpGPX expression, either in transgenic lines with lower total cpGPX expression (GPX1 and GPX7) or in a gpx7 insertion mutant, leads to compromised photooxidative stress tolerance but increased basal resistance to virulent bacteria. Depletion of both GPX1 and GPX7 expression also caused alterations in leaf cell and chloroplast morphology. Leaf tissues were characterized by shorter and more rounded palisade cells, irregular spongy mesophyll cells, and larger intercellular air spaces compared with the wild type. Chloroplasts had larger and more abundant starch grains than in wild-type and gpx7 mutant plants. Constitutively reduced cpGPX expression also led to higher foliar ascorbic acid, glutathione, and salicylic acid levels in plants exposed to higher light intensities. Our results suggest partially overlapping functions of GPX1 and GPX7. The data further point to specific changes in the chloroplast ascorbate-glutathione cycle due to reduced cpGPX expression, initiating reactive oxygen species and salicylic acid pathways that affect leaf development, light acclimation, basal defense, and cell death programs. Thus, cpGPXs regulate cellular photooxidative tolerance and immune responses.

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Survival under stress depends on the plant’s ability to perceive multiple external stimuli and adjust metabolism and growth accordingly (Rao et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). Reactive oxygen species (ROS) such as hydrogen peroxide (H$_2$O$_2$), singlet oxygen, and superoxide anion radical (O$_2^{-}$) are generated during photosynthesis. While enhanced production of ROS can be destructive to cells, ROS also serve as substrates in metabolism and as signaling molecules in acclimation and defense responses (Foyer and Noctor, 2000; Apel and Hirt, 2004). Plant cells have thus evolved effective mechanisms to modulate steady-state levels of ROS inside and also to some extent outside cells (Mittler, 2002). The redox buffers ascorbate and glutathione and associated enzymes (including superoxide dismutases [SODs], catalases [CATs], ascrobate peroxidases [APXs], monodehydroascorbate, glutathione reductases, peroxiredoxins, and glutathione peroxidases [GPXs]) are crucial for such control (Kliebenstein et al., 1998; Asada, 1999; Rey et al., 2007; Ślesak et al., 2007).

In the natural environment, plants normally experience multiple simultaneous stresses. For example,
drought often occurs together with heat, or high light with chilling. These abiotic stresses commonly involve the generation of excess excitation energy (EEE), which arises as a consequence of absorbed light energy being in excess of the amount required to drive compromised photosynthetic metabolism under such conditions (Baker, 2008). Failure to dissipate EEE can result in programmed cell death (Karpinski et al., 1999; Mühlenbock et al., 2008). Such a situation is not necessarily detrimental to the plant, since this can alter responses to pathogens and wounding (Mullineaux et al., 2000; Karpinski et al., 2003; Chang et al., 2004; Mühlenbock et al., 2007, 2008; Slesak et al., 2007). Accordingly, extensive cross-regulation of pathways governing responses to abiotic and biotic stress stimuli has been observed. For example, responses to biotrophic pathogens, acclimation to conditions that promote EEE, or root hypoxia induce local and systemic reactions resembling those typically observed in response to pathogens (Mateo et al., 2004; Mühlenbock et al., 2007, 2008). Recently, it was demonstrated that systemic acquired resistance (Métraux et al., 1990) and systemic acquired acclimation (Karpinski et al., 1999) share some common metabolic and genetic components (Karpinski et al., 2003; Kiddle et al., 2003; Mateo et al., 2004, 2006; Bechtold et al., 2005; Mühlenbock et al., 2008) and some specific components (Rossel et al., 2007).

GPXs (EC 1.11.1.9) are important ROS scavengers because they have broad substrate specificities and a high affinity for H$_2$O$_2$ (Brigelius-Flohe´ and Flohe´, 2008). Such a situation is not necessarily detrimental to the plant, since this can alter responses to pathogens and wounding (Mullineaux et al., 2000; Karpinski et al., 2003; Chang et al., 2004; Mühlenbock et al., 2007, 2008). Failure to dissipate EEE can result in programmed cell death (Karpinski et al., 1999; Mühlenbock et al., 2008). Such a situation is not necessarily detrimental to the plant, since this can alter responses to pathogens and wounding (Mullineaux et al., 2000; Karpinski et al., 2003; Chang et al., 2004; Mühlenbock et al., 2007, 2008; Slesak et al., 2007). Accordingly, extensive cross-regulation of pathways governing responses to abiotic and biotic stress stimuli has been observed. For example, responses to biotrophic pathogens, acclimation to conditions that promote EEE, or root hypoxia induce local and systemic reactions resembling those typically observed in response to pathogens (Mateo et al., 2004; Mühlenbock et al., 2007, 2008). Recently, it was demonstrated that systemic acquired resistance (Métraux et al., 1990) and systemic acquired acclimation (Karpinski et al., 1999) share some common metabolic and genetic components (Karpinski et al., 2003; Kiddle et al., 2003; Mateo et al., 2004, 2006; Bechtold et al., 2005; Mühlenbock et al., 2008) and some specific components (Rossel et al., 2007).

GPXs (EC 1.11.1.9) are important ROS scavengers because they have broad substrate specificities and a high affinity for H$_2$O$_2$ (Brigelius-Flohe´ and Flohe´, 2003). Their principal activity is thought to catalyze the reduction of H$_2$O$_2$ and lipid hydroperoxide to water and alcohol, respectively, using glutathione as the electron donor (Ursini et al., 1995; Fu et al., 2002). In plants, complementary DNAs encoding proteins with significant amino acid homology to animal GPXs have been isolated and characterized. Their roles in ROS homeostasis and stress signaling are unclear (Criqui et al., 1992; Sugimoto and Sakamoto, 1997; Mullineaux et al., 1998; Li et al., 2000; Jung et al., 2002). However, plant GPX genes are responsive to abiotic stresses, hormone treatments (Roxas et al., 1997; Avsian-Kretchmer et al., 1999; Milla et al., 2003; Sreenivasulu et al., 2004; Miao et al., 2006), pathogens (Criqui et al., 1992), aluminum toxicity (Milla et al., 2002), and wounding (Depege et al., 1998), and a role for GPXs in limiting an oxidative burst and programmed cell death was reported in Arabidopsis (Arabi-
dopsis thaliana; Chen et al., 2004).

