The Mitochondrial Type II Peroxiredoxin F Is Essential for Redox Homeostasis and Root Growth of Arabidopsis thaliana under Stress*

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Peroxiredoxins (Prx) have recently moved into the focus of plant and animal research in the context of development, adaptation, and disease, as they function both in antioxidant defense by reducing a broad range of toxic peroxides and in redox signaling relating to the adjustment of cell redox and antioxidant metabolism. At-PrxII F is one of six type II Prx identified in the genome of Arabidopsis thaliana and the only Prx that is targeted to the plant mitochondrion. Therefore, it might be assumed to have functions similar to the human 2-Cys Prx (PRDX3) and type II Prx (PRDX5) and yeast 1-Cys Prx that likewise have mitochondrial localizations. This paper presents a characterization of PrxII F at the level of subcellular distribution, activity, and reductive regeneration by mitochondrial thioredoxin and glutaredoxin. By employing tDNA insertion mutants of Arabidopsis thaliana lacking expression of AtprxII F (KO-AtPrxII F), it is shown that under optimal environmental conditions the absence of PrxII F is almost fully compensated for, possibly by increases in activity of mitochondrial ascorbate peroxidase and glutathione-dependent peroxidase. However, a stronger inhibition of root growth in conditions correlated with its down-regulation (8). Likewise, PRDX5 inhibited p53-dependent apoptosis (9). Of the five peroxiredoxins identified in the Saccharomyces cerevisiae genome, the 1-Cys Prx mTPx (Sc-mTPx) has been localized in the mitochondrion. Its expression is controlled by redox- and carbon-dependent signaling pathways (10), being highly expressed during the stationary phase. Sc-mTPx is thought to protect yeast mitochondria from oxidative stress (11). Of the 10 Prx genes identified in the Arabidopsis genome, 5 are targeted to organelles, i.e. 4 to the chloroplast, and only 1 PrxII F to the mitochondrion (12). The in silico prediction of mitochondrial location of PrxII F was verified in proteomic approaches (13, 14). The function of the peroxiredoxin PrxII F in plant mitochondria is unknown. However, the general presence of a peroxiredoxin in mitochondria in all eukaryotes on the one hand and the presence of a set of peroxiredoxins in chloroplasts with their highly active photosynthetic energy metabolism on the

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The first peroxiredoxin was isolated from yeast 16 years ago by identifying a protein fraction that protected DNA from oxidative breakage and sensitive enzymes from oxidative inactivation in vitro. In these studies, the authors (1) employed a mixed function oxidation system containing Fe²⁺, O₂, and di-thiothreitol (DTT)³ to initiate oxidative damage to the macromolecules. Supplementation of the assay with thiol-specific antioxidant protein (now known as peroxiredoxin) suppressed damage development (1). Prx was shown to detoxify hydrogen peroxide, alkyl hydroperoxide, and peroxynitrite. By cloning and site-directed mutagenesis of the yeast thiol-specific antioxidant, the catalytic cysteiny1 residue (Cys-47) was identified as the primary site of peroxide turnover (2, 3). Peroxiredoxins are grouped into four clans according to sequence similarities, the presence of an additional resolving Cys group at variable positions, the mechanisms of catalysis, and regeneration (4, 5). Human, rat, and mice genomes contain six Prx genes prdx1 to 6, i.e. four 2-Cys Prx, one type II Prx, and one 1-Cys Prx. They are cytosolic, secreted, or targeted to the mitochondrion or peroxisome, respectively. The mammalian 2-Cys Prx (PRDX3) and type II Prx (PRDX5) are localized to the mitochondrion, and the latter was also detected in other subcellular compartments, namely the cytosol, nucleus, and peroxisome (6). In stably overexpressing thymoma cells, PRDX3 was related to the regulation of cellular H₂O₂ concentrations and protection from apoptosis (7); in addition, Alzheimer disease and Down’s syndrome correlated with its down-regulation (8). Likewise, PRDX5 inhibited p53-dependent apoptosis (9). Of the five peroxiredoxins identified in the Saccharomyces cerevisiae genome, the 1-Cys Prx mTPx (Sc-mTPx) has been localized in the mitochondrion. Its expression is controlled by redox- and carbon-dependent signaling pathways (10), being highly expressed during the stationary phase. Sc-mTPx is thought to protect yeast mitochondria from oxidative stress (11). Of the 10 Prx genes identified in the Arabidopsis genome, 5 are targeted to organelles, i.e. 4 to the chloroplast, and only 1 PrxII F to the mitochondrion (12). The in silico prediction of mitochondrial location of PrxII F was verified in proteomic approaches (13, 14). The function of the peroxiredoxin PrxII F in plant mitochondria is unknown. However, the general presence of a peroxiredoxin in mitochondria in all eukaryotes on the one hand and the presence of a set of peroxiredoxins in chloroplasts with their highly active photosynthetic energy metabolism on the

³ The abbreviations used are: DTT, dithiothreitol; Apx, ascorbate peroxidase; AOX, alternative oxidase; Asc, ascorbate; DHA, dehydroascorbate; ETC, electron transport chain; Gpx, glutathione peroxidase; Grx, glutaredoxin; ORP, open reading frame; NTRA, NADPH-dependent thioredoxin reductase A; PDH, pyruvate dehydrogenase; Prx, peroxiredoxin; ROS, reactive oxygen species; RT, reverse transcription; SHAM, salicylhydroxamic acid; Trx, thioredoxin; WT, wild type; TBS, Tris-buffered saline; BSA, bovine serum albumin; CHES, 2-cyclohexylaminoethanesulfonic acid; KO, knock-out; SOD, superoxide dismutase; MOPS, 4-morpholinopropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethylamino]ethanesulfonic acid; m, mitochondria.
other hand (5) are suggestive of an essential role for PrxII F in organellar redox homeostasis.

Mitochondria are the sites of high rate energy metabolism, conversion reactions such as glycine decarboxylation, and the provision of carbon skeletons for a number of biosynthetic pathways, including nitrogen assimilation. They link redox metabolism to ATP synthesis through the mitochondrial electron transport chain (ETC). Reactive oxygen species are released when electron carriers become over-reduced (15). Therefore, the primary line of defense against oxidative stress in mitochondria involves mechanisms to keep the electron transport chain oxidized, i.e., by balancing substrate oxidation with ATP requirement, as well as activation of alternative oxidase (AOX), uncoupling proteins, and rotenone-insensitive NAD(P)H dehydrogenase (15–18). With increasing reduction of AOX, uncoupling proteins, and rotenone-insensitive ATP requirement, as well as activation of alternative oxidase (AOX), uncoupling proteins, and rotenone-insensitive NAD(P)H dehydrogenase (15–18). With increasing reduction of the ETC, mitochondria release ROS at rates probably exceeding those measured in animal mitochondria where 1–5% of consumed O2 was diverted into ROS production (15). In plants, ROS production decreased with activation of AOX (19), which has been shown to be activated by the reduction of their regulatory thiol groups (20). Likewise, a search for mitochondrial thiol proteins interacting with thioredoxin has produced a list of 50 putative targets for redox regulation by thiolsulfine transition, including various enzymes of the Krebs cycle and the permeability transition pore (21). Thus, similar to chloroplasts, the component activities of an intricate network of redox systems are modulated depending on the redox environment of the mitochondrial matrix. Mitochondrial metabolism with a basic activity under conditions of oxidized ETC may undergo a phase of reductive activation with increasing electron pressure, before oxidative damage develops when excess reducing power generates ROS. Changes in the mitochondrial redox state will not only affect mitochondrial activities but also other cellular processes such as photosynthesis, stress defense, and initiation of programmed cell death (22–24).

