Decrease in Manganese Superoxide Dismutase Leads to Reduced Root Growth and Affects Tricarboxylic Acid Cycle Flux and Mitochondrial Redox Homeostasis1[C][W]


Superoxide dismutases (SODs) are key components of the plant antioxidant defense system. While plastidic and cytosolic isoforms have been extensively studied, the importance of mitochondrial SOD at a cellular and whole-plant level has not been established. To address this, transgenic Arabidopsis (Arabidopsis thaliana) plants were generated in which expression of AtMSD1, encoding the mitochondrial manganese (Mn)SOD, was suppressed by antisense. The strongest antisense line showed retarded root growth even under control growth conditions. There was evidence for a specific disturbance of mitochondrial redox homeostasis in seedlings grown in liquid culture: a mitochondrially targeted redox-sensitive green fluorescent protein was significantly more oxidized in the MnSOD-antisense background. In contrast, there was no substantial change in oxidation of cytosolically targeted redox-sensitive green fluorescent protein, nor changes in antioxidant defense components. The consequences of altered mitochondrial redox status of seedlings were subtle with no widespread increase of mitochondrial protein carbonyls or inhibition of mitochondrial respiratory complexes. However, there were specific inhibitions of tricarboxylic acid (TCA) cycle enzymes (aconitase and isocitrate dehydrogenase) and an inhibition of TCA cycle flux in isolated mitochondria. Nevertheless, total respiratory CO2 output of seedlings was not decreased, suggesting that the inhibited TCA cycle enzymes can be bypassed. In older, soil-grown plants, redox perturbation was more pronounced with changes in the amount and/or redox poise of ascorbate and glutathione. Overall, the results demonstrate that reduced MnSOD affects mitochondrial redox balance and plant growth. The data also highlight the flexibility of plant metabolism with TCA cycle inhibition having little effect on overall respiratory rates.

Plant mitochondria are important not only for respiration but also for several other metabolic activities (Sweetlove et al., 2007), including photosynthesis (Raghavendra and Padmasree, 2003). A key feature of mitochondrial biochemistry is the unavoidable produc-

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reactive hydroxyl radical that directly reacts with available molecules at its site of formation, the superoxide anion radical can diffuse a considerable distance before it reacts with a suitable target. It has a selective reactivity with some biological important targets, such as iron (Fe)-sulfur clusters of enzymes, which makes it cytotoxic to living cells (Fridovich, 1995). The Fe that is released from the oxidized enzymes mediates the production of hydroxyl and alkoxyl radicals. At neutral pH superoxide can also oxidize polyphenols, thiols, ascorbate, and sulfate (Halliwell, 2006). Superoxide reacts at very high rates with nitric oxide to form the toxic product peroxynitrite. Moreover, in nonpolar environments, it is a powerful base, nucleophile, and reducing agent and can be extremely damaging to membrane systems (Halliwell and Gutteridge, 1984). In eukaryotic cells, superoxide dismutases (SODs) are the only enzymes capable of catalyzing the dismutation of two superoxide radicals to H$_2$O$_2$ and molecular oxygen. SODs are ubiquitous metalloenzymes in prokaryotic and eukaryotic cells with aerobic metabolism. The Arabidopsis (Arabidopsis thaliana) genome encodes eight SOD genes, comprising all three types of isoenzymes, Fe-, manganese (Mn)-, and copper/zinc-SOD. These are ubiquitously found in plants and differ by the named metal cofactor and their subcellular localization (Kliebenstein et al., 1998; del Rio et al., 2003). Plant mitochondria possess a highly conserved MnSOD (Fridovich, 1995; Gutteridge and Halliwell, 2000), which assembles as a homotetramer and contains one Mn atom per tetramer (Sevilla et al., 1982). The H$_2$O$_2$ produced as catalytic by-product of the MnSOD is further reduced to water by a variety of peroxidases including a type II peroxiredoxin (PrxII F; Finkemeier et al., 2005), an ascorbate peroxidase (APX; Chew et al., 2003), as well as a glutathione (GSH) peroxidase that was recently shown to be a functional peroxiredoxin (Navrot et al., 2006). Although the mitochondria are one source of ROS in the cell, the amount of ROS they produce is rather minor in comparison to the chloroplasts and peroxisomes (Foyer and Noctor, 2003). As a consequence, the impact of mitochondrial ROS production at the cellular level remains to be clarified. Nevertheless, the redox state of the mitochondrial may be important in setting whole cell redox homeostasis (Noctor et al., 2007). There are also several lines of evidence suggesting that mitochondrial function is sensitive to oxidative stress. Proteomic and biochemical analysis of the response of Arabidopsis to exogenous menadione, H$_2$O$_2$, and antimycin A treatment indicated that the tricarboxylic acid (TCA) cycle enzymes are particularly sensitive to oxidative inactivation (Sweetlove et al., 2002), and key TCA cycle enzymes are also known to be inhibited during abiotic stresses (Taylor et al., 2004). Metabolic studies are also consistent with a rapid inactivation of the TCA cycle and respiration during oxidative stress (Baxter et al., 2007) and many TCA cycle enzymes contain readily oxidized amino acid side groups (Winger et al., 2005; Möller et al., 2007).