In Arabidopsis, GPXs are encoded by a gene family of eight members (AtGPX1 to AtGPX8). Based on primary sequence analysis and transit peptide predictions, AtGPX family members have been assigned to the cytosol (AtGPX2, AtGPX4, and AtGPX6), chloroplast (AtGPX1 and AtGPX7), mitochondria (AtGPX3), and endoplasmic reticulum (AtGPX5; Milla et al., 2003). AtGPX8 has not been reported previously and is most likely a cytosolic enzyme, since it lacks a signal peptide (Supplemental Fig. S1, A and B). The different predicted intracellular locations of these GPXs raised the question of whether individual isoforms have particular functions. GPX1 and GPX7 mRNAs that were used as expression markers for the induction of resistance against Pseudomonas syringae pv glya infection (Levine et al., 1994) and encoded enzymes were indeed subsequently shown to be chloroplastic en-
zymes (Mullineaux et al., 1998). AtGPX1 resides on the thylakoid membrane (Ferro et al., 2003; Peltier et al., 2004) or stroma (Zybailov et al., 2008), while the precise location of AtGPX7 is unclear (Meyer et al., 2005). These two chloroplastic (cp) GPX enzymes have 82% amino acid identity and, therefore, were thought to have overlapping activities (Milla et al., 2003).

We aimed to investigate the contribution of cpAtGPXs to the control of plant stress responses. Here, we have measured the effects of reducing both AtGPX1 and AtGPX7 expression as well as the specific loss of AtGPX7. Our results suggest that cpGPXs have an important regulatory and protective role during acclimation to photoxidative stress conditions and in limiting programmed cell death in response to infection by virulent and avirulent biotrophic pathogens.

RESULTS
Reduced Expression of cpGPXs Compromises Photoxidative Stress Tolerance

To characterize the functions of AtGPX1 and AtGPX7, we generated a series of antisense (AS) transgenic lines in Arabidopsis accession Columbia. Three AS-cpGPX lines (denoted 71-90, 71-92, and 71-93) were selected that exhibited reduced expression of both AtGPX1 and AtGPX7, with line 71-93 showing the most depletion (Fig. 1A). A T-DNA insertion mutant of AtGPX7 (gpx7) that was deficient only in AtGPX7 transcripts was also selected (Fig. 1A). The total GPX activity measured using two substrates, H$_2$O$_2$ and t-butyl hydroperoxide, in the crude foliar extract was not diminished in AS-cpGPX lines 71-90, 71-92, and 71-93 or in the gpx7 null mutant when compared with wild-type plants (Fig. 1B). However, GPX activity in isolated intact chloroplasts was reduced in the AS lines and the gpx7 mutant, with AS-cpGPX 71-93 exhibiting the strongest reduction (up to 75%) of wild-type chloroplast activity (Fig. 1C). The extent of cpGPX depletion was also evaluated at the protein level using anti-cpGPX antibody (Fig. 1D; Supplemental Fig. S2). In order to produce antibodies against cpGPX, we expressed and purified from Escherichia coli a 58-amino acid region between Ser-175 and the C-terminal Ala of GPX7 that is most different from the cytosolic GPX isoforms and has 74% identity to AtGPX1. The anti-cpGPX antibodies recognized an approximately 20-kD band on a western blot corre-
sponding to the size of AtGPX1 and AtGPX7 (Supplemental Fig. S2). Antibodies were purified and tested on western blots of total and chloroplastic protein extracts from wild-type plants and the transgenic 71-90,
71-92, and 71-93 lines. In an ELISA, amounts of cpGPX from total extracts were reduced by 9%, 40%, 44%, and 75% in AS-cpGPX lines 71-90, 71-92, gpx7, and 71-93, respectively, relative to wild-type cpGPX protein levels (Fig. 1D). Since these lines were not affected in the expression of other GPX family members (Supplemental Fig. S1C), they were considered suitable for analysis specifically of cpGPX functions.

Depletion of cpGPX expression did not alter rosette size or plant biomass. Regulation of photooxidative and photoinhibitory stress responses and associated acclimation are closely linked and can be monitored by changes in the maximum quantum efficiency of PSII (Fv/Fm). Fv/Fm of the AS-cpGPX lines was similar to that of wild-type plants when cultivated under low-light conditions (75 ± 10 μmol m⁻² s⁻¹; Fig. 2A). It has been shown that induction of several oxidative stress-related genes (including GPXs and SODs) occurred only under combined cold/high-light treatments that would cause photooxidative stress (Soitamo et al., 2008). This prompted us to test whether the AS-cpGPX lines 71-90, 71-92, and 71-93 or the gpx7 null mutant were more susceptible to photooxidative stress by combined high light and chilling temperature. When

Figure 1. Selected transgenic AS-cpGPX lines and gpx7 have reduced cpGPX activity and protein levels. A, Transcript abundance of AtGPX1 and AtGPX7 in selected transgenic Arabidopsis AS-cpGPX lines (71-90, 71-92, 71-93, and gpx7) measured by reverse transcription-PCR assay using 18S RNA as an internal standard. B, GPX activity measured in crude cell extracts of selected lines. C, GPX activity in extracts of isolated intact chloroplasts. H₂O₂ and t-butyl hydroperoxide were used as substrates and expressed as a ratio compared with wild-type (WT) plants. D, cpGPX protein level measured by ELISA and expressed as a ratio to wild-type plants. The values are represented as percentages of control. Mean values (±SD) of six different plants from three independent experiments (n = 18) are shown. The asterisks indicate the significance of differences from wild-type plants (* P < 0.05, ** P < 0.01).

