Running Head:
MnSOD: mitochondrial protection and cellular redox balance

Corresponding author
Dr. Lee Sweetlove
University of Oxford
Department of Plant Sciences
South Parks Road
Oxford OX1 3RB
UK

Tel: +44 (0)1865 275000
Fax: +44 (0)1865 275074
E-mail: lee.sweetlove@plants.ox.ac.uk

Journal research area: Environmental Stress and Adaptation
Decrease in manganese superoxide dismutase leads to reduced root growth and affects TCA cycle flux and mitochondrial redox homeostasis

Megan J. Morgan¹, Martin Lehmann², Markus Schwarzländer¹, Charles J. Baxter¹*, Agata Sienkiewicz-Porzucek², Thomas C. R. Williams¹, Nicolas Schauer², Alisdair R. Fernie², Mark D. Fricker¹, R. George Ratcliffe¹, Lee J. Sweetlove¹**, and Iris Finkemeier¹

Addresses
¹Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, UK
²Max-Planck-Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Potsdam-Golm, Germany
*current address: Syngenta, Jealott’s Hill International Research Centre, Bracknell, Berkshire, RG42, 6EY, UK
This work was supported by the Biotechnology and Biological Sciences Research Council UK (M.J.M., C.J.B, T.C.R.W., M.D.F., R.G.R, and L.J.S), the Gatsby Charitable Foundation (M.S.), the Alexander von Humboldt Foundation (I.F.), the Max-Planck-Gesellschaft (A.S., M.L., and A.R.F.) and by the BMBF in the framework of Deutsch Israeli Projekt award (N.S.).

** Corresponding author
E-mail: lee.sweetlove@plants.ox.ac.uk
Tel: +44 (0)1865 275000
Fax: +44 (0)1865 275074
ABSTRACT
Superoxide dismutases (SODs) are key components of the plant antioxidant defence 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 plants were generated in which expression of *AtMSD1*, encoding the mitochondrial MnSOD 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 GFP (roGFP) was significantly more oxidised in the MnSOD antisense background. In contrast, there was no substantial change in oxidation of cytosolically-targeted roGFP, nor changes in antioxidant defence 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 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.

KEYWORDS: superoxide dismutase, reactive oxygen species, mitochondria, Arabidopsis, organic acids, respiration, redox homeostasis


INTRODUCTION
Plant mitochondria are important not only for respiration and several other metabolic activities (Sweetlove et al., 2007) but also play a key role in influencing photosynthetic metabolism in the chloroplast via numerous metabolite exchanges between the two organelles (Raghavendra and Padmasree, 2003). A key feature of mitochondrial biochemistry is the unavoidable production of reactive oxygen species (ROS) by the redox centres of the respiratory chain (Noctor et al., 2007). ROS are known to act in redox signalling to regulate plant metabolism and development especially under unfavourable environmental conditions (Foyer and Noctor, 2003; Navrot et al., 2007), and they are also regarded as potential candidates for retrograde and inter-organelar signalling molecules to co-ordinate plastid and mitochondrial biochemistry through the regulation of nuclear transcription (Pesaresi et al., 2007). Moreover, the exact chemical identity as well as the intracellular location of ROS production has a bearing on gene expression (op den Camp et al., 2003; Laloi et al., 2004). Gadjev et al. (2006) identified marker transcripts that were specifically regulated by hydrogen peroxide, superoxide or singlet oxygen, demonstrating that different ROS lead to different responses in plants.