Detoxification of ROS and repair of oxidative damage in the mitochondrion counteract the expression of oxidative stress. A set of antioxidant enzymes has been identified and (partially) characterized in plant mitochondria. Mn-SOD converts superoxide anion radicals to hydrogen peroxide that may be detoxified by ascorbate peroxidase; oxidized monodehydroascorbate is regenerated through the ascorbate/glutathione cycle. Chew et al. (25) demonstrated dual targeting of ascorbate peroxidase (At4g08390), monodehydroascorbate reductase (At1g63940), and glutathione reductase (At3g54660) to both the plastid and mitochondrion, whereas a specific dehydroascorbate reductase (At3g54660) was solely imported into the mitochondria. The work extended previous investigations that suggested dual targeting of glutathione reductase (26) and solved the contradiction between reported activities of the respective enzyme activities in mitochondrial preparations and the lack of corresponding genes with unequivocal information for mitochondrial targeting in the Arabidopsis thaliana genome (27). Recently, PrxII F was identified as mitochondrial type II peroxiredoxin. Many peroxiredoxins have a broad activity toward peroxide substrates (12, 28–30) but may also act as reductors (4, 31). Therefore, the aim of the study was to characterize the function of the mitochondrial PrxII F in plant cell metabolism. To this end and in addition to the determination of its basic catalytic activity, subcellular and tissue distribution, Arabidopsis lines lacking PrxII F proteins should be analyzed in order to characterize PrxII F function in mitochondrial antioxidant metabolism and redox homeostasis and evaluate its importance for plant phenotype and fitness.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

For growth on hydroponic cultures, Arabidopsis seedlings (ecotype Columbia) of wild type, KO-PrxII F-1, and KO-PrxII F-2 were germinated and grown on nutrient solution as described in detail (32). The hydroponic medium contained 1.5 mM KNO3, 1.5 mM Ca(NO3)2, 0.25 mM MgSO4·7H2O, 0.5 mM Ca(H2PO4)2, 5 μM Fe-EDTA, 0.4 μM ZnSO4, 1 μM KCl, 25 μM H3BO3, 2 μM MnSO4, 0.1 μM CuSO4, 0.1 μM CaCl2, 0.5 mM MES (pH 5.25). Five-week-old plants were treated for 1 week with 10 μM CdCl2 or 25 μM SHAM, respectively. Nutrient solution was replaced every 2nd day. Growth chamber conditions were adjusted to a 10:14-h day/night cycle (22:20 °C) at light intensities of 120 μmol quanta m−2 s−1 with 50% relative humidity. Plants were harvested 3 h after the onset of illumination. Entire roots of 17 plants per experiment were pooled, immediately frozen in liquid nitrogen, and stored at −80 °C.

For growth on plates, seedlings were grown for 10 days in a vertical position on 0.5% of phytagel with nutrient solution, where the macroelements KNO3 and Ca(NO3)2 were replaced with equimolar amounts of KCl and CaCl2, to enhance root elongation. The plates were supplemented with 7.5 μM CdCl2 or 25 μM SHAM.

**Identification of tDNA Insertion Lines**

The first mutant allele KO-AtPrxII F-1 (GABI-Kat line114G01) was obtained from the GABI-Kat collection of tDNA insertion lines (33), the second mutant allele KO-AtPrxII F-2 was obtained from the Sail collection (34). Individual plants of the T3 generation were screened for tDNA insertion by PCR using gene-specific primers and primers anchored in the tDNA borders. Homozygous plants were identified by the absence of PrxII F-PCR product obtained from gene-specific primers, which compose the tDNA insertion site. The following primers were used for the prxII F ORF: prxII F-5′-TCAAAAGCTCAGGAAAGGACT-3′; prxII F-3′-TAGATCTGCTCCTAAGA-3′; and the following primers were used for the tDNA left borders: GABI tDNA, 5′-ATATTGACCATGACTACCTGAC-3′; Sail tDNA, 5′-TGACATCGTAATTTTCATACCCATCGATAC-3′.

**Heterologous Expression and Purification of Recombinant Proteins**

Δ28-AtPrxII F was cloned as described previously (35). For cDNA amplification of Atgrx-CxxS510, the following primers were used to obtain a product that lacks the N-terminal 7-amino acid signal peptide: grx-Cxxs510 F, 5′-gaggtcagaaagacgtgctc-3′, and grx-Cxxs510 R, 5′-taaggccaaagcaccagctt-3′. The cDNA was cloned in-frame into the pCR® II/NT-TOPO vector (Invitrogen), which allows expression and purification of an N-terminally His6-tagged recombinant protein. Expression and purification of the recombinant proteins were performed as described (12).

**Peroxiredoxin Activity Assays**

In vitro peroxidase activity of recombinant PrxII F was measured in two different assays.

**Xylenol Orange Assay—PrxII F (1–30 μM) was maintained in its reduced state by 10 mM DTT or 0.5–5 mM GSH in presence or absence of 14 μM Grx-CxxS10, respectively. The reduction of H2O2, t-butyl hydroperoxide, cumene hydroperoxide, phospholipid hydroperoxide, and phosphatidylcholine dilinoleoyl hydroperoxide was measured in a time course over 2 min, in 20-s intervals. The remaining peroxides were detected by ferrous-dependent oxidation of xylenol orange (FOX). 20 μl of the reaction was incubated with 1 ml of FOX reagent for 5–15 min for different substrates, respectively. The absorbance was measured and compared with standard curves established for each substrate at 560 nm. The FOX reagent contained 25 mM H2SO4, 100 mM sorbitol, 250 μM Fe((NH4)2(SO4)2), and 125 μM xylene orange, respectively. The activity test was repeated 5–10 times with enzymes from two different preparations. Synthesis of phosphatidylcholine dilinoleoyl hydroperoxide and linoleic acid hydroperoxide was performed as described (36).

**Ttx-dependent Activity Assay—**The rates of H2O2 reduction were determined in a coupled assay with mitochondrial Arabidopsis thiooxidan-t (Ttx-0) and NADPH-dependent thioredoxin-reductase (NTRA) by monitoring because rease in A560 because of NADH oxidation. The assay contained 100 mM MOPS-KOH (pH 7.4), 1 μM PrxII F, 3 μM AtTtx-0, 0.3 μM NTRA, and 1 mM NADPH in a final volume of 1 ml. The reaction was started by the addition of H2O2 to a final concentration of 1 mM and the change in A560 followed.