Figure 2. Analysis of photooxidative stress responses in the AS-cpGPX lines and the gpx7 mutant. A, Maximal photochemical efficiency parameter (Fv/Fm) in lines during cold and photooxidative stress (4 d, HLC) and after 3 d in recovery in laboratory growth conditions. Prior to measurements, plants were dark adapted for 30 min. The arrow indicates Fv/Fm initial values for control plants at time 0 growing at 75 ± 10 μmol m⁻² s⁻¹. B, The difference between Fv/Fm on the leaf abaxial side (Fv/Fm_ab) and the adaxial side (Fv/Fm_ad) is defined as ΔFv/Fm = Fv/Fm_ab - Fv/Fm_ad of LL-acclimated leaves exposed to 1 h of EL. The adaxial and abaxial leaf sides represent palisade and spongy cells in the leaf tissue. Mean values (±SD) of six different plants from three independent experiments (n = 18) are shown. The asterisks indicate the significance of differences from wild-type (WT) plants (* P < 0.05, ** P < 0.01, *** P < 0.001).
2-week-old plants were exposed to a combination of high-light stress (650 ± 50 μmol m⁻² s⁻¹) at chilling temperature (4°C) for 4 d (hereafter called HLC), transient photoinhibition of PSII occurred (Fig. 2A). Reduced \( F_v/F_m \) is indicative of photooxidative stress and photoinhibition (Karpinski et al., 1999; Mateo et al., 2004; Baker, 2008). Decreases in \( F_v/F_m \) were observed in all plant lines after 1 d of exposure to HLC. However, AS-cpGPX lines 71-93 and 71-92 and the \( gpx7 \) mutant displayed a more acute response than the wild type (Fig. 2A). Full recovery in overall \( F_v/F_m \) occurred when plants were returned to ambient low light and room temperature conditions for 3 d. The extent of \( F_v/F_m \) reduction correlated with cpGPX activities and protein levels in the different lines (Fig. 1). The difference between \( F_v/F_m \) on the abaxial side (\( F_v/F_m^{ab} \)) and the adaxial side (\( F_v/F_m^{ad} \)) is defined as \( \Delta F_v/F_m = F_v/F_m^{ab} - F_v/F_m^{ad} \) (Fig. 2B). A substantial \( \Delta F_v/F_m \) increase was observed in low light (LL)-acclimated AS-cpGPX line 71-93 and \( gpx7 \) compared with the wild type after excess light (EL) for 1 h (Fig. 2B). This result further supports the involvement of cpGPXs in the protection of the photosynthetic apparatus and thus the whole plant response to photooxidative stress, with higher photooxidative stress sensitivity on the adaxial side of the leaf in lines with depleted AtGPX1 and AtGPX7 expression. These data suggest that plants depleted in cpGPX expression are more susceptible to conditions that promote moderate photooxidative stress but nevertheless are able to adjust to such a stress.

This conclusion was further supported by the inhibition of the rate of photosynthetic O₂ evolution (photoinhibition) over a range of photosynthetically active photon flux densities in recovery phase (Fig. 3). Significantly lower photosynthetic O₂ evolution saturation rates were evident in AS-cpGPX lines 71-92 and 71-93 (data not shown) and in the \( gpx7 \) mutant grown either in LL or high light (HL; Fig. 3, A and D). For direct comparison, we choose line 71-92 and the \( gpx7 \) mutant, since they have similar reduction of the cpGPX activity (Fig. 1), although they responded in different ways to variable light conditions. After 1 h of exposure to EL (2,000 ± 100 μmol m⁻² s⁻¹) and a 1-h recovery in LL, the LL-acclimated AS-cpGPX 71-92 line and the \( gpx7 \) mutant exhibited similar degrees of photosynthesis inhibition compared with wild-type plants (Fig. 3, B and C). Because the \( F_v/F_m \) remained unchanged in LL-acclimated plants (Fig. 2A), this transient decrease in quantum efficiency suggests that cpGPXs play an important role in protecting PSII function during transient increases in excitation energy (in EEE conditions). This was further illustrated in HL-acclimated plants (Fig. 3, E and F), in which inhibition of photosynthesis after EL exposure was significantly different (as indicated by ANOVA and Tukey’s posttest) in AS-cpGPX line 71-92 compared with the \( gpx7 \) mutant and wild-type plants, even after a 1-h recovery in HL (Fig. 3F). On the other hand, lower oxygen evolution could also result from higher inhibition of the Calvin cycle enzymes due to higher ROS production rates in lines with depleted cpGPX activity under photooxidative stress conditions.

Nevertheless, both the \( F_v/F_m \) and oxygen evolution data (Figs. 2 and 3) suggest that sensitivity to and ability to recover from photooxidative stress and photoinhibition are impaired in plants with diminished cpGPX activity. The most affected plants were plants with diminished expression of both AtGPX1 and AtGPX7 genes. We reasoned that the capacity for sensing or regulating these stresses must be altered in leaves with reduced cpGPX activities.

Transgenic Lines with Reduced cpGPX Activity Have Higher Foliar H₂O₂ Levels

If depleting cpGPXs in Arabidopsis increases plant sensitivity to photooxidative stress by altering PSII activity, what is the underlying mechanism? The principal biological activity of GPXs is to catalyze the reduction of H₂O₂ and/or lipid hydroperoxides and other organic hydroperoxides (Ursini et al., 1995; Fu et al., 2002). However, we did not observe significant differences in lipid peroxidation between LL-cultivated AS-cpGPX and \( gpx7 \) lines and wild-type plants exposed to EL (Fig. 4A). We reasoned that H₂O₂ may be overproduced without any significant change in lipid peroxidation. H₂O₂ content was measured in leaves from plants grown under LL conditions (LL) and LL-acclimated plants after 1 h of EL (LL + EL) using a fluorometric assay. Significantly higher foliar H₂O₂ levels were found in the AS-cpGPX lines (71-92 and 71-93) and the \( gpx7 \) mutant after exposure to EL (Fig. 4B). It is notable that basal foliar H₂O₂ levels of the AS-cpGPX lines 71-92 and 71-93 grown under LL were also higher than in wild-type plants and \( gpx7 \) (Fig. 4B). Increased photooxidative stress in the AS-cpGPX lines 71-92 and 71-93, but not in 71-90 and the \( gpx7 \) mutant, under HL was associated with a significantly higher accumulation of anthocyanins (Fig. 4C). These results further support our conclusion that depletion of cpGPX leads to increased photooxidative stress.

The intensity, duration, and localization of ROS level changes (such as H₂O₂) require a tight regulatory network (Mittler et al., 2004). In plants, this network includes SODs, CATs, APXs, and GPXs together with other antioxidants for effective cellular ROS homeostasis. We first assessed the effect of GPX depletion on SOD activities, since they are major scavengers of O₂⁻ and H₂O₂ producers during photooxidative stress.

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Activity of FeSOD (a chloroplastic form) was not altered in the AS-cpGPX lines 71-92 and 71-93 or gpx7, whereas activities of cytosolic Cu/ZnSOD I, chloroplastic Cu/ZnSOD II, and mitochondrial MnSOD (Kliebenstein et al., 1998) were reduced in AS-cpGPX activity. In the gpx7 mutant, we observed a significant reduction only in cytosolic Cu/ZnSOD I and chloroplastic Cu/ZnSOD II (Fig. 4D). The depletion of cpGPXs in AS-cpGPX lines and the gpx7 mutant was not compensated for by higher expression of CAT, which acts as a cytosolic scavenger of H2O2 (Supplemental Table S1). However, we observed slightly higher total APX activity in line 71-93 and the gpx7 mutant, probably caused by induction of the AtAPX1 gene (Supplemental Table S2).