However, all these studies rely on the addition of an exogenous agent to induce oxidative stress and it is not possible to assess the extent to which extramitochondrial processes contribute to the response. An Arabidopsis knockout mutant of the prxII F has demonstrated the importance of mitochondrial H$_2$O$_2$ detoxification for root growth especially under oxidative stress conditions (Finkemeier et al., 2005). However, the effect was complicated by elevated activities of mitochondrial ascorbate and GSH peroxidases that in part compensated for the absence of prxII F under control conditions. The specific effect of mitochondrial superoxide production on cellular function has not yet been examined. Moreover, since no other mitochondrial enzyme can compensate for the MnSOD activity, an antisense suppression strategy should give clear insight into the importance of superoxide detoxification. To date, the role of plant mitochondrial MnSOD has been solely investigated in the context of oxidative stress tolerance in transgenic plants in which the enzyme was overexpressed in various compartments (Bowler et al., 1991; Van Camp et al., 1994; Slooten et al., 1995; Van Breusegem et al., 1999). However, the requirement for mitochondrial MnSOD during either optimal or stress conditions has not been properly investigated and the consequences of increased mitochondrial superoxide production are not fully understood. Here, we have investigated the consequences of mitochondrial superoxide production for plant metabolism and development by characterization of MnSOD antisense plants at the phenotypic level as well as in detail at the molecular-biochemical level.

RESULTS
Generation of MnSOD-Antisense Plants
To investigate the role of MnSOD in the antioxidant system of plant mitochondria we designed a cauliflower mosaic virus 35S:msd1 antisense construct to generate transgenic Arabidopsis plants with repressed levels of the mitochondrial MnSOD protein MSD1. MSD1 (At3g10920) encodes a 25 kD protein found in the mitochondrial matrix (Kruft et al., 2001; Millar et al., 2001; Herald et al., 2003). From 18 independent transformants selected for the kanamycin resistance marker from the T1 seed pool, two antisense lines (AS-5 and AS-7) showed greater than 80% decrease in msd1 transcript levels compared to wild type (Fig. 1A). Both lines showed a strong decrease in MnSOD protein level compared to wild type, with a 70% and 60% decrease in lines AS-5 and AS-7, respectively, detected in purified mitochondria by immunoblotting using a MnSOD-specific antiserum (Fig. 1B). Homozygous seed batches were produced in T3 and T4 generations for both lines, which were used for subsequent analysis. Unless otherwise stated, 10-d-old seedlings grown in a sterile liquid culture medium under continuous shaking were used for most experiments.
This growth system was chosen because it allows for highly reproducible growth conditions and facilitates the production of large quantities of seedlings for isolation of mitochondria.

Decreased MnSOD Protein Level Affects Seedling Growth

Alterations in cellular antioxidant levels are known to affect the growth of plants, especially the root system (Finkemeier et al., 2005; Olmos et al., 2006; Miller et al., 2007). To screen the MnSOD-antisense lines for growth phenotypes, we monitored the root growth of lines AS-5 and AS-7 on vertical agar plates under control growth conditions as well as after treatment with abiotic stressors known to induce oxidative stress (Fig. 2). Corresponding to the MnSOD protein level, line AS-5 showed an overall more pronounced phenotype than AS-7, which was reflected in a significantly decreased root growth and seedling dry weight under standard growth conditions (Fig. 2, A–C). However, the seedling dry weight and root growth of AS-7 were not significantly affected under control growth conditions (Fig. 2, A–C). Root growth of AS-7 as compared to wild type was significantly decreased after 7-d growth on media containing 50 mM sorbitol or 50 μM Fe (Fig. 2B). Most stress treatments, with the exception of 0.05 μM methyl viologen, had no further inhibiting effect on root growth of AS-5 (Fig. 2B). Interestingly, both antisense lines seemed to be more tolerant to salinity stress (25 mM NaCl) than the wild type. A similar growth phenotype was observed for the apx1/tylapx mutants investigated by Miller et al. (2007).

Mitochondrial Redox Status Is Shifted to More Oxidizing Conditions in the MnSOD-Antisense Seedlings

To investigate whether the reduced MnSOD level affected the cellular redox status we used the recently developed redox-sensing GFP (roGFP1; Jiang et al., 2006), which reports, like roGFP2 (Meyer et al., 2007), the redox status of the GSH pool in vivo (Schwarzländer et al., 2007).

Morgan et al.

et al., 2008). The measurement of the 405-/488-nm fluorescence ratio of the roGFP1 allows the percentage oxidation of the roGFP1 to be estimated. We crossed Arabidopsis plants expressing the roGFP1 targeted to either the mitochondria or cytosol, respectively (Schwarzländer et al., 2008), with either wild-type or AS-5 plants. Progeny expressing the roGFP1 in each subcellular compartment (Fig. 3, A–D) and the msd1-antisense construct (as determined by kanamycin resistance) were analyzed. Strikingly, the mitochondrial roGFP1 was significantly more oxidized in the AS-5 line in comparison to wild type (Fig. 3E). In contrast, the cytosolic roGFP remained highly reduced in both wild-type and AS-5 line (Fig. 3F) at the limit of the dynamic range (Schwarzländer et al., 2008). Thus, it seems that the major shift in redox status of the GSH pool occurred in the mitochondrion but not the cytosol in AS-5 seedlings. Consistent with this statement, there was no evidence of a change in the redox poise of the total cellular ascorbate pool (Fig. 4A).