**Antibody Production and Western Blot Analyses**

An antisera against PrxII F protein heterologously expressed in E. coli was generated in rabbit (Pineda). For Western blot analysis,
frozen plant material was ground to a fine powder in liquid N₂, and proteins were extracted in a buffer containing 250 mM Tris-Cl (pH 6.8). The protein contents of the aqueous extracts were quantified spectrophoto-
metrically as described (Bio-Rad) according to the manufacturer's instructions. SDS-PAGE and Western blot analysis were performed as described (12).

**Immunocytochemistry**

Immunocytochemical analysis was performed as described (37). Sections of 1 mm² were cut from 4-week-old Arabidopsis leaves of wild type and KO-PrxII F-1 plants and immediately fixed in 2.5% (v/v) glutar-
dehyde in EM buffer (50 mM Bio-Rad, pH 7.4, 0.1% NP-40, (pH 7.0) for 45 min. After dehydration in acetone gradients, the samples were embedded stepwise in TransMit EM resin (TAAB Laboratories Equipment). Ultrathin sections of 60–70 nm were cut with a diamond knife (DuPont) on an ultratract microtome (Reichert Ultracut E) and placed onto 400 mesh gold grids.

Samples were immunolabeled with rabbit anti-PrxII F antisera (1:100 diluted in bovine serum albumin-containing Tris-buffered saline (BSA/TBS)) supplemented with 0.05% (w/v) NaN₃ for 1 h. Grids were rinsed five times with TBS and incubated for 1 h with goat-conjugated (15 nm) anti-rabbit IgG (Sigma) at a 1:30 dilution in BSA/TBS. Samples were counterstained with 0.1% (w/v) uranyl acetate (5 s) followed by 2% (w/v) lead citrate (5 s). Preparations were examined with a Hitachi H500 electron microscope at 75 kV.

**Isolation and Subfractionation of Mitochondria**

Mitochondria were isolated from Arabidopsis rosette leaves as fol-
lows. All procedures were done at 4 °C; all apparatus and plasticware were detergent-free. Rosette leaves (20–50 g fresh weight) were harvested from 6-week-old Arabidopsis plants grown under short day conditions (8-h photoperiod), washed in sterile distilled water, and blotted dry on tissue paper. Mitochondria were extracted by homogenizing leaves using a pestle and mortar containing 4 volumes of extraction medium (0.4 M mannitol, 50 mM sodium pyrophosphate (pH 8.0), 0.5% (w/v) BSA, 1.0% (w/v) polyvinylpyrrolidone-40, 2 mM EGTA, 20 mM cytochrome). A small amount of acid-washed sand. The extract was filtered through Miracloth (Merck), and unbroken cells and chloroplasts were removed by low speed centrifugation (1000 × g, 5 min). Mitochon-
dria were pelleted by high speed centrifugation (18,000 × g, 15 min) and resuspended in wash buffer (0.3 M mannitol, 10 mM TES-KOH (pH 7.5)). The rounds of low and high speed centrifugation were repeated. The resulting mitochondrial pellet was resuspended in 1 ml of wash buffer and layered onto a Percoll (Amersham Biosciences) gradient consisting of 0–5% polyvinylpyrrolidone-40 in 25% (v/v) Percoll in wash buffer. After centrifugation at 43,000 × g for 30 min, mitochondria were visible as a white/brown band toward the lower part of the gradient. Mitochon-
dria were collected by aspiration and washed twice in wash medium. Mitochondria were further subfractionated into outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, and matrix according to Ref. 38.

**Respiratory Measurements and Enzyme Assays**

Measurements of mitochondrial respiration were done as described previously (14). Activities of aconitase, fumarase, NAD-malic enzyme, and pyruvate dehydrogenase were assayed according to Ref. 39. Apx was assayed according to Ref. 40. Gpx was assayed according to Ref. 41.

**Root O₂ Uptake**

Root O₂ measurements were performed similar to Ref. 42. Total roots from hydroponically cultivated plants were excised and directly trans-
ferred to a high grade stainless steel net in EM buffer (5 mM Bio-Rad, 10 mM K₂CO₃, 0.5 ml of an air-saturated 2 mM Ca(NO₃)₂ solution at 20 °C). SHAM was used as a respiratory inhibitor at a final concentration of 7.5 mM. Respiratory activity from single plant roots was recorded over 10 min.

**Metabolite Measurements**

For metabolite analysis 100 mg of frozen leaf and root tissue from hydroponic plants, respectively, were ground in liquid nitrogen. ATP contents were determined luminometrically according to Ref. 43. The frozen tissue was homogenized in 1 ml of ice-cold 5% HClO₄ and cen-
trifuged at 13,000 rpm at 4 °C. 10 μl of 2 M Tris was added to 500 μl of the supernatant, and the pH was adjusted to 7.4 with 7.5 μl of K₂CO₃. The cleared extract was diluted in 50 mM HEPS/KOH, 5 mM MgCl₂ (pH 7.4). 10 μl of crude firefly lantern extract (Sigma) was added, and emitted light intensities were measured in a luminometer, which al-
lowed for direct quantification of ATP when compared with authentic standards. For ascorbate and dehydroascorbate measurements, the tissue was extracted in 600 μl of ice-cold 1 M HClO₄. Asc contents were determined spectrophotometrically via the oxidation of ascorbate with potassium ferricyanide as described (Bio-Rad) according to the manufacturer’s instructions. SDS-PAGE and Western blot analysis were performed as described (12).

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2. A. Kandlbinder, unpublished.
Arabidopsis genome, and the only Prx targeted to Arabidopsis mitochondria (12, 29, 30). The alignment of Fig. 1 includes six PrxII F amino acid sequences from diverse higher plants, and one each from the moss Physcomitrella patens, Homo sapiens, and the cyanobacterium Synechocystis. Furthermore, two sequences encoding cytosolic Arabidopsis type II Prx were also compared as more distantly related genes. In addition to the amino acid sequence motifs typical for all type II Prx, such as the sequences adjacent to Cys-64 and Cys-89, PrxII F proteins from plants were characterized by three highly conserved domains not found in other type II Prx, suggesting early separation of the mitochondrial isoform from the other type II Prx. All PrxII F-like proteins possess a mitochondrial targeting sequence. These unique PrxII F sequence sites are positively charged and hydrophilic and are suggested to be important for function and interaction of PrxII F with other proteins, respectively. The plant PrxII F proteins are highly similar, for example the Orzya sativa PrxII F shows 73% identity to Arabidopsis PrxII F and from P. patens there is still a remarkable identity of 62%. It is interesting to note that up to now no expressed sequence tags of PrxII F homologues were reported from unicellular eukaryotes.

**Statistical Analysis**

Statistical analyses were carried out with STATISTICA for WINDOWS software (version 5.5, Stat software). The significance of differences was tested at $p < 0.05$ by using analysis of variance with post-hoc LSD or Student's $t$ test, as indicated.