Leaf and Chloroplast Morphology Is Altered in AS-cpGPX and gpx7 Mutant Plants

Leaf anatomy between the adaxial and abaxial surfaces, consisting of several layers of palisade and spongy mesophyll cells, is highly specialized for light absorption. The increased sensitivity of AS-cpGPX 71-92 and 71-93 lines to photooxidative stress led us to hypothesize that leaf morphology becomes adapted to a lower activity of PSII. Light microscopy of leaf sections from LL-acclimated 8-week-old AS-cpGPX lines revealed shorter and more rounded palisade cells, irregular spongy mesophyll cells, and larger intercellular air spaces compared with the wild type (Fig. 5, A, C, E, G, and I). The altered morphology was particularly pronounced in AS-cpGPX lines 71-92 and 71-93 with larger air spaces (Fig. 5, compare E and G).

We investigated whether there was also a change in chloroplast morphology. Electron microscopy imaging revealed that chloroplasts with reduced cpGPX activity in the AS-cpGPX 71-90 line and the gpx7 mutant had larger and more abundant starch grains than in wild-type and gpx7 mutant plants (Fig. 5, B, D, F, H, and J; samples were taken directly after the dark period). The distribution and number of starch grains in chloroplasts from the AS-cpGPX 71-90 line and the gpx7 mutant were similar to those in the wild type (Fig. 5, compare B and J). By contrast, no differences in the ultrastructure of endoplasmic reticulum, mitochondria, or nucleus were observed among the AS-cpGPX 71-90, 71-92, and 71-93 lines, gpx7 mutant, and wild-type plants (data not shown).

Depletion of cpGPX Reveals Changes in Foliar Levels of Antioxidants and Salicylic Acid

We have shown that the AS-cpGPX lines and the gpx7 mutant have increased sensitivity to transient photooxidative stress (Figs. 2–4). We reasoned that cpGPX depletion likely causes compensatory changes in antioxidant levels in order to cope with the stress; therefore, we measured the accumulation of the major redox buffers ascorbate, glutathione, and salicylic acid (SA). There were no differences in foliar levels of...
ascorbate, glutathione, and SA in plants cultivated in LL conditions (Table I). However, in plants cultivated in HL, foliar ascorbate, glutathione, and SA levels were significantly higher in AS-cpGPX lines 71-92 and 71-93 compared with the wild type. By comparison, only ascorbate levels were higher in gpx7 mutant leaves under HL conditions (Table I).

**R Gene-Triggered Host Cell Death and Basal Defense Responses Are Enhanced in the cpGPX AS Lines**

Current evidence suggests a high degree of coregulation of plant responses to abiotic (EEE) and biotic stresses. We investigated whether the cpGPX AS lines or the gpx7 mutant were altered in their response to isolates of the bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) strain DC3000. Leaves were infiltrated with avirulent *Pst* DC3000 strain expressing *avrRpm1*, and the extent of hypersensitive plant cell death was measured by lactophenol-trypan blue staining. While hypersensitive cell death was restricted to the area around pathogen infection sites in the wild type and the gpx7 mutant, lesions expanded in the AS-cpGPX lines over a period of 72 h after infection (Fig. 6A). We measured the extent of ion leakage as a quantitative indicator of cell death (O’Donnell et al., 2001) by monitoring electrical conductivity of leaf discs 4 d after inoculation with *Pst* DC3000/*avrRpm1*. Similar to the trypan blue staining, the AS-cpGPX lines but not gpx7 displayed more ion leakage than wild-type plants (Fig. 6B). However, growth of *Pst* DC3000/*avrRpm1* was not different in any of the AS plants compared with the wild type. We also measured growth of the virulent strains *Pst* DC3000 (without the *avr* gene) and *Pseudomonas syringae* pv *maculicola* (*Psm*) ES4326. The initial rate of bacterial growth was similar in all tested lines (data not shown). At day 4 after inoculation, however, bacterial titers were at least 10 times lower in the AS-cpGPX lines compared with the wild type (Fig. 6C). Therefore, we concluded that depletion of cpGPX activity enhances basal resistance to virulent bacteria.

**Meta-Analysis of AtGPX Gene Expression Profiles**

In order to identify potentially informative expression patterns for particular GPX-encoded isoforms, a meta-analysis of AtGPX gene expression profiles obtained from publicly available microarray databases (Nottingham Arabidopsis Stock Centre [http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl] and The Arabidopsis Information Resource [http://www.arabidopsis.org/]) was performed (Supplemental Ta-

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Figure 4. Transgenic lines with reduced cpGPX activity have higher foliar H2O2 and anthocyanin levels and reduced SOD activities. A, Quantification of malondialdehyde (MDA) as lipid peroxidation in LL (100 ± 50 μmol m⁻² s⁻¹)-grown plants exposed to 1 h of EL (2,000 ± 100 μmol m⁻² s⁻¹). B, Quantification of foliar H2O2 by a fluorometric assay (Guilbault et al., 1967) from LL-acclimated plants and after 1 h of EL. C, Quantification of anthocyanin content from HL-acclimated plants (450 ± 50 μmol m⁻² s⁻¹). Mean values (±sd) of pooled leaf samples from three different plants from two or three independent experiments (n = 10–15) are shown. The asterisks indicate the significance of differences from wild-type (WT) plants (* P < 0.05). D, Relative activity of four distinct forms of SOD from LL-acclimated plants. Values are expressed in percentages of corresponding SOD activity in wild-type plants (mean ± sd; n = 3; * P < 0.05). FW, Fresh weight.
We found that \textit{AtGPX1} is responsive to most pathogen treatments (11 of 13 samples tested). Moreover, \textit{cpAtGPX1} expression was suppressed at 2 h after infection with \textit{Pst} DC3000 (no expression changes at 24 h), while \textit{cpAtGPX7} was induced at 24 h after inoculation with the same strain (no expression changes at 2 h). These meta-data support a role of \textit{cpAtGPX1} and \textit{cpGPX7} during pathogen infection and support our and other (Levine et al., 1994) experimental observations.