At the same time ROS levels have to be tightly controlled by antioxidant systems, because their positive redox potential poses a major risk for the cellular molecular machinery. In mitochondria, superoxide is initially generated at the univalent electron carriers of the mitochondrial electron transport chain (Møller, 2001; Camacho et al., 2004). In contrast to the very reactive hydroxyl radical which 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-sulfur clusters of enzymes, which makes it cytotoxic to living cells (Fridovich, 1995). The iron which 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 sulfite (Halliwell, 2006). Superoxide reacts at very high rates with nitric oxide to form the toxic product peroxynitrite. Moreover, in non polar 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 (SOD) are the only enzymes capable of catalysing the dismutation of two superoxide radicals to hydrogen peroxide and molecular oxygen. Superoxide dismutases are ubiquitous metalloenzymes in prokaryotic and eukaryotic cells with aerobic metabolism. The Arabidopsis genome encodes eight SOD genes, comprising all three types of isoenzymes, Fe-, Mn-, and Cu/Zn-SOD. These are ubiquitously found in plants and differ by the named metal cofactor and their subcellular
localisation (Kliebenstein et al., 1998; del Rio et al., 2003). Plant mitochondria possess a highly conserved Mn-containing superoxide dismutase (MnSOD) (Fridovich, 1995; Gutteridge and Halliwell, 2000), which assembles as a homotetramer and contains one Mn atom per tetramer (Sevilla et al. 1982). The hydrogen peroxide 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 (Chew et al., 2003), as well as a glutathione peroxidase which 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 mitochondrion 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 oxidised amino acid side groups (Winger et al., 2005; Møller 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 extra-mitochondrial processes contribute to the response. An Arabidopsis knock-out mutant of the peroxiredoxin II F (prxII F) has demonstrated the importance of mitochondrial hydrogen peroxide 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 glutathione peroxidases which 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 over-expressed 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 CaMV35S:msd1 antisense construct to generate transgenic Arabidopsis plants with repressed levels of the mitochondrial MnSOD protein. MSD1 (At3g10920) encodes a 25 kDa protein found in the mitochondrial matrix (Millar et al., 2001; Kruft et al., 2001; Herald et al., 2003). From eighteen 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 (WT) (Fig. 1A). Both lines showed a strong decrease in MnSOD protein level compared to WT, with a 70 % and 60 % decrease in line AS-5 and AS-7, respectively, detected in purified mitochondria by immunoblotting using an 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, 10d-old seedlings grown in a sterile liquid culture media 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. 2A-C). However, the seedling dry weight and root growth of AS-7 were not significantly affected under control growth conditions (Fig. 2A-C). Root growth of AS-7 as compared to wild type was significantly decreased after 7d growth on media containing 50 mM sorbitol or 50 µM Fe (Fig. 2B). Most stress treatments, with the exception of 0.05 µM methylviologen, 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 WT. 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 green fluorescent protein roGFP (Jiang et al., 2006) which reports, like roGFP2 (Meyer et al., 2007), the redox status of the glutathione pool in vivo, (Schwarzländer et al., submitted). 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., submitted) with either WT or AS-5 plants. Progeny expressing the roGFP1 in each subcellular compartment (Figs. 3 A-D) and the msd1 antisense construct (as determined by kanamycin resistance) were analysed. Strikingly, the mitochondrial roGFP1 was significantly more oxidized in the AS-5 line in comparison to WT (Fig. 3E). In contrast, the cytosolic roGFP remained highly reduced in both WT and AS-5 line (Fig. 3F) at the limit of the dynamic range (Schwarzländer et al., submitted). Thus, it seems that the major shift in redox status of the glutathione 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 defence systems (Foyer & Noctor, 2003). We therefore measured the activity or mRNA transcript abundance of a number of key antioxidant defence related proteins. The activity level of mitochondrial APX was significantly reduced in the AS-5 mitochondria compared to wild type, while the 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 defence-related
transcripts tested, there were few major changes. The level of ferritin-1 transcript, which is a
marker for hydrogen peroxide (Op den Kamp et al. 2003), was slightly decreased in both
MnSOD-antisense lines, whereas transcript levels from the organellar monodehydroascorbate
reductase (mdhar) and the mitochondrial peroxiredoxin were slightly increased (Fig. 4D).

To investigate whether the reduced mitochondrial antioxidant capacities resulted in
higher levels of 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 1h 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 glutathione 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-
catalysed 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, C) and
probed with an anti-DNP antibody (Fig. 5 B, D). The normalised quantity of protein spots was
established from images of Coomassie-stained 2D-gels and Western blots, respectively, using
PDQuest software (vs. 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 MALDI-TOF mass spectrometry
(Table 1). The only protein spot which showed a strong 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, C; Table 1). 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 aspartate aminotransferase (Spot 7, 8:
Fig. 5 A, C; Table 1). 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 (Spot 1-4: Fig. 5 B, D; Table 1). Among them are the mitochondrial monodehydroascorbate reductase (MDHAR) and the heat shock 70-like protein (Table 1). However, 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 as 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 6h. In accordance with the decreased mitochondrial IDH activity (Fig. 6A), the rate of 13C incorporation into 2-oxoglutarate was decreased by about 60 % in the AS-5 line compared to wild type, whereas 13C incorporation into citrate occurred with the same rate (Fig. 6B, C).

Respiratory CO2 release is not inhibited in antisense MnSOD plants

To investigate the respiratory pathways in more detail in vivo we measured the 14CO2 release from leaf discs of wild type and MnSOD-antisense plants incubated in 14C-glucose labelled at C1, C2, C3:4 and C6 positions, respectively. The 14CO2 release was monitored hourly over a period of 6h. Carbon dioxide that is released from the C1, but also the C2 position of glucose 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 labelled glucose \textit{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 labelled position at the pyruvate dehydrogenase step whereas CO₂ is
released from the C6 labelled 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 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 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. 8B, C).