**RESULTS**

**PrxII F Protein Sequences Contain Plant-specific Elements**—PrxII F is one of six type II peroxiredoxins encoded in the *Arabidopsis* genome, and the only Prx targeted to Arabidopsis mitochondria (12, 29, 30). The alignment of Fig. 1 includes six PrxII F amino acid sequences from diverse higher plants, and one each from the moss *Physcomitrella patens*, *Homo sapiens*, and the cyanobacterium *Synechocystis*. Furthermore, two sequences encoding cytosolic *Arabidopsis* type II Prx were also compared as more distantly related genes. In addition to the amino acid sequence motifs typical for all type II Prx, such as the sequences adjacent to Cys-64 and Cys-89, PrxII F proteins from plants were characterized by three highly conserved domains not found in other type II Prx, suggesting early separation of the mitochondrial isoform from the other type II Prx. All PrxII F-like proteins possess a mitochondrial targeting sequence. These unique PrxII F sequence sites are positively charged and hydrophilic and are suggested to be important for function and interaction of PrxII F with other proteins, respectively. The plant PrxII F proteins are highly similar, for example the *Orzya sativa* PrxII F shows 73% identity to *Arabidopsis* PrxII F and from *P. patens* to *Arabidopsis* there is still a remarkable identity of 62%. It is interesting to note that up to now no expressed sequence tags of PrxII F homologues were reported from unicellular eukaryotes.

**In Vitro Peroxidase Activity Is Preferential for Reduction of Hydrogen Peroxides Rather than Reduction of Alkyl Hydroperoxides**—Many Prx possess a broad specificity toward various peroxide substrates (36, 45, 46). In a previous study (12), a heterologously expressed PrxII F protein catalyzed hydrogen peroxide reduction. However, a cytosolic type II Prx from popular reduced complex lipid hydroperoxides almost as efficiently as hydrogen peroxide (28). Both peroxide detoxification activities are important for mitochondria, because the generation of...
Mitochondrial Atypical Grx Are Electron Donors for AtPrxII F

In vitro peroxide reducing activity of heterologously expressed PrxII F protein. A, PrxII F activity with various substrates was analyzed in a nonenzymatic activity assay using 10 mM DTT as electron donor for regeneration of oxidized Prx protein: 500 μM hydrogen peroxide (H₂O₂), 250 μM t-butyl hydroperoxide, 250 μM cumene hydroperoxide (COOH), 250 μM linoleic acid hydroperoxide (LOOH), and 300 μM phosphatidylcholine dilinoleoyl hydroperoxide (PLOOH). B, H₂O₂ (250 μM) reducing activity of 6.8 μM PrxII F using 0.5 or 5 mM glutathione in the presence and absence of 14 μM Grx-CxxS10 as an electron donor. C, peroxidase activity of PrxII F using the Arabidopsis mitochondrial thioredoxin system as an electron donor. PrxII F was incubated with H₂O₂, NADPH, and components of the Arabidopsis mitochondrial thioredoxin system as indicated. Activity was measured by following the oxidation of NADPH. D, bandshift of recombinant PrxII F protein in a Coomassie Blue-stained SDS-PAGE in the presence of H₂O₂. 10 μg of PrxII F protein was incubated for 30 min with either 10 mM DTT and 25 mM or 50 mM H₂O₂, respectively.

Glutathione as Well as the Mitochondrial Trx System and Mitochondrial Atypical Grx Are Electron Donors for AtPrxII F—Oxidized Prx must be reduced prior to the next catalytic cycle. In vitro electron donors for Prx are thioredoxins, glutaredoxins, cyclophilins, and glutathione, respectively (37, 48, 49). Although for poplar Prx II, a preference for the glutaredoxin system was shown (29, 49), the Prx II of S. cerevisiae was reduced by thioredoxin (50). Arabidopsis mitochondria contain a thioredoxin system, consisting of Trx and a NADH-dependent thioredoxin reductase (NTRA) (51). Bioinformatic analyses of the Arabidopsis genome indicated that there are two glutaredoxins (28) and two cyclophilins (52) in Arabidopsis mitochondria, which could act as reduction partners of PrxII F. Therefore, in vitro activity tests were performed employing either recombinant AtTrx-o, AtNTRA, and NADPH on the one hand or recombinant AtGrx-CxxS10 (A3g21460; nomenclature according to Ref. 28) and glutathione on the other hand (Fig. 2, B and C) as reduction partners for PrxII F. Both systems regenerated reduced PrxII F. Glutathione alone was also capable of acting as an efficient electron donor to PrxII F.

Substrate-dependent dihydrothiol-disulfide transition involving the Cys group in the active site of Prx induces major conformational changes with concomitant modification of quaternary structure and an electrophoretic mobility shift (36, 53). For type II Prx, the formation of an intramolecular disulfide bond could be observed, whereas 2-Cys Prx form intermolecular dimers. For recombinant mature Δ-28AtPrxII F, both forms were observed, similar to the results obtained with recombinant AtPrxII B and AtPrxII E of Breihelin et al. (29). Oxidation with H₂O₂ produced a bandshift after loading recombinant PrxII F protein on an SDS-polyacrylamide gel (Fig. 2D). At lower H₂O₂ concentrations, the formation of the intramolecular disulfide bridge was predominantly observed, which could be distinguished from the reduced form by its slightly increased electrophoretic mobility. At higher H₂O₂ concentration, the presence of the dimeric form increased.

AtPrxII F Is Predominantly Located in the Mitochondrial Matrix—Beside the mitochondrial targeting peptide of 28 amino acids, the mitochondrial localization of the PrxII F protein can also be independently inferred from three different Arabidopsis mitochondria proteome projects (13, 14, 54) and one rice proteome project (55). An antiserum against PrxII F was generated by immunizing rabbits with purified recombinant protein from Arabidopsis. The antiserum was tested for cross-reactivity with other peroxiredoxins and was found to be specific for PrxII F (data not shown). In immunocytochemical studies using that antibody, a low number of clear signals were observed only for mitochondria of Arabidopsis leaves and roots (Fig. 3A). In order to address the question further, whether the PrxII F is attached to the membrane, as observed previously for the chloroplastic 2-Cys Prx (37), or whether it is located in the matrix, isolated Arabidopsis mitochondria were subfractionated to isolate outer mitochondrial membrane, the intermembrane space, the inner mitochondrial membrane, and the matrix. An antibody against fumarase (56) was used to exemplify the distribution of a matrix-localized enzyme. As shown in Fig. 3B, the dominant signal for PrxII F is in the matrix. Some signal was also observed in the inner mitochondrial membrane and intermembrane space fractions, but this most likely represents a small degree of cross-contamination during subfrac-
ionation, because a similar pattern is also seen with fumarase.

Identification of Two Independent Arabidopsis tDNA Insertion Lines—Two independent Arabidopsis tDNA insertion lines (accession Columbia) were obtained from GABI-KAT (KO-PrxII F-1) (33) and from the SAIL collection (KO-PrxII F-2) (34), both with a tDNA insertion in the third intron of the prxII F gene (At3g06050) (Fig. 4A). The presence of the tDNA was confirmed by PCR with genomic DNA from both lines (Fig. 4, B and C). Segregation analysis for the proof of a single tDNA insertion was performed for both lines. For KO-PrxII F-1 from 98 germinated heterozygote T2 seeds, 73 seedlings, or 74.5%, were resistant to phosphinotricin, indicating a high probability of only one insertion locus. RNA was isolated from homozygous resistant to sulfadiazine, indicating a high probability of only one insertion locus in the genome. For KO-PrxII F-2, from 92 germinated heterozygote T2 seeds, 59 seedlings, or 64.1%, were resistant to phosfinotricin, indicating a high probability of only one insertion locus. RNA was isolated from homozygous T3 plants, and RT-PCR analysis confirmed the absence of PrxII F transcript in both lines (Fig. 4, B and C). Western blot analysis with the antiserum against PrxII F and fumarase, respectively.