\section*{DISCUSSION}

In this study, we aimed to define the role of \textit{AtGPX1} and \textit{AtGPX7} encoding cGPXs in abiotic and biotic stress responses. Chloroplastic \textit{AtGPX1} and \textit{AtGPX7} have 82\% sequence identity and likely have overlapping activities (Supplemental Fig. S1, A and B; Milla et al., 2003), although their responses to different stimuli differ considerably based on meta-analysis of gene expression data (Supplemental Table S1; Figure 5).

**Figure 5.** Alterations of leaf and chloroplast morphology of \textit{AS-cpGPX} and \textit{gpx7} lines. A, C, E, G, and I, Leaf section images were obtained from light microscopy of 8-week-old plants with LL acclimation (100 ± 50 \mu mol m\(^{-2}\) s\(^{-1}\)): the wild type (WT; A), \textit{AS-cpGPX} lines 71-90 (C), 71-92 (E), and 71-93 (G), and \textit{gpx7} (I). B, D, F, H, and J, Transmission electron microscopy images of chloroplasts from wild-type plants (B), \textit{AS-cpGPX} lines 71-90 (D), 71-92 (F), and 71-93 (H), and \textit{gpx7} (J) with LL acclimation. S, Starch grains. Bars = 25 \mu m in A, C, E, G, and I and 1 \mu m in B, D, F, H, and J.

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Zimmermann et al., 2004). Thus, GPX1 and GPX7 may have some distinct functions in stress responses. Our results lead us to conclude that AtGPX1 and AtGPX7 expression is important for fine-tuning cellular ROS metabolism, photosynthesis, and regulation of light acclimatory and immunodefense responses. Additional unexpected consequences of depletion of cpGPX expression were found in leaf mesophyll and chloroplast morphology.

Fine-Tuning of the Photooxidative Stress Tolerance by cpGPXs

Depletion of AtGPX1 and AtGPX7 expression compromises the plant’s ability to tolerate acute photooxidative stress (Figs. 2–4; Table I). Notably, AtGPX7 was not fully redundant with AtGPX1, reinforcing the notion that there may be a degree of functional specificity between particular chloroplast GPX enzymes. Importantly, we found that increased acute sensitivity to such a stress is proportional to the extent of AtGPX1 and AtGPX7 depletion. Our results suggest that cpGPXs help to regulate photooxidative stress tolerance and are important for optimization of the photosynthetic apparatus performance in conditions that promote EEE, such as chilling temperature combined with HL or EL alone (Soitamo et al., 2008). In addition, different photoinhibitory reactions of wild-type plants and transgenic or mutated lines acclimated to LL or HL and then exposed to EL support the idea of some distinct roles of GPX1 and GPX7 in protection of PSII activity, Calvin cycle activity, and chloroplast-nucleus retrograde signaling (Figs. 2–5).

Lipid peroxidation is a major indicator of oxidative damage in cells and is a marker for membrane perturbation and inactivation of membrane proteins leading ultimately to cell death (Avery and Avery, 2001). We did not observe significant differences in lipid peroxidation among AS-cpGPX lines, the gpx7 mutant, and wild-type plants under EL stress (Fig. 4A). Also HL-acclimated leaves of the ascorbate-deficient Arabidopsis mutant vitamin C defective2 did not exhibit higher levels of lipid peroxidation (Müller-Moulé et al., 2004). These data suggest that cpGPXs predominantly control ROS levels in the hydrophilic phase of the chloroplasts. This was reflected by significantly higher H$_2$O$_2$ levels in both LL-acclimated and EL-exposed AS-cpGPX lines 71-92 and 71-93 compared with wild-type plants (Fig. 4B), increased foliar levels of anthocyanins (Fig. 4C), depleted Cu/ZnSOD and MnSOD activities (Fig. 4D), and slightly induced APX1 gene expression (Supplemental Table S2). Thus, lowered cpGPX activity is associated with higher foliar H$_2$O$_2$, possibly of chloroplast origin, and the results support close cooperation between cpGPXs and Cu/ZnSODs in the ascorbate-glutathione (water-water) cycle (Asada, 1999; Foyer and Noctor, 2000).

### Table I. Foliar levels of conjugated SA, ascorbate, and total glutathione expressed in nanomoles per gram fresh weight of foliar tissue in LL (100 μmol m$^{-2}$ s$^{-1}$)-acclimated and HL (450 ± 50 μmol m$^{-2}$ s$^{-1}$)-acclimated wild-type, As-cpGPX, and gpx7 mutant plants

| Treatment          | Wild Type | 71-90 | 71-92 | 71-93 | gpx7 |
|--------------------|-----------|-------|-------|-------|------|
| Conjugated SA (nmol g$^{-1}$ fresh weight) | LL 110 ± 27 | 122 ± 31 | 97 ± 23 | 118 ± 26 | 121 ± 22 |
|                     | HL 168 ± 32 | 173 ± 35 | 211 ± 25* | 237 ± 38** | 202 ± 36 |
| Ascorbate (nmol g$^{-1}$ fresh weight) | LL 264 ± 39 | 302 ± 41 | 251 ± 36 | 293 ± 31 | 307 ± 43 |
|                     | HL 483 ± 53 | 416 ± 48 | 621 ± 62*** | 643 ± 61*** | 568 ± 69* |
| Glutathione (nmol g$^{-1}$ fresh weight) | LL 67 ± 21 | 85 ± 26 | 82 ± 17 | 75 ± 21 | 91 ± 23 |
|                     | HL 141 ± 24 | 152 ± 35 | 189 ± 27** | 209 ± 31*** | 167 ± 25 |

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**Conjugated SA and total glutathione were measured with HPLC as described by Mateo et al. (2006), and ascorbate was measured spectroscopically as described by Klenell et al. (2005). Results are representative for three independent experiments and pooled samples of three to five leaves from different plants (mean ± SD; n = 3). Asterisks indicate significance (*P < 0.05, **P < 0.01, ***P < 0.001).**
Figure 6. Transgenic lines with reduced cpGPX expression displayed enhanced cell death and resistance to Pseudomonas bacteria. LL (100 ± 50 μmol m⁻² s⁻¹)-acclimated leaves of 4-week-old plants were infiltrated with a bacterial suspension containing 10⁵ colony-forming units (cfu) mL⁻¹ in 10 mM MgCl₂. A, Trypan blue staining was done at 0 (T0), 24 (T24), and 72 (T72) h after inoculation with avirulent Pst DC3000 expressing avrRpm1. B, Electrical conductivity (EC) was measured 4 d after infiltration of leaves with Pst DC3000/avrRpm1 or with 10 mM MgCl₂ (as a nonspecific wound control). C, Bacterial growth at 4 d after inoculation of virulent strains Psm ES4326 and Pst DC3000. Mean values ± SD of n = 45 are shown. Results were obtained from three independent experiments. Asterisks indicate significant differences from wild-type (WT) plants at P < 0.05.