The mitochondrial redox state is thought to be an important signal that sets global antioxidant defense systems (Foyer and Noctor, 2003). We therefore measured the activity or mRNA transcript abundance of a number of key antioxidant defense-related proteins. The activity level of mitochondrial APX was significantly reduced in the AS-5 mitochondria compared to wild type, while the monodehydroascorbate reductase (MDHAR) activity level was unaffected (Fig. 4B). In addition, protein levels of the plastidic FeSOD were strongly increased as detected by immunoblots using a FeSOD specific antiserum (Fig. 4C). Of the antioxidant defense-related transcripts tested, there were few major changes. The level of ferritin-1 transcript, which is a marker for H2O2 (Op den Kamp et al., 2003), was strongly increased as detected by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS; Table I). The only protein spot that showed a 1.5-fold change) in AS-5 as compared to wild type superoxide in the MnSOD-antisense seedlings, we performed in situ nitroblue tetrazolium (NBT) stains. For both wild-type and antisense lines no staining was observed in seedlings grown under control condition after 1-h incubation with NBT in the dark (Fig. 4E). This shows that the antioxidant capacities are not overwhelmed by superoxide production in antisense plants with lower MnSOD levels under control growth conditions. A stronger staining compared to wild type was observed for the antisense lines grown on 50 and 100 μM Fe2+ (Fig. 4E).

Mitochondrial Protein Carbonylation

The decreased mitochondrial APX activity, as well as the significantly more oxidized mitochondrial GSH pool indicated an oxidative stress response occurs in mitochondria of the AS-5 seedlings. To assess the extent of oxidative damage the degree of oxidative modification of mitochondrial proteins was determined. Protein carbonyls can be detected after derivatization with 2,4-dinitrophenyl hydrazine (DNP) and are markers for metal-catalyzed protein oxidation (O’Brien et al., 2004). Mitochondria were isolated from three independent replicate batches of 10-d-old wild-type and AS-5 seedlings. DNP-derivatized mitochondrial proteins were separated by two-dimensional electrophoresis (Fig. 5, A and C) and probed with an anti-DNP antibody (Fig. 5, B and D). The normalized quantity of protein spots was established from images of Coomassie-stained two-dimensional gels and western blots, respectively, using PDQuest software (version 7.3.1, Bio-Rad). Spots that showed a statistically significant change in abundance in Coomassie gels (t test, P < 0.05) or carbonyl western blots (>1.5-fold change) in AS-5 as compared to wild type in all three replicates are highlighted (Fig. 5). The spots were excised from the Coomassie-stained gels and identified by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS; Table I).

**Figure 3.** RoGFP1 redox status in mitochondria and cytosol of wild-type and AS-5 seedlings. A to D, Expression of roGFP1 in mitochondria (A and C) and cytosol (B and D) in leaf epidermis cells of wild-type and AS-5 seedlings, respectively. E and F, Degree of oxidation of roGFP1 in mitochondria (E) and cytosol (F) calculated from the measured mean intensity ratios (405/488 nm) following in situ calibration using 10 mM dithiothreitol and 100 mM H2O2, as described by Schwarzländer et al. (2008; n = 5, mean ± se). Asterisks indicate significant differences between wild type and AS-5 (t test, P < 0.05).
decrease in abundance (by approximately 60%) in the Coomassie-stained gels from the AS-5 line compared to wild type was identified as the mitochondrial MnSOD (spot 5: Fig. 5, A and C; Table I). Six other protein spots showed an increased abundance. Two spots showing more than 2-fold increases in abundance in AS-5 were identified as mitochondrial Asp aminotransferase (spots 7, 8: Fig. 5, A and C; Table I). The overall degree of protein carbonylation changed rather little in the AS-5 mitochondria. However, four proteins could be identified in the anti-DNP western blots that show a more than 1.5-fold increased signal intensity (spots 1–4: Fig. 5, B and D; Table I). Among them are the mitochondrial MDHAR and the heat shock 70-like protein (Table I). However, the
The activity of the mitochondrial MDHAR was unaffected by the higher degree of carbonylation in the AS-5 line (Fig. 4B).