Elevated Antioxidant Enzyme Activity in KO-PrxII F Mitochondria—The first phenotypical analysis of KO-PrxII F mutants was done in soil culture under standard growth conditions. No appreciable differences in biomass production, leaf size, or flowering between wild type (WT) and mutants could be observed (data not shown). There are two possible explanations for this lack of a growth phenotype. First, that absence of PrxII F had no major effect on mitochondrial biochemistry. Second, other mechanisms were induced to compensate for the loss of PrxII F. To address this issue, mitochondria from mature leaves of wild type and KO-PrxII F mutants were isolated, and respiratory activity as well as enzyme activities of selected trichloroacetic acid cycle enzymes and antioxidant enzymes were measured (Table I). The respiratory activity of the four complexes of the respiratory chain did not show a major alteration as a result of the absence of PrxII F protein, and only the activities of complex I and II were slightly decreased. Some enzymes of the trichloroacetic acid cycle, such as aconitase, the E2 subunit of the PDH and NAD-malic enzymes, are very sensitive to oxidative stress (47, 57). However, the activities of NAD-malic enzyme, aconitase, and PDH were unchanged in the KO-PrxII F mutants in comparison to wild type. The activity of fumarase was significantly elevated in the KO-PrxII F-2 mutant. Taking a closer look at the antioxidant enzymes, it is very remarkable that the activities of the Apx as well as that of a Gpx are highly increased in the KO-PrxII F mitochondria in comparison to the wild type. Apparently, the loss of PrxII F protein decreases detoxification of peroxides in mitochondria, a process that is compensated for by increased activities of Apx and Gpx under control conditions.

**Growth of KO-PrxII F Plants Indicates an Important Role of PrxII F in Root Growth under Conditions of Oxidative Stress**—Respiratory energy production is particularly important in heterotrophic tissue with high metabolic activity. Accordingly, mitochondria are highly active in meristems and root growth zones and essential for rapid root growth. Root extension of KO-PrxII F was tested by growing seedlings vertically on sterile phytagel plates. Root growth was further stimulated by omitting nitrogen sources from the media. Among the stresses tested, like sodium chloride and copper, the strongest inhibitory effects on root growth of KO-PrxII F mutants as compared with wild type were seen in response to cadmium, to salicylhydroxamic acid (SHAM), an inhibitor of alternative oxidase (Fig. 5), and to antimycin A (data not shown). Root growth of KO-PrxII F-1 and KO-PrxII F-2 was stronger inhibited under cadmium and SHAM treatment than the root growth of wild type. In the case of cadmium stress, the degree of inhibition in KO mutants was increased by 60 and 27% as compared with wild type, and in the case of SHAM treatment by 41 and 19% for F-1 and F-2, respectively, indicating that fitness of the knock-out plants is reduced in oxidative stress conditions. In addition, on media supplemented with nitrogen, both treatments had stronger inhibitory effects on the KO-PrxII F mutants than on wild type (data not shown). To increase root biomass for subsequent biochemical analyses, the plants were grown on hydroponic culture supplemented with nitrogen for 5 weeks and then stressed with 10 μM cadmium or 25 μM SHAM for 1 week, respectively.

**Root Respiration and ATP Status—** Cd²⁺ is highly toxic to cell metabolism because of its high affinity to sulfhydryl and amino groups. Furthermore, Cd²⁺ may substitute for Ca²⁺ and other metal cofactors in reaction centers of enzymes (58). In relation to mitochondrial function, inhibitory as well as stimulatory effects of Cd²⁺ on respiration and increased permeabilization of mitochondrial membranes have been reported for plants and animals (59). Particularly, at low Cd²⁺ concentrations, a stimulating effect on respiration and ATP production was often observed in plants (e.g. Refs. 60 and 61). Root respiration of total roots was measured in a Clark-type O₂ electrode. After measuring total respiration for 10 min, 7.5 mM SHAM was added to completely inhibit AOX, and residual respiration was determined. Both Cd²⁺ and SHAM stimulated respiration in wild type and KO-PrxII F-1 roots, and the ATP content increased (Fig. 6, A and B). This enhancement was stronger in the SHAM than in the Cd²⁺-exposed plants. The percentage of alternative respiration amounted to about 16–20% under control conditions. The figure increased to 30–44% in treated roots. Respiration of wild type and KO-PrxII F-1 roots was not significantly altered under control conditions nor in the presence of 10 μM Cd²⁺. In a converse manner, respiration of roots grown in 25 μM SHAM was significantly induced in KO-PrxII F-1 compared with wild type. Alternative respiration was similar in SHAM-treated WT and KO-PrxII F-1 plants, indicating that cytochrome oxidase-dependent respiration was stimulated more in KO-PrxII F-1 plants (by about 75%) than in WT.
Although residual respiration was higher in KO-PrxII F-1, the ATP content was unchanged in comparison to WT plants treated with SHAM (Fig. 6B), probably due to elevated alternative NAD(P)H dehydrogenase activities or uncoupling leading to a reduction of proton motive force. Another possibility is that the stimulated respiration resulted from other O₂-consuming enzymes. In plasma membranes of plant roots, a rapid SHAM-stimulated NADH oxidation was often observed, which is accompanied by O₂ consumption and H₂O₂ production and can stimulate root respiration by up to 40% (62, 63). However, these effects were not observed under concentrations below 1 mM SHAM (63).

### Table 1

Activity of respiratory complexes, trichloroacetic acid cycle enzymes, and mitochondrial antioxidant enzymes in WT and KO-PrxII F

Mitochondria were isolated from mature Arabidopsis leaves 6 h into the photoperiod. O₂ consumption was assayed with various substrates in the presence of ADP (state III). Trichloroacetic acid cycle enzymes and antioxidant enzymes were assayed in lysed mitochondria. All activities were corrected for mitochondrial yield based on the recovery of fumarase activity. ND, not determined.