explain effects on MnSOD, which is generally more resistant toward higher H₂O₂ and predominantly located in mitochondria (Dutilleul et al., 2003). On the other hand, no significant reduction of FeSOD activity was observed in AS-cpGPX lines and gpx7 mutant plants showing higher than wild-type accumulation of foliar H₂O₂ (Fig. 4, B and D). This could be due to the fact that FeSOD in Arabidopsis is more resistant to H₂O₂ than Cu/ZnSODs. Alternatively, this result leads us to speculate that FeSOD gene regulation in plants
with deregulated expression of cpGPXs is altered compared with that in wild-type plants.

Anthocyanins act as a powerful antioxidant that helps protect plants from ROS damage (Teng et al., 2005). The higher anthocyanin levels in AS-cpGPX lines under HL conditions (Fig. 4C) are consistent with a further metabolic link between cpGPX activity and anthocyanin synthesis, reinforcing earlier observations (Vanderauwera et al., 2005). Thus, plants with reduced AtGPX1 and AtGPX7 expression are more susceptible to moderate photooxidative stress, and these genes play a specific role in protection of the photosynthetic apparatus and regulation of chloroplastic and mitochondrial ROS homeostasis during EEE conditions. By contrast, Rey et al. (2007) demonstrated that compromised H$_2$O$_2$ scavenging in the chloroplast, as a result of inhibition of expression of a gene encoding chloroplastic sulfiredoxin, leads to an increased tolerance to moderate photooxidative stress, probably because of compensatory changes in other antioxidant defenses (Rey et al., 2007). This suggests that the opposite situation results from what appears to be a similar disruption of H$_2$O$_2$ homeostasis in the chloroplast, reflecting the complexity of the ROS scavenging system and redox homeostasis in the chloroplast.

Chloroplast and mitochondrial homeostasis and retrograde chloroplast-to-nucleus signaling have been shown to control light acclimatory and defense responses (Kiddle et al., 2003; Ball et al., 2004; Mateo et al., 2004, 2006; Mühlenbock et al., 2007, 2008). Our results also suggest that cpGPX activity could be somehow coupled with Cu/ZnSOD and MnSOD activities, for example, through feedback mechanisms that control chloroplastic and mitochondrial ROS homeostasis and, in consequence, photooxidative stress responses (Fig. 4; Table I). Nevertheless, more experimental data are needed to conclude the presence or absence of such a putative feedback mechanism.

Significantly, we found that the adaxial side of a leaf is more prone to photooxidative stress than the abaxial side in the AS-71-93 transgenic line and gpx7 mutant plants (Fig. 2B). This correlates with anatomical changes in the shape of spongy mesophyll cells and the size of air spaces through which CO$_2$ and O$_2$ circulate as well as with starch grain size in chloroplasts (Fig. 2, A, C, E, G, and I) in 71-93 but not in gpx7 plants. To our knowledge, such morphological traits have not been shown to be associated with cpGPXs. These observations reinforce the idea that AtGPX1 and AtGPX7 may have distinct functions, since such traits were not observed in the gpx7 mutant. Moreover, it suggests that AtGPX1 and AtGPX7 expression may be particularly important during natural photooxidative stress in plants growing, for example, in springtime in a temperate climate when reflected sunlight, such as from snow, induces additional stress on the abaxial side of a leaf (Karpinski et al., 1993). Chloroplasts of AS-cpGPX lines, but not the gpx7 mutant, have more starch grains (Fig. 5, B, D, F, H, and J), which could be due to altered redox status of the photosynthetic electron carriers and chloroplast sugar metabolism, especially recycled soluble sugars during photosynthesis. Soluble sugars have been implicated in the regulation of ROS-producing/scavenging metabolic pathways, and this is supported by our previous observation that many different DNA cis-regulatory elements, including those in GPX1 and GPX7 promoter sequences, have dual functions in ROS and Suc signaling (Geisler et al., 2006). These results suggest that cpGPX activity is required for optimization of chloroplast and cellular metabolism under conditions that promote EEE.

Fine-Tuning of the Immunodefenses by cpGPXs

The phenylpropanoid pathway generates complex secondary metabolites such as flavonoids and isoflavonoids (Dixon et al., 2002). Anthocyanins and benzoic acids also derive from phenylpropanoids. The C$_6$C$_6$ benzoic acids include SA as an important defense signal (Winkel, 2004; Strawn et al., 2007). Increased levels of SA were detected in the AS-cpGPX 71-92 and 71-93 lines compared with wild-type plants, correlating with higher foliar ascorbate and glutathione levels (Table I) and enhanced disease resistance (Fig. 6). It may be significant that the highest levels of SA, glutathione, and ascorbate (Table I) were observed in AS-cpGPX line 71-93, which consistently displayed the highest basal resistance to virulent Pst DC3000 and Psm ES4326 bacteria (Fig. 6). The enhanced resistance may be associated with specific redox changes of the plastoquinone pool (Karpinski et al., 1999; Mühlenbock et al., 2008) and subsequent activation of the phenylpropanoid pathway and its related products under EEE conditions (Table I; Mateo et al., 2006).

Ascorbate and glutathione play an essential role in chloroplastic protection against the potentially deleterious effects of ROS, acting as direct antioxidants and fulfilling other functions related to redox sensing and signaling (Wingsle and Karpinski, 1996; Karpinski et al., 1997, 1999; Karpinska et al., 2000; Kiddle et al., 2003; Mateo et al., 2006). Recently, we demonstrated that glutathione and SA signaling are functionally integrated (genetically and physiologically) in acclimation to conditions that promote EEE and in plant immune responses (Mateo et al., 2004, 2006; Mühlenbock et al., 2007, 2008). Moreover, retrograde chloroplast-to-nucleus signaling in response to conditions that evoke EEE in Arabidopsis is regulated by LESION SIMULATING DISEASE1, PHYTOALEXIN DEFICIENT4, ENHANCED DISEASE SUSCEPTIBILITY1, and ETHYLENE INSENSITIVE2 (Mateo et al., 2004; Mühlenbock et al., 2007, 2008). The results presented here point to an important role of cpGPXs in chloroplastic ROS homeostasis and redox signaling between cellular compartments that may coordinate acclimatory and defense responses. Probably, plants have evolved defense mechanisms that depend on light acclimatory responses and retrograde chloroplast sig-
naling that are likely to be crucial to Darwinian fitness in the natural environment, where acclimatisation to prevailing stresses needs to be integrated. Rapid changes in light intensity and quality, humidity, and temperature make acclimatisation to the natural environment an imperative.