**Mitochondrial TCA Cycle Activity Is Strongly Affected by a Decreased MnSOD Level**

To assess whether the observed redox shift in mitochondria of the AS-5 line was reflected in an altered mitochondrial metabolism, we determined the activities of key mitochondrial respiratory enzymes, such as the mitochondrial electron transport chain complexes and several TCA cycle enzymes, in purified mitochondria from wild-type and AS-5 seedlings (Fig. 6A). The activities of the mitochondrial respiratory complexes were not significantly decreased in the AS-5 line. Interestingly, there was a marginal decrease in the alternative oxidase activity in the AS-5 line. A substantial reduction (approximately 50%) of aconitase and NADH-dependent isocitrate dehydrogenase (IDH) activity was detected in the AS-5 line compared to wild type (Fig. 6A). To further investigate whether the decreased activities of the mitochondrial isoforms of aconitase and IDH in the AS-5 line had consequences on the rate of TCA cycle organic acid production, we monitored the flux through the TCA cycle in isolated mitochondria using real-time 13C-NMR. Coupled mitochondria were incubated in 3-13C-pyruvate under simulated cytosolic conditions as described in detail in Smith et al. (2004). 13C-NMR spectra were recorded over a time period of 6 h. Carbon dioxide that is released from the C1, but also the C2 position of Glc, is derived from decarboxylation processes in the oxidative pentose phosphate pathway as well as from TCA cycle, whereas release from C3:4 and C6 positions is mainly associated with mitochondrial CO2 release (ap Rees and Beevers, 1960). Given the inhibition of TCA cycle activity in isolated mitochondria (Fig. 6), it was anticipated that there would be a decrease in CO2 release from labeled Glc in vivo. However, there was no significant difference in CO2 release from the C6 position in either of the two antisense lines (Fig. 7). Nor was there a significant difference in CO2 release from the C3:4 position for the AS-7 line but there was a significant increase in the AS-5 line. It is not immediately obvious why there should be this disparity between C3:4 and C6 CO2 release in the AS-5 line. CO2 is released from the C3:4 labeled position at the pyruvate dehydrogenase step, whereas CO2 is released from the C6 labeled position after two turns of the TCA cycle. One possible explanation lies in the sensitivity of the pyruvate dehydrogenase enzyme to the NADH/NAD+ ratio (Tovar-Mendez et al., 2003); an increased oxidation of the mitochondrial NADH

### Table I. Identification of mitochondrial protein spots fractionated by IEF-SDS-PAGE with altered abundance or protein carbonyl content in AS-5 line compared to wild type

<table>
<thead>
<tr>
<th>Spot Identification</th>
<th>Entry</th>
<th>Details of Match</th>
<th>Average Increase in Carbonylation Relative to Wild Type</th>
<th>Average Change in Abundance Relative to Wild Type</th>
<th>Gel MM/pl</th>
<th>Match MM/pl</th>
<th>No. MP</th>
<th>% Protein Coverage</th>
<th>Error ppm</th>
<th>TP</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>At3g07770</td>
<td>Heat shock protein 89-1 (HSP90 related)</td>
<td>2.4</td>
<td>0.84</td>
<td>90,000/4.65</td>
<td>90,537/5.26</td>
<td>9</td>
<td>12</td>
<td>41</td>
<td>M</td>
</tr>
<tr>
<td>2</td>
<td>At4g37910</td>
<td>Heat shock protein 70-like protein</td>
<td>2.4</td>
<td>0.79</td>
<td>70,000/4.8</td>
<td>73,030/5.51</td>
<td>14</td>
<td>26</td>
<td>84</td>
<td>M</td>
</tr>
<tr>
<td>3</td>
<td>At1g63940</td>
<td>MDHAR, putative</td>
<td>1.7</td>
<td>1.38</td>
<td>48,000/5.9</td>
<td>52,469/7.05</td>
<td>12</td>
<td>32</td>
<td>68</td>
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<tr>
<td>4</td>
<td>At4g27585</td>
<td>Stomatin; band 7 family protein, similar to stomatin-like protein</td>
<td>2.3</td>
<td>1.45</td>
<td>46,000/5.95</td>
<td>44,992/6.35</td>
<td>9</td>
<td>22</td>
<td>134</td>
<td>M</td>
</tr>
<tr>
<td>5</td>
<td>At3g10920</td>
<td>MSD1</td>
<td>–</td>
<td>0.36</td>
<td>25,500/6.3</td>
<td>25,428/8.47</td>
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<td>41</td>
<td>52</td>
<td>M</td>
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<tr>
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<td>At2g33150</td>
<td>Acetyl-CoA C-acyltransferase</td>
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<td>1.55</td>
<td>44,500/9.0</td>
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<td>At2g30970</td>
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<td>42,000/6.95</td>
<td>47,727/8.36</td>
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<td>23</td>
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<tr>
<td>8</td>
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<td>AAT1</td>
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</tbody>
</table>

*Chew et al. (2003).*
pool in the AS-5 line could relieve this product inhibition. There were no significant differences in the ratio of CO₂ released from the C1 and C6 positions, suggesting that flux through the oxidative pentose phosphate pathway was not altered in the MnSOD-antisense seedlings.

One possible interpretation of these data is that an inhibition of mitochondrial TCA cycle flux between citrate and 2-oxoglutarate is compensated for by the cytosolic isoforms of aconitase and NADP-dependent isocitrate dehydrogenase (ICDH). To investigate whether the various isocitrate dehydrogenase isoforms are differentially regulated on transcript and protein level in the MnSOD-antisense lines, we performed semiquantitative reverse transcription (RT)-PCR and western-blot analysis. Apart from the organellar icdh transcript, the transcript abundances of citrate synthase, aconitase, as well as cytosolic icdh and various organellar idh isoforms, did not show a strong regulation in both MnSOD-antisense lines (Fig. 8A). Interestingly, the protein levels of the mitochondrial isocitrate dehydrogenase (IDH) isoforms increased in both MnSOD-antisense lines (Fig. 8B) despite the reduced activity of this enzyme in mitochondrial extracts (Fig. 6, A–C). The increase in protein amount is presumably a response to oxidative inactivation of existing IDH protein. Moreover, no increase in the amount and activity of ICDH was observed in extracts from whole seedlings (Fig. 8, B and C).