| Enzyme Activity (corrected for mitochondrial yield based on fumarase recovery) (n = 3) | SAIL line | GABI line |
|---|---|---|
| | WT | KO-PrxII F-2 | WT | KO-PrxII F-1 |
| External NADH dehydrogenase | 7.3 ± 0.4 | 6.2 ± 0.5 | ND | ND |
| Complexes I and II (malate/pyruvate) | 2.3 ± 0.1 | 1.8 ± 0.1* | ND | ND |
| Complex II (succinate) | 3.4 ± 1.1 | 2.2 ± 0.1 | ND | ND |
| Complex IV (cyclooxygenase) | 5.4 ± 1.0 | 5.8 ± 2.1 | ND | ND |
| Fumarase | 13.5 ± 0.1 | 16.0 ± 0.8* | 15.4 ± 0.6 | 16.7 ± 0.2 |
| Pyruvate dehydrogenase | 0.7 ± 0.3 | 0.7 ± 0.1 | ND | ND |
| Aconitase | 4.2 ± 0.7 | 5.0 ± 0.2 | 4.9 ± 1.1 | 3.5 ± 0.3 |
| NAD-malic enzyme | 13.0 ± 0.5 | 12.2 ± 0.3 | 8.6 ± 0.2 | 9.3 ± 0.4 |
| Ascorbate peroxidase | 2.7 ± 0.2 | 6.2 ± 0.2* | 4.7 ± 0.3 | 8.3 ± 1.0* |
| Glutathione peroxidase | 4.0 ± 0.2 | 22.9 ± 4.9* | 2.7 ± 0.7 | 4.8 ± 0.5* |

*Values indicate significant differences from wild type (t test, p < 0.05).
redox homeostasis. Enzymes of the ascorbate/glutathione cycle for H$_2$O$_2$ reduction have been found in the chloroplast, cytosol, and mitochondrion. In addition, the peroxide detoxification activity of PrxII F was sustained with glutathione as an electron donor (Fig. 2B). Because activities of mitochondrial Apx and Gpx were increased in KO-PrxII F (Table I) and the last step of ascorbate synthesis is linked to the electron transport chain (64), the question was addressed whether there is a change in ascorbate and glutathione pools in the roots of wild type and KO-PrxII F-1. An increase in total root Asc was seen upon Cd$^{2+}$/SHAM treatment, both in WT (54%) and KO-PrxII F-1 (71%); Asc contents in SHAM-treated roots was unaltered. The reduction state of ascorbate was significantly increased from 65 to 73% reduced ascorbate in control conditions to 90–94% in Cd$^{2+}$/SHAM treatment. Asc contents of WT and KO-AtPrxII F were not significantly different in any treatment; however, there was a regular trend to decreased total Asc levels in KO-PrxII F-1, i.e. by 13–15% in control and SHAM-treated and 5% in Cd$^{2+}$-treated plants (Fig. 6C). Moreover, DHA contents were 6–11% higher in control and SHAM treatment and 78% higher in Cd$^{2+}$/SHAM-treated roots. The ratio of Asc to DHA characterizes the cellular redox status and indicates elevated oxidative stress in KO-PrxII F-1 plants. All Asc/DHA ratios were decreased in the KO-PrxII F-1 roots compared with wild type (Fig. 6C). Root glutathione contents of KO-PrxII F-1 were significantly decreased by about 20% as compared with WT (Fig. 6D). Furthermore, the GSH/GSSG ratio was lower in KO-PrxII F-1 under control conditions and in SHAM-treated roots. Cd$^{2+}$ and SHAM affected glutathione contents differently; total glutathione was unchanged by Cd$^{2+}$ and increased in the presence of SHAM. The stress effects were opposite those seen on Asc contents. Whereas in Cd$^{2+}$-treated plants the total glutathione content was unchanged, the GSH/GSSG ratio increased. Nevertheless, significant differences between WT and KO-Prx II F-1 were not seen upon Cd$^{2+}$ and SHAM treatment.

Alterations in Nuclear Gene Expression in KO-PrxII F Mutants—To investigate whether the loss of PrxII F protein alters gene expression, transcript levels were analyzed by using macroarrays hybridized with root samples from standard growth conditions or following cadmium and SHAM treatment. The “redoxin array” was comprised of 143 redox-related transcripts with assumed importance in cellular antioxidant defense or redox regulation and included members of the gene families of Prx (10 genes), Grx (29 genes), cyclophilins (29 genes), Trx (41 genes), Apx (8 genes), superoxide dismutases (9 genes), and catalases (3 genes). The “chondriome array” on the other hand

![Fig. 5. Phenotypical analysis of KO-PrxII F mutants. Effects of treatment with 7.5 μM Cd$^{2+}$ and 25 μM SHAM on root growth of KO-PrxII F-1 (A). Root lengths of wild type KO-PrxII F-1 (B) and wild type and KO-PrxII F-2 (C) were determined from 10 plants each (mean ± S.E.) and were grown on the same plate at days 6, 8, and 10 after sowing. * indicates significant differences to wild type of the same day and treatment (t test, p < 0.05).](image1)

![Fig. 6. Root respiration (A), ATP contents (B), ascorbate contents (C) and glutathione contents (D) in WT and KO-PrxII F-1 roots under control conditions, as well as Cd$^{2+}$ and SHAM treatment, respectively. The different letters indicate significant differences (analysis of variance, post-hoc LSD, p < 0.05). The percentage values in A represent the alternative respiratory activities, which were inhibited by 7.5 mM SHAM. The composite bars in C and D indicate the reduced forms at the bottom and the oxidized forms on top. The data are means of n = 3–6, ± S.E. from three independent experiments.](image2)
was fabricated with 114 cDNAs encoded by mitochondrial DNA and an additional 50 cDNAs from the nuclear encoded genes.

72 and 77 transcripts on the redoxin and chondriome arrays, respectively, were reliably detected with root-derived RNA probes. Fig. 7 summarizes all significant (p < 0.05, t test) or marginally significant differences (p < 0.1, t test) between KO-PrxII F and wild type. Semi-quantitative RT-PCR for selected transcripts essentially verified the results obtained from
the arrays (Fig. 8, A and B). Moreover, transcripts of some genes that were not detected on the array could be quantified by RT-PCR.

Upon cadmium treatment, 39% of these transcripts were distinctly regulated in the KO-PrxII F lines in comparison to WT, even though the magnitude of differences was relatively low. Under control conditions and SHAM treatment, this was still the case for about 10 and 14% of the transcripts, respectively. Because of this fact, the chondriome array was hybridized only with cDNAs from control and cadmium treatments. Gene families did not show uniform responses. Most interestingly, in all three treatments two cyclophilins, cyp40 and cyp63, were inversely regulated in the KO-PrxII F mutants compared with wild type. Expression of the cytosolic cyp40 declined from control to cadmium and SHAM treatment in the wild type roots. In a converse manner, its expression was relatively low under control conditions in the KO-PrxII F plants and increased strongly under cadmium and SHAM treatment (Fig. 8A). The transcript level of the nuclear cyp63 increased in the stressed wild type, whereas in the KO-PrxII F it was more than 2-fold higher under control conditions and decreased with cadmium and SHAM treatment. A similar but less pronounced trend was also seen for cyp71.

The transcript levels of the large gene family of Grx behaved very differently in the KO-PrxII F lines. Although under conditions of control and cadmium treatment, three and six members, respectively, were differentially regulated, none was found to be differentially regulated under SHAM treatment. Catalase transcripts were down-regulated under cadmium treatment in the KO-PrxII F lines. This effect was strongest for the cat1 transcript (Fig. 8A).