MATERIALS AND METHODS

Plant Material

Arabidopsis (Arabidopsis thaliana) plants were germinated and grown in conventional soil (Topstar-Economia Garden) with a thin layer of autoclaved clay pebbles. All experiments were performed in greenhouse conditions transgenic lines photoperiod at 22°C in a relative humidity of 50% to 60% (LL of 100 ± 50 μmol m⁻² s⁻¹ or HL of 450 ± 50 μmol m⁻² s⁻¹ at 22°C). In all experiments, unless otherwise stated, the accession Columbia was used. All mutants used in this study were obtained from the Nottingham Arabidopsis Stock Centre.

EL conditions were described previously (Karpinski et al., 1999). For high light and cold stress (denoted as HLC), 2-week-old seedlings from a long-day photoperiod at 22°C and 50%–60% humidity of 50%–60%) were transferred to continuous high light (650 μmol m⁻² s⁻¹) for 4 d. At day 4, plants were returned to the initial growth conditions and analyzed after 3 d of recovery (called the “recovery period”). The measurements of Fᵥ/Fₘ were done using six technical repeats on six leaves from each line of plants during the HLC and the recovery period in the initial growth conditions. Three independent experiments were performed.

AS Constructs and Selection of Transgenic Plants

The cDNA clone (1810M7) encoding chloroplast GPX (AtGPX1) in plasmid SKII+ (Alting-Mees and Short, 1989) was ordered from the European Molecular Biology Laboratory (http://www.ebi.ac.uk/embli/). A cDNA clone was used to amplify the 500-bp PCR fragments carrying the nonconserved part of the genes (forward, 5'-CGTACGATCCTTCCTACAAGCTGGC-3'; reverse, 5'-GGTCCATTCAC-GTGCAACCTTATCA-3'). The fragment was cloned in the AS orientation in the vector pIT60 (Guerineau and Mullineaux, 1993) between the double cauliflower mosaic virus 35S promoter and the cauliflower mosaic virus 35S terminator. The whole 35S cassettes were subsequently cut out using SstI and HindIII and inserted between StfI and SalI restriction sites of pBIN19 binary vector (Bevan, 1984). Agrobacterium tumefaciens strain GV3101 was used for transformation. The integrity of the construct was verified by sequencing after isolation from Agrobacterium and amplification in Escherichia coli.

Twelve primary transformant lines were isolated and self-pollinated. About 200 plants from the T2 generation for each line were analyzed for kanamycin resistance. Lines segregating with a ratio of 3:1 were kept, and the whole 35S cassettes were selected to create comparable genetic backgrounds. Finally, three transgenic AS-AtGPX lines (71-90, 71-92, and 71-93) and one transgenic knockout of AtGPX7 (gpx7) obtained from the Sainsbury Laboratory Arabidopsis Transposants population but genetically cleaned up in our laboratory and later compiled into an Arabidopsis database (Tissier et al., 1999) were chosen for further experiments.

Isolation of Chloroplasts

Intact chloroplasts were isolated from Arabidopsis leaves according to Weigel and Glazebrook (2002). The leaves were homogenized with a blender in precooled medium containing 50 mM HEPES (pH 7.5), 0.33 M sorbitol, 2 mM EDTA, and 1 mM MgCl₂. The homogenate was squeezed through two layers of Miracloth (Calbiochem) and cheesecloth (SeletaTrade), and the filtrate was centrifuged at 1,300g for 5 min. The pellets were resuspended in the same medium and loaded onto a two-step density gradient with 40% and 80% Percoll (Sigma-Aldrich). After centrifugation at 3,750g for 8 min, the intact chloroplast layer between the 40% and 80% Percoll fractions was collected and verified with a microscope.

cpGPX Antibody Production and ELISA

In order to produce antibodies against cpGPX, a 58-amino acid region between Ser-175 and the C-terminal Ala of AtGPX7, which is the most different from the cytosolic form of GPXs, was chosen (Supplemental Fig. S1). This sequence has been expressed in fusion with thioredoxin protein and His-Tag using the pET32a vector (Novagen) in E. coli BL21DE3 strain. For purification of the fusion protein, we used the Chelating Sepharose Fast Flow (Amersham Biosciences) charged with nickel ions. Purified protein was loaded on a preparative polyacrylamide gel, visualized by Pierce Gel Code staining (Pierce), and then cut out from the gel and used to immunize two rabbits. Immunization of rabbits was conducted by Agrisera on a commercial base. Obtained sera were purified and tested in western blot experiments on total and chloroplastic protein extracts from wild-type plants and transgenic Arabidopsis lines. Both sera gave specific signals of expected molecular mass of approximately 20 kD in the crude protein extract.

For further quantification of cpGPX protein levels in Arabidopsis, ELISA with modification (Mett et al., 2000) was used. The samples, previously quantified using the Bradford assay to ensure equal loading, were transferred to 96-well polystyrene microtiter plates (Nunc-Immuno-Plate, Maxisorb; Nunc) at room temperature for 1 h with shaking. After adding blocking solution (0.5% in phosphate-buffered saline + 0.1% Tween), primary antibodies, secondary antibody, and the color substrate ortho-phenylenediamine (Sigma-Aldrich), the reaction was stopped after 15 min of incubation by the addition of 50 μL of 5 μL sulfuric acid. The optical density was determined at 450 nm on a microplate reader (Bio-Tek Instruments). A pool of samples from six plants for each line was collected, five technical repeats were measured, and three independent experiments were performed.