**Antisense Plants Adapt to Lower MnSOD Levels during Their Life Cycle**

In contrast to the reduced root growth and shoot dry weight of seedlings (Fig. 1), 4- to 5-week-old MnSOD-antisense plants grown on soil had an 8% (0.82 ± 0.06 mg, AS-7) and 28% (0.97 ± 0.08 mg, AS-5) increased leaf dry weight in comparison to wild type (0.76 ± 0.06 mg). This was not due to a change in antisense suppression as the MnSOD protein levels in leaves were still reduced by 60% to 70% in the antisense lines compared to wild type. This implies that the plants have adapted to the loss of MnSOD. To examine the metabolic capacities of older plant leaves in more detail, we measured photosynthetic rates, chlorophyll content, metabolite content, and respiration rates. No significant differences in the rate of photosynthesis between wild-type and the MnSOD-antisense lines were observed under various light conditions up to 1,000 μE (Fig. 9A). Similarly there were no significant differences in chlorophyll contents between the lines and wild type (Fig. 9B). Respiratory CO₂ release remained similar or slightly higher than wild type as was observed for seedlings (Fig. 9C). To make a broader assessment of the metabolic consequences that result from MnSOD-antisense suppression we performed a gas chromatography (GC)-MS metabolic profile analysis (Roessner et al., 2001). Strikingly, from more than 60 analyzed metabolites only TCA cycle
organic acids (Fig. 9D) and ethanolamine (Supplementary Table S1) were significantly altered in the two MnSOD-antisense lines as compared to wild type. This provides further evidence that the effects of reduced MnSOD are mainly felt in the mitochondrion. However, there was some evidence of extramitochondrial changes in 4- to 5-week-old soil-grown leaves and in particular there was a substantial up-regulation of total cellular antioxidant defences. Activities of total cellular APX and GSH peroxidase, as well as total pools of ascorbate and GSH, were increased in the MnSOD-antisense leaves as compared to wild type (Fig. 10, A–D). Total ascorbate and GSH levels in lines AS-7 and AS-5 were increased by 40% and 50%, respectively, when compared to wild type (Fig. 10, C and D). Moreover, it should be noted that with more than 40% dehydroascorbate, the ascorbate pool in AS-5 and AS-7 was highly oxidized (Fig. 10D).

Figure 7. 14CO2 evolution from seedlings in the light. A to D, 10-d-old seedlings were incubated in 10 mM MES-KOH, pH 6.5, supplemented with [1,14C]-, [2,14C]-, [3,4,14C]-, or [6-14C]-labeled Glc (n = 3, mean ± se). Different letters indicate significant differences between the different lines at time point t = 6 h (ANOVA posthoc LSD, P < 0.05).

Figure 8. Transcript levels of TCA cycle enzymes, IDH and ICDH protein content, and ICDH activity in 10-d-old wild-type and MnSOD-antisense seedlings. A, Semiquantitative RT-PCR analysis of nuclear-encoded gene products from the TCA-cycle enzymes citrate synthase, aconitate, and NAD(P)-isocitrate dehydrogenase. Transcripts were amplified by gene-specific RT-PCR, and EtBr-stained gels were scanned and densitometrically analyzed. Numbers are given as average induction factors from three independent experiments. B, Western-blot analysis of IDH and ICDH contents in protein extracts from whole seedlings. C, ICDH enzyme activity (n = 3, mean ± se). Different letters indicate significant differences between the different lines (ANOVA posthoc LSD, P < 0.05).
Protein levels of the plastidic FeSOD were strongly increased as was seen in younger seedlings (Figs. 4C and 10E). MnSOD levels showed a more than 60% decrease in total leaf extracts as seen before in isolated mitochondria from seedlings (Figs. 3E and 10E).

DISCUSSION

This article presents the first characterization of plants with suppressed MnSOD level, providing new insights into the central role of MnSOD in the mitochondrial antioxidant defense system. Constitutive expression of an msd1-antisense construct in Arabidopsis transformants allowed us to select two lines, AS-5 and AS-7, which possessed an 80% decrease in the msd1 transcript level leading to a 70% and 60% decrease in MnSOD protein level, respectively (Fig. 1; Table I). We assumed that a decrease in MnSOD protein level would result in higher steady-state levels of superoxide and H$_2$O$_2$ as predicted from computational simulation (Pollé, 2001), which in turn can give rise to other reactive molecules such as the hydroxyl radical and cause oxidative stress in the mitochondrion. Increased ROS production is sensed by the plant and leads to an activation of the antioxidant defense system (Noctor and Foyer, 1998), and it is the precise balance between antioxidants and the rate of ROS production that sets the level of ROS. The phenotypic and molecular characterization of the MnSOD-antisense plants has demonstrated the extent to which MnSOD is important in regulating this balance and has provided a new perspective on the metabolic consequences of altered mitochondrial redox status.