Superoxide dismutase transcripts were similar in KO-PrxII F and wild type. However, upon cadmium treatment, the plastidial csd2 and ftd3 transcripts increased in KO-PrxII F lines as compared with wild type (Figs. 7A and 8A). Transcripts of the three stress-inducible cytosolic type II prxII B, C, and D (12, 31) were found to be partially increased in all three treatments in the KO-PrxII F lines (Figs. 7A and 8A). Unexpectedly, the cytosolic apx1 was not affected by the loss of PrxII F, and the transcript level of the strong stress-inducible apx2 was slightly decreased with SHAM treatment (Figs. 7A and 8A). In all three treatments, redox-related transcripts were differentially up- and down-regulated in the KO-PrxII F mutants as compared with the wild type.

By taking a look at mitochondrial antioxidant and redox-related transcripts, it is remarkable that the prxII F transcript was up-regulated under cadmium treatment and down-regulated under SHAM treatment in wild type roots (Fig. 8B). SHAM also decreased PrxII F protein levels (Fig. 8C). Although both treatments induce oxidative stress, they evoke totally different responses. Transcript levels of mitochondrial Mn-SOD (msd1), which converts superoxide anions produced from the ETC to H2O2, were unaffected by cadmium and SHAM treatment in wild type but dropped by a factor of 0.4 and 0.8 in the KO lines, respectively (Fig. 8B). As shown above, Apx activity was strongly increased in KO-PrxII F mitochondria. This increase was not observed at the transcript level under control and cadmium treatment, and only a strong increase of the s-apx (At4g08390) transcript was found in the SHAM treatment, which is doubly targeted to plastid and mitochondrion (25).

As demonstrated above, the mitochondrial Trx and Grx systems act as reducing partners of PrxII F. Other interacting partners could be the mitochondrial Cyp 21-3 and Cyp 21-4. cyp21-3 transcripts decreased with cadmium and SHAM treatment, whereas this decrease was stronger in the KO-PrxII F lines (Fig. 8B). The grx-CxxS10 transcript was induced under control conditions in the KO-PrxII F lines as compared with wild type (Fig. 7A), whereas trx o-1 transcript decreased in cadmium treatment and the transcript abundance of the NADPH-dependent thioredoxin reductase (ntr) increased (Fig. 8B).

The mitochondrial uncoupling protein (Ucp) was involved in the prevention of oxidative stress in mitochondria, by uncoupling ATP synthesis and dissipating excess reducing energy (18). Most interestingly, the ucp2 transcript was reduced in abundance in the KO-PrxII F lines in comparison to wild type.

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**Fig. 8.** Semiquantitative RT-PCR analysis of antioxidant (A) and mitochondrial (B) gene transcript abundance in the wild type and in KO-PrxII F mutants. A and B, the figure shows the gel documentation of a representative experiment. Numbers are given as induction factors, which indicate significant changes of transcripts in the KO-PrxII F mutants compared with the wild type for the corresponding treatment. Similar changes were seen in two independent experiments of KO-PrxII mutants compared with the wild type for the corresponding treatment. Numbers are given as induction factors, which indicate significant changes of transcripts in the KO-PrxII F of a representative experiment. Numbers are given as induction factors, which indicate significant changes of transcripts in the KO-PrxII F mutants compared with the wild type for the corresponding treatment. Numbers are given as induction factors, which indicate significant changes of transcripts in the KO-PrxII F mutants compared with the wild type for the corresponding treatment.
under control conditions and cadmium treatment. Although its transcript level decreased in wild type under SHAM treatment, it showed an increase in the KO-PrxII F lines (Fig. 8B).

Modification of Mitochondrial Gene Expression in KO-PrxII F Plants—Among the 77 transcripts reliably detected on the chondriome array, there were 59 mitochondria-encoded and 18 nuclear encoded transcripts for proteins with mitochondrial localization. 10 and 11 mitochondria-encoded genes were differentially regulated in the KO-PrxII F under control and cadmium treatment, respectively. The function of most of the proteins encoded by these ORFs is unknown.

Under control conditions, three transcripts of the F-ATP synthase were down-regulated, and one was up-regulated in the KO-PrxII F lines as compared with wild type (Fig. 7B). With the exception of orf 25, which is part of the FO subunit of the F-ATPase, none of these was affected by the cadmium treatment. The orf 25 transcript was drastically induced by cadmium treatment in the wild type and to a much lesser extent in the KO-PrxII F lines. Mitochondria-encoded subunits of NADH dehydrogenases (65) were also up-regulated in the KO-PrxII F lines under control (nad 6, nad 9, and orf 131) and cadmium treatment (nad 7), as well as two different transcripts of ribosomal proteins (rpl 16 and rps 4). A set of genes with unknown function, i.e. orf 100 b, orf 106 f, orf 159, and orf 167, were up-regulated more than 1.6-fold in the cadmium treatment. For orf 167, this effect was more pronounced in RT-PCR analysis with a 12-fold induction as compared with 3-fold obtained in arrays.

Among the nuclear encoded mitochondrial transcripts, some enzymes of primary metabolism were affected by the loss of PrxII F protein. Transcript levels of the oxidative stress-susceptible aconitase were elevated in the KO-PrxII F lines in control and SHAM but not in cadmium treatment (Fig. 8B). The transcripts of the E1 and E3 subunits of the pyruvate dehydrogenase complex and the P protein of the glycine decarboxylase were slightly down-regulated in the KO-PrxII F under cadmium treatment (Fig. 7B), whereas the transcript level of the succinate dehydrogenase (sdhI-1) was found to be induced (Figs. 7B and 8B).

DISCUSSION

Mitochondria, particularly the respiratory chain, are the sites of continuous generation of reactive oxygen species (15). In the bacterial respiratory chain, about 0.6% of the consumed O₂ is converted into O₂⁻ and H₂O₂ (66, 67). Likewise, at least 1% of O₂ is converted into O₂⁻ and H₂O₂ into H₂O₂ and O₂. For bacteria, the homologue molecules of O₂⁻ and H₂O₂ production in plant mitochondria (15). SOD converts two of endogenously generated H₂O₂ in the mitochondria of Arabidopsis thaliana (14). Under these particular conditions of oxidative stress, the authors observed that 16 mitochondrial proteins were partly broken down and 12 strongly decreased in abundance. Despite the alteration in mitochondrial proteome, the cells maintained their viability (14). These data suggest a role in oxidative defense and prompted us to investigate the role of PrxII F in mitochondrial and cell metabolism by using mutants devoid of PrxII F.