GPX Activity

The fast chlorophyll a induction kinetics were measured as described previously (Karpinski et al., 1999) using an FMS1 portable modulating fluorimeter, the manufacturer’s software (Hansatech Instruments), and a protocol similar to that described by Gentry et al. (1989). The chlorophyll a fluorescence parameter Fₐ/Fₘ was calculated according to Maxwell and Johnson (2000). Six leaves were used for each time point, and three independent experiments were done. Oxygen-exchange rates were measured in several leaves in gas phase with saturated CO₂ (0.12%) using a Clark-type oxygen electrode (LDZ/3 oxygen electrode chamber) connected to an Oxylab control unit (Hansatech Instruments) and recorded online with a computer using Hansatech software.

Chlorophyll Fluorescence and Oxygen Evolution Measurements

The fast chlorophyll a induction kinetics were measured as described previously (Karpinski et al., 1999) using an FMS1 portable modulating fluorimeter, the manufacturer’s software (Hansatech Instruments), and a protocol similar to that described by Gentry et al. (1989). The chlorophyll a fluorescence parameter Fₐ/Fₘ was calculated according to Maxwell and Johnson (2000). Six leaves were used for each time point, and three independent experiments were done. Oxygen-exchange rates were measured in several leaves in gas phase with saturated CO₂ (0.12%) using a Clark-type oxygen electrode (LDZ/3 oxygen electrode chamber) connected to an Oxylab control unit (Hansatech Instruments) and recorded online with a computer using Hansatech software.

Preparation of RNA and Quantitative Reverse Transcription-PCR

Harvested leaf tissue or other specified parts of the plant were frozen and ground to a fine powder in liquid nitrogen. RNA extraction was performed using a Qiagen RNaseasy plant mini kit (Qiagen) followed by a DNA-free kit (Ambion). The first cDNA strand was synthesized with the RETROscript kit (Ambion). PCRs were performed using specific primers custom made by Invitrogen and combined with 18S RNA as an internal standard (QuantumRNA 18S; Ambion). The accession numbers for the GPX family in Arabidopsis are as follows: AtGPX1 (At2g25080), AtGPX2 (At2g31570), AtGPX3 (At2g30350), AtGPX4 (At2g48150), AtGPX5 (At3g03080), AtGPX6 (At4g11600), AtGPX7 (At4g31870), and AtGPX8 (Atig03460). A pool of samples from three plants for each line was collected, and two independent experiments were performed.

Pathogen Assays

Pseudomonas syringae strains were cultured and prepared for inoculations as described previously (Bartsch et al., 2006). Bacterial dilutions in 10 mm
MgCl₂ was inoculated into the underside of intact leaves of 4-week-old Arabidopsis plants using a syringe without needle. To determine bacterial growth in leaves, leaf discs (1 cm²) were harvested and bacteria were extracted by macerating discs with a plastic pestle in 0.5 mL of 10 mM MgCl₂. Serial dilutions were plated on peptone-yeast extract-glycerol medium plates containing selective antibiotics. Bacterial titers were determined at 0 and 4 d after inoculation. Three leaf discs were taken per plant, and five plants were used for each line and time point. Three independent experiments were performed.

Cell Death Measurements

Development of the hypersensitive cell death response in leaf tissues was monitored by staining with lactophenol-trypan blue and destaining in saturated chloral hydrate as described (Koch and Slusarenko, 1990). Samples were mounted on slides in 60% glycerol and examined using a light microscope. Cell death was quantified by cellular electrolyte leakage from rosette leaves as described by Overmeyer and colleagues (2003). Conductivity was determined after incubating three leaves for 1 h in 5 mL of distilled water with an inolab Cond Level 1 conductivity meter (Wissenschaftlich-Technische Werkstätten GmbH). Three samples were taken per time point from three different plants for each line, and three independent experiments were performed.

H₂O₂ Quantifications

Quantification of H₂O₂ was performed by a fluorometric assay accordingly to the method described by Guibault et al. (1967). Approximately 100 mg of leaf material was collected and immediately frozen in liquid nitrogen. The extraction was carried out on ice in 1 mL of extraction medium (50 mM HEPES, pH 7.5, 1 mM EDTA, and 5 mM MgCl₂). The homogenized tissue was centrifuged for 10 min at 21,000 g and 4°C. Supernatant was mixed (1:1) and vortexed for 30 s with a chloroform:methanol mixture (2:1). Afterward, the mixture was centrifuged for 10 min at 11,000g, and the water phase was collected. This procedure was repeated twice. A total of 400 µL of the water phase was mixed with 2.6 mL of reaction mixture (50 mM HEPES, pH 7.5, 0.5 mM NADPH, 100 µM luminol, 0.25 µM horseradish peroxidase) in a fluorometric cuvette and incubated for 10 min. The measurement of H₂O₂ was made in a Hitachi F2500 fluorometer at 315 and 425 nm for excitation and emission, respectively. A standard line was made using 0 to 33 nmol mL⁻¹ H₂O₂. The values were calculated in nmol g⁻¹ fresh weight. Two independent experiments were performed.

Lipid Peroxidation Quantifications

Malondialdehyde was determined using Bioxytech kit LPO-S86 following the manufacturer's instructions. A pool of leaves from three different plants for each line was taken, five technical repeats were measured, and three independent experiments were performed.

Anthocyanin Quantification

Anthocyanin extraction and spectrophotometric quantification were performed as described by Noh and Spalding (1998). The amount of anthocyanin is presented as the values of A₅₃₅ – 2(A₆₅₃) per gram fresh weight. A pool of leaves from three different plants for each line was taken, five technical repeats were measured, and two independent experiments were performed.

Preparation of Soluble Proteins, Native PAGE, Staining, and Evaluation of SOD Activity

To isolate fractions of soluble proteins, two to three frozen leaves were homogenized in 150 µL of the medium (100 mM Tricine, adjusted with Tris to pH 8, 3 mM MgSO₄, 1 mM diithiothreitol, and 3 mM EDTA). Nonsoluble material was removed by centrifugation for 1 min at 10,000g. Native PAGE was performed at 4°C and 180 V on 12% polyacrylamide gels according to Miszalski et al. (1998). For each lane, 10 or 5 µg of protein extract was applied. Protein concentration was determined according to Bradford (1976) using the Bio-Rad protein assay kit with bovine serum albumin as the standard. Activity of SOD on the gels was visualized by activity staining according to a modified method of Beauchamp and Fridovich (1971). After 20 to 25 min of incubation in the dark at room temperature in staining medium (50 mM potassium phosphate, pH 7.8, containing 1 mM EDTA, 2.8 mM N,N,N′,N′-tetramethyle-
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