MnSOD Suppression Alters Cellular Redox Homeostasis

The decrease in MnSOD level caused specific perturbations in mitochondrial redox status in seedlings and more general cellular redox shifts in older, soil-grown plants. A disturbance in the redox balance of ascorbate and GSH is normally regarded as oxidative stress that can potentially lead to oxidative damage.
The higher oxidation status of the mitochondrial roGFP in AS-5 seedlings and the inhibited TCA cycle flux showed that the remaining MnSOD protein is not sufficient to detoxify mitochondrial superoxide produced during respiration and that superoxide and most likely other ROS are also quenched by the GSH pool. Moreover, in the AS-5 seedlings the mitochondrial APX activity decreased. This might be due to APX inactivation through depletion of mitochondrial ascorbate pools, since, when ascorbate is depleted, chloroplastic APX is known to be sensitive to inactivation in the presence of H₂O₂ (Nakano and Asada, 1987). Therefore, it would be very interesting to monitor the redox state and pool size of mitochondrial ascorbate. However, to our knowledge, to date there is no method available to accurately determine mitochondrial ascorbate pools.

In older plant leaves the reduced mitochondrial superoxide detoxification capacities caused an increase in the total cellular antioxidant capacity, with higher ascorbate and GSH peroxidase activities, increased FeSOD levels, and an increased pool size of the redox buffers GSH and ascorbate in both MnSOD-antisense lines (Fig. 10). Oxidative stress is known to induce the accumulation of ascorbic acid (Noctor and Foyer, 1998; Nagata et al., 2003), and increased levels of GSH were also found in plants with decreased levels of plastidic copper/zinc-SOD (Rizhsky et al., 2002). Overcompensation of defects in antioxidant enzymes seems to be a general response to the loss of antioxidant enzymes and was also observed in double knockout mutants of apx1/cat1 (Rizhsky et al., 2002) and apx1/thylapx (Miller et al., 2007). However, the highly oxidized pool of total ascorbate reflects the shift in redox homeostasis and the higher oxidative load in both of the 5-week-old MnSOD-antisense lines. These lines are a good example of the flexibility of the plant antioxidant defense network. The system is able to adapt to a situation of decreased superoxide detoxification capacities and therefore also to adapt to changing environmental conditions, which allows the plant to fulfill its life cycle.

**MnSOD-Antisense Seedlings Show Inhibited Growth**

A common response to oxidative stress is that plants redirect their growth, which may be part of a direct acclimation strategy or may just be due to growth inhibition of oxidatively damaged plant tissues (Miller et al., 2007; Potters et al., 2007). However, ROS can be produced in a number of different subcellular locations and mitochondrial ROS are often regarded as insignificant in terms of growth effects because of the relatively low rate of ROS production in comparison to other organelles. However, we have shown that root growth was significantly reduced by the lower MnSOD protein level, even under nonstress growth conditions. Shoot growth was not obviously affected, although this was not quantified in seedlings. In older

**Figure 10.** Effects of decreased MnSOD levels on leaf antioxidant defense systems from 5-week-old plants. APX activity (A), GSH peroxidase activity (B), GSH content (C), and ascorbate content (D). E, FeSOD and MSD protein levels were detected by western-blot analysis in protein extracts from whole leaves. Different letters indicate significant differences between the different lines (n = 4, mean ± si, ANOVA posthoc LSD, P < 0.05).
soil-grown plants, where it was possible to quantify leaf mass, there was actually a significant increase in leaf mass in the transgenic lines, but this was most likely related to the late flowering phenotype that has the effect of extending the period of vegetative growth. Inhibition of root growth was also reported in Arabidopsis mutants lacking the mitochondrial PrxIIF (Finkemeier et al., 2005). Thus, a consistent picture is emerging that mitochondrial ROS production does have an important impact upon plant growth and development.

Relationship between Mitochondrial ROS Production and Plant Growth

There are two conceivable ways in which a deficiency in MnSOD might impinge upon plant growth: first, by oxidative inhibition of mitochondrial function (Sweetlove et al., 2002) and second, by perturbation of redox signaling (Foyer and Noctor, 2003). From the data presented here, the first possibility seems unlikely. Despite the fact that TCA cycle flux was reduced in the transgenic lines, as might be expected given the known sensitivity of TCA cycle enzymes to oxidative inactivation and damage (Verniquet et al., 1991; Flint et al., 1993; Sweetlove et al., 2002), the overall rate of respiratory CO₂ production in vivo was not decreased and was even slightly increased in AS-5. There was also a significant accumulation of TCA cycle organic acids. The most likely explanation for this is the considerable flexibility and redundancy that exists in the plant metabolic network. For example, it is possible that inhibited mitochondrial TCA cycle enzymes such as aconitase and IDH are bypassed in favor of the cytosolic isoforms of these enzymes. Exported citrate from the mitochondrion may also lead to an increase in the accumulation of organic acids in the vacuole. A mitochondrial citrate valve is thought to occur under oxidative stress conditions, when aconitase is inhibited (Igamberdiev and Gardestrom, 2003) and was even slightly increased in AS-5. There was no significant reduction of TCA cycle flux in the antisense lines, as might be expected given the small number of respiratory enzymes affected and no evident general increase in mitochondrial protein carbonyls, making it unlikely that there is a direct oxidative inhibition of growth.