Lack of PrxII F Affects Redox Homeostasis under Optimum Conditions and Is Strongly Inhibitory under Stress—In the GABI KAT and the SAIL collection, respectively, two independent lines with T-DNA insertions in the prxII F gene were identified, both totally lacking the PrxII F protein. It was anticipated that absence of PrxII F protein would lead to an increase in the activity of hydrogen peroxide and downstream ROS and a consequent oxidative stress within the mitochondrion. However, we could find no evidence for decreases in the activity of respiratory complexes or trichloroacetic acid cycle enzymes that have been shown previously to be sensitive to oxidative damage. There are two possible explanations for this apparent lack of oxidative stress in the absence of PrxII F: either PrxII F does not function as an antioxidant protein or the activity of other proteins compensate for the lack of PrxII F. The fact that a significant increase in the activity of mitochondrial ascorbate peroxidase- and glutathione-dependent peroxidase was observed in the PrxII F knock-out lines supports the latter possibility. Both peroxidases detoxify H₂O₂. Additionally, Gpx has a broad peroxide substrate specificity that in-
cludes lipid peroxides (70). Based on their catalytic activity, Apx and Gpx have the enzymatic ability to metabolically replace the missing peroxidase activity of PrxII F. Because both in soil and in aseptic cultures on phytagel-solidified medium, growth performance was not different between WT and KO-PrxII F plants, apparently the up-regulation of antioxidant enzymes and possibly other metabolic readjustments allowed for full compensation of the defect under optimum growth conditions. Up-regulation of antioxidant enzymes in relation to mitochondrial dysfunction is also reported for tobacco plants with impaired complex I function (22). In fact, H$_2$O$_2$ concentrations were lower in the mutant tobacco than in WT. The authors (22) concluded that antioxidant cross-talk and acclimation maintained cellular redox balance.

However, under stress conditions the compensatory mechanisms were insufficient to sustain adequate redox homeostasis, which led to significantly reduced growth rates of KO-PrxII F roots. Under the tested stress conditions, root growth of KO-PrxII F-1 was 2-fold stronger inhibited than the root growth of KO-PrxII F-2 (Fig. 5), which might be explained by a higher activity of APX and GPX in the second mutant (Table I). Cadmium is one of the most toxic of the heavy metals. Root growth sensitively responds to cadmium at submicromolar or low micromolar concentrations, and tissue respiration is often stimulated under mild heavy metal stress (58). Toxic cadmium concentrations cause ROS production and also induce expression of prxII F.

Alternative oxidase helps to minimize mitochondrial ROS production by draining electrons from an over-reduced ubiquinone pool. Transgenic plants with enhanced expression of AOX showed decreased levels of ROS, and those with antisense-suppressed AOX showed increased levels of ROS (71). Addition of low levels of SHAM, an AOX inhibitor, to the hydroponics medium increased the respiration rate in both WT and KO-PrxII F-1 plants (Fig. 6). The portion of alternative respiration sensitive to high SHAM remained unaltered. It is reported that SHAM exerts side effects on other enzymes, like the inhibition of horseradish peroxidase, xanthine oxidase, and lipooxygenase on the one hand and the strong stimulation of plasma membrane NADH oxidases on the other hand (63). Therefore, treatment with 25 μM SHAM might have causes for the formation of oxidative stress supplementary to that by inhibition of AOX. Despite the increased respiration, ATP levels showed an insignificant trend to decrease in both the Cd$^{2+}$- and SHAM-treated roots of KO-PrxII F-1 pointing at a metabolic imbalance. Redox regulation of alternative oxidase has been confirmed recently (72) by identifying a novel thioredoxin Trx h2 as electron donor and efficiently reduced H$_2$O$_2$ but not the mitochondrial Trx h2 and o1 (72). The redox coupling of Gpx remains unclear. Nevertheless, Gpx is likely to contribute to antioxidant defense in PrxII F-deficient mitochondria. Most interestingly, the ascorbate pool showed a general trend to increased oxidation in KO-PrxII F plants (Fig. 6C), indicating some oxidative stress.

**Changes in Transcript Levels Indicate Disturbance of Redox Homeostasis and Involvement of Redox Signaling**—In addition to their function in antioxidant defense, a role of Prx in signaling events and regulation of stress-induced gene expression has been proposed (4). The suggested triggers for that kind of function of 2-Cys Prx are mechanisms of inactivation with subsequent flooding of the cell with ROS (6), oligomerization (37), and direct interaction with a stress-activated protein kinase or other target proteins (75). Recently, deficiency of the human mitochondrial 2-Cys Prx was recognized as a proapoptotic stimulus as indicated by increased collapse of mitochondrial membrane potential and cytochrome c release in HeLa cells (76). Up to now, a similar function of type II Prx has not been addressed. To be able to adapt mitochondrial metabolism and to prevent cellular damage under different environmental conditions, cells depend on a functional mitochondrial-nucleus communication for coordinated gene expression. A targeted transcript analysis performed here has allowed us to identify significant changes in the expression of both nuclear and mitochondrial genes. Apparently, despite the presence of a complete ascorbate-glutathione cycle with an efficient Apx as H$_2$O$_2$ quencher in the plant mitochondrion (25), the lack of PrxII F had a severe impact on signaling within the mitochondrion as well as from the mitochondrion to the nucleus. It is not yet clear at present whether the altered transcript regulation apparent in the KO-PrxII F mutants is the direct consequence of the loss of PrxII F as a signaling element (for instance by its functioning as a static redox sensor (5)) or whether it is an indirect effect caused by alterations in potent redox signals such as H$_2$O$_2$ or Asc, as seen in catalase- or ascorbate-deficient mutants of Arabidopsis (77, 78). However, the inverse regulation of cyp40 and cyp63 transcripts in the KO-PrxII F under all conditions can hardly be explained by ROS signaling alone. Alterations in transcript regulations were also observed for diverse Prx deletion mutants. In Prx-null yeast cells, a compensatory expression of other antioxidant enzymes, like Gpx 2, catalase 1, and Trx 1 was detected even under control growth conditions; moreover, expression of SOD 1, Trx 2, and glutathione reductase 1 increased markedly under conditions of oxidative stress (74). Expression of the γ-glutamylv cysteine synthetase 1 was increased in Δ-mTpxI, Δ-cTpxI, and Δ-cTpxII (73). For the 2-Cys Prx (Tpx1) of Schizosaccharomyces pombe, it was shown that it is directly involved in the signaling pathway of Gpx 1 and catalase 1, which leads to a transcript accumulation after prooxidant treatment; Δ-Tpx 1 cells did not show any induction of these transcripts after exposure to H$_2$O$_2$ (75).

Many genes represented on the redoxin array are known to be implicated in redox signaling and acclimation. Distinct modulation of their expression between wild type and KO-PrxII F plants, as described above, suggests that PrxII F is involved in modulating redox-dependent signaling between the mitochondrion and the cytosol. In future work it will be important to separate H$_2$O$_2$-dependent and redox-dependent signaling pathways by a more detailed analysis of gene expression, for example in comparison with A. thaliana signaling mutants that are defective in catalase (77) or mitogen-activated protein kinase activity (79).

In conclusion, we want to point out that PrxII F is an impor-
tant H$_2$O$_2$-scavenging enzyme, localized in the mitochondrial matrix. Its loss has a major impact on plant fitness, particularly in non-green tissue under conditions of oxidative stress. Because it possesses reductase activity and interacting with various redox-related proteins as well as antioxidants like glutathione, PrxII F is a definite candidate for perception of changes in the redox state in the mitochondrion. Based on these facts and due to changes in nuclear and mitochondrial gene expression in the knock-out plants of PrxII F, we postulate that, beside its antioxidant function, PrxII F has got a role in redox signaling. Furthermore, a more detailed analysis of the KO-PrxII F plants at the cell level, for example during pollen development, possibly will allow the identification of the crucial roles of PrxII F in plant development and adaptation in addition to its function in redox homeostasis, particularly under oxidative stress.

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