The other possible link between reduced MnSOD and root growth is redox signaling. The induction of antioxidant defense genes under oxidative stress conditions is well known and often observed (Noctor and Foyer, 1998; Gadjev et al., 2006). While the precise nature of the signaling molecules and the molecular components of the signal transduction pathway remain poorly defined, it is now well accepted that ROS themselves are important signaling molecules. And increasingly, the mitochondrion is thought of as a key player in setting cellular redox balance and homeostasis (Noctor et al., 2007). The induction of antioxidant genes and perturbation of the redox state of the main cellular redox buffers in response to decreased MnSOD activity adds further weight to the notion that the redox status of the mitochondrion is sensed and that resultant redox signaling is important in setting cellular redox balance. Any number of signal molecules could be involved, including superoxide itself, other reactive oxygen and nitrogen species, ascorbate or GSH, as well as organic acids. All of these are candidate molecules for mitochondrial retrograde signaling (Rhoads and Subbaiah, 2007) and have been implicated in redox signaling. Furthermore, given that plant growth is known to be genetically constrained during stress conditions (Achard et al., 2006), the intriguing possibility emerges that the reduced root growth in MnSOD-deficient plants is a result of interaction between redox signaling pathways and the hormonal pathways that govern growth inhibition (Alvey and Harberd, 2005; Pasternak et al., 2005).

Ultimately, the signaling and oxidative-damage effects of ROS are difficult to separate by crude manipulation of the antioxidant system through constitutive mutation or transgenesis. Not only will an inducible approach be needed to avoid acclimatory and adaptive responses, but a controlled alteration of ROS production rates will be needed such that signaling is perturbed but oxidative damage is not induced. Technologies such as redox-sensitive GFPs that allow plant redox status to be quantitatively monitored at subcellular resolution and in real time (Meyer et al., 2007; Schwarzlander et al., 2008) will be essential in this regard.

MATERIALS AND METHODS

Antisense Constructs and Plant Transformation

The open reading frame of msd1 (At3g10920) was amplified from Arabidopsis (Arabidopsis thaliana; Columbia 0) cDNA using the following primers: msd1-attB, 5’-GGGGACAAATTCGTCAGAAGAATTCTCATGCGGATTCGTTGTGTAGC-3’ and 5’-GGGGACACCTCTTGCAGAAAAGCTGCTGTTCTACATCTCCTATATGCATCG-3’ (attB site is underlined), and cloned into the pK2WG7 vector for antisense expression in plants (Karimi et al., 2002) using the Gateway Technology system (Invitrogen). The vector construct (p35S:antisense-msd1) was verified by sequencing and transformed into Agrobacterium tumefaciens strain C58 followed by floral dip transformation of Arabidopsis (Columbia 0) plants (Clough and Bent, 1998). Transformants were selected by germination of seeds on Murashige and Skoog agar plates containing kanamycin (50 μg/mL). Resistant plants were transferred to soil and propagated.

Plant Material and Growth Conditions

Arabidopsis seeds were surface sterilized and layered onto a sterile liquid culture medium (4.4 g/L Murashige and Skoog, 10 g/L Suc, 0.4 g/L MES, 0.12 g/L agar, pH 5.8). Seedlings were grown for 10 d under continuous shaking (60 rpm) in a photoperiod of 16 h and a light intensity of 80 μE m⁻² s⁻¹. Soil-grown plants were grown for 4 to 5 weeks on compost supplemented with Vermiculite at 22°C and a photoperiod of 16 h and a light intensity of 150 to 200 μE m⁻² s⁻¹. For root-growth assays seeds were surface sterilized and
grown on vertical 1% agar plates supplemented with 0.5× Murashige and Skoog media and effectors as stated.

Isolation of Mitochondria

Mitochondria were isolated from 50 g fresh weight of 10-d-old Arabidopsis seedlings (Day et al., 1985). Seedlings were disrupted in a Waring blender by three successive 15-s bursts in 250 mL of grinding medium (0.3 M Suc, 25 mM tetrasodiumpyrophosphate, 1% [w/v] bovine serum albumin, 1% [w/v] PVPP, 20 mM Na₂EDTA, 10 mM KH₂PO₄, 20 mM ascorbate, 5 mM Cys pH 7.5). The filtered cell extract was separated by differential centrifugation and mitochondria were purified on a PVPP-Percoll gradient. The isolated mitochondria were washed twice and resuspended in 0.5 M mannitol, 10 mM TES, pH 7.5.

Respiratory Measurements and Enzyme Assays

Measurements of mitochondrial respiration were done as described in Sweetlove et al. (2002). Activities of citrate synthase, aconitate, IDH, 2-oxoglutarate dehydrogenase, NAD-malic enzyme, and pyruvate dehydrogenase were assayed as described in Jener et al. (2001). ICDH activity assay was performed after Iagramsberg and Gardestrom (2003). 2-Oxoglutarate dehydrogenase was assayed after Dry and Wiskich (1987) with the following modifications. The standard assay contained 70 mM TES (pH 7.0), 2 mM MgCl₂, 0.05% (v/v) Triton X-100, 0.2 mM ADP, 0.2 mM thiamine pyrophosphate, and 0.12 mM CoA. The reaction was initiated with 1 mM 2-oxoglutarate. AIF was assayed according to Janda et al. (1999), MDHAR according to Miyake and Asada (1992), and GSH peroxidase according to Yoshimura et al. (2004). NBT stains of excised leaves were performed as described in Duttileu et al. (2003).

One- and Two-Dimensional Gel Electrophoresis, Immunodetection, and MALDI-TOF-MS

Proteins were either extracted from frozen leaf tissue in a buffer containing 100 mM Tris, 1 mM EDTA (pH 6.8), or by acetone extraction from purified mitochondria. One-dimensional SDS-PAGE was performed according to standard protocols using 12% (w/v) polyacrylamide 0.1% (w/v) SDS gels. Two-dimensional gel electrophoresis was performed as described in Sweetlove et al. (2002). Two-dimensional gel electrophoresis of carbonylated proteins was carried out according to O’Brien et al. (2004). For immunodetection, separated proteins were transferred onto a nitrocellulose membrane and incubated with a 1:5,000 dilution of anti-MnSOD (Kliebenstein et al., 1998), or with a 1:5,000 dilution of anti-DNP antibodies (Sigma-Aldrich). ICDH and IDH proteins were detected with anti-idh antisera (Sigma-Aldrich), and FeSOD according to Janda et al. (1999), MDHAR according to Miyake and Asada (1992), and GSH peroxidase according to Yoshimura et al. (2004). NBT stains of excised leaves were performed as described in Dutittleu et al. (2003). Mass spectra were acquired by MALDI-TOF using a Shimadzu Axima CFR + (Shimadzu Biotech) in positive ion reflectron mode and mass lists matched against a translation of the Homozygous AS-5 plants were crossed with heterozygous lines expressing roGFP in the cytosol and in the mitochondria (Schwarzelander et al., 2008). The progeny were screened for kanamycin resistance (indicating presence of the msd1-antisense construct) and for expression of the roGFP in the appropriate subcellular compartment by confocal laser-scanning microscopy. Confocal laser-scanning microscopy and data processing were carried out as described in Schwarzelander et al. (2008). Whole leaves of seedlings were placed in a closed perfusion chamber RC-21BR (Warner Instruments LLC). Images were collected with a 25× lens (Zeiss 25 × 0.8 N.A. Plan-NEOFLUAR multimmersion lens) in multitrack mode of a Zeiss confocal microscope LSM510META equipped with lasers for 405- and 488-nm excitation. The 405-/488-nm laser power was kept constant at 1.4. Leaf samples from seedlings were perfused with one-half-strength Murashige and Skoog medium, pH 5.8, for approximately 2.5 min. Each experiment included an internal calibration at the end of the experiment by perfusion with 10 mM dihydrothreitol for approximately 10 min, washing with one-half-strength Murashige and Skoog medium for approximately 1.5 min, and perfusion with 100 mM H₂O₂ for approximately 1 min for in situ calibration to drive the roGFP to a fully reduced and fully oxidized form, respectively. The photometric analysis of the image time series was performed with a custom MatLab analysis suite (The MathWorks) available on request from M.D. Fricker. Representative diagrams of the photometric analysis are shown in Supplemental Figure S1.

RNA Isolation, CDNA Synthesis, and Semiquantitative RT-PCR

RNA was extracted using Trizol Reagent (Invitrogen) followed by chloroform extraction, isopropanol precipitation, and spectrophotometric quantification. cDNA was synthesized from DNase-treated RNA with SuperscriptII-reverse transcriptase (Invitrogen) following the manufacturer’s protocol. cDNA products were standardized for semiquantitative RT-PCR using ubq10 (At4g05320) primers as reference. Cycle numbers were optimized for each template using cDNA from wild-type plants to assure that the amplification reaction was tested in the exponential phase. Primers: citrate synthase (At2g44450) fwd: 5′-GGGATATGCTACCGTGTTTC-3′, rev: 5′-CTCTTGGCTTCTACAGTTGC-3′; aconitate (At2g05710) fwd: 5′-CCAAAGAACAGCCAGGCTTG-3′; rev: 5′-AAGCATCGGTCTCGCACTAC-3′; idh (At3g52680) fwd: 5′-AATTCAGTGCTCGCTGTCCTG-3′, rev: 5′-AATTCAGTGCTCGCTGTCCTG-3′; idhV (At4g0550) fwd: 5′-GGTCTCTTCGCTTTCTGC-3′, rev: 5′-CATTGCTCTGTCCTGC-3′; idhV (At1g03280) fwd: 5′-CATCAGGAAACACGAGAAG-3′, rev: 5′-AAGGGCTACACCACTTCC-3′; idhVI (At3g09810) fwd: 5′-AGGCCATCTGTTGATCATCCTC-3′, CGCCAAAACATGACAGCA-3′; NADPH-idh (Atg5) 112 Plant Physiol. Vol. 147, 2008

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Statistical Analysis

Student’s t tests (P < 0.05, significant; P < 0.1, marginally significant) were performed using Microsoft Excel. Significant differences (P < 0.05) are highlighted with an asterisk. Multifactorial analysis of variance was carried out using ANOVA with posthoc LSD (P < 0.05) with STATISTICA for WIN-DOWS software (version 5.5, Stat Soft). Different letters in the figures indicate significant differences (P < 0.05).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Representative diagrams of ratiometric analysis of redGFP oxidation in cytosol and mitochondria of wild-type and AS-5 seedlings followed by in vivo calibration with 10 mM dithiothreitol and 100 mM H2O2.

Supplemental Table S1. GC-MS metabolite data.

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LITERATURE CITED


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