\textbf{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), 4-5
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 WT (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 - 70% in the antisense lines compared to WT. 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 WT and the MnSOD-antisense lines were observed under various light conditions up to 1000 µE (Fig. 9A). Similarly there were no significant differences in chlorophyll contents between the lines and WT (Fig. 9B). Respiratory CO₂ release remained similar or slightly higher than WT 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 GC-MS metabolic profile analysis (Roessner et al., 2001). Strikingly, from more than 60 analysed metabolites only TCA cycle organic acids (Fig. 9D) and ethanolamine (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 extra-mitochondrial changes in 4-5 week old soil-grown leaves and in particular there was a substantial upregulation of total cellular antioxidant defences. Activities of total cellular ascorbate peroxidase (APX) and glutathione peroxidase, as well as total pools of ascorbate and glutathione (GSH) were increased in the MnSOD -antisense leaves as compared to wild type (Fig. 10A-D). Total ascorbate and GSH levels in line AS-7 and AS-5 were increased by 40 and 50 %, respectively, when compared to wild type (Fig. 10C, 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). Protein levels of the plastidic FeSOD were strongly increased as was seen in younger seedlings (Figs 4C, 10E). MnSOD levels showed a more than 60 % decrease in total leaf extracts as seen before in isolated mitochondria from seedlings (Figs. 3E, 10E).

DISCUSSION

This paper presents the first characterization of plants with suppressed MnSOD level providing new insights into the central role of MnSOD in the mitochondrial antioxidant defence 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 1). We assumed that a decrease in MnSOD protein level would result in higher steady state levels of superoxide and hydrogen peroxide as predicted from computation simulation (Polle et al., 2001), which in turn can given 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 defence 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 glutathione is normally regarded as oxidative stress which can potentially lead to oxidative damage (Halliwell 2006). 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 glutathione 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 hydrogen peroxide (Nakano and Asada, 1987). Therefore, it would be very interesting to monitor the redox state and pool size of mitochondrial ascorbate. However, 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 glutathione peroxidase activities, increased FeSOD levels and an increased pool size of the redox buffers glutathione 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 glutathione were also found in plants with decreased levels of plastidic Cu/Zn-SOD (Rizhsky et al., 2003). Overcompensation of defects in antioxidant enzymes seem to be a general response to the loss of antioxidant enzymes and were also observed in double-knock out mutants of apx1/cat1 (Rizhsky et al., 2002), 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 5week-old MnSOD-antisense lines. These lines are a good example of the flexibility of the plant...
antioxidant defence 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 fulfil 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 non-stress growth
conditions. Shoot growth was not obviously affected, although this was not quantified in
seedlings. In older 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 which 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 signalling (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 \textit{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
favour 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 (Igamberidiev and Gardetrom, 2003) and the resulting higher level of cytosolic NADPH can be used by antioxidant systems (de Carvalho et al., 2003). Thus, despite specific restrictions on TCA cycle flux in the mitochondrion, the flexibility of the metabolic system ensures that the overall respiratory flux is unaffected. Moreover, the extent of oxidative damage in the antisense lines is rather limited, with only a small number of respiratory enzymes affected and no evidence of a 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 signalling. The induction of antioxidant defence genes under oxidative stress conditions is well known and often observed (Noctor and Foyer, 1998; Gadjiev et al., 2006). While the precise nature of the signalling molecules and the molecular components of the signal transduction pathway remain poorly defined, it is now well accepted that ROS themselves are important signalling 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 signalling 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 glutathione, as well as organic acids. All of these are candidate molecules for mitochondrial retrograde signalling (Rhoads and Subbaiah, 2007) and have been implicated in redox signalling. Furthermore, given that plant growth is known to be genetically constrained during stress conditions (Archard et al., 2006), the intriguing possibility emerges that the reduced root growth in MnSOD-deficient plants is a result of interaction between redox signalling pathways and the hormonal pathways that govern growth inhibition (Alvey et al., 2005; Pasternak et al., 2005).

Ultimately, the signalling 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 signalling 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 (Schwarzländer et al., submitted; Meyer et al., 2007) will be essential in this regard.

MATERIALS AND METHODS

Antisense Constructs and Plant Transformation

The open reading frame of msd-1 (At3g10920) was amplified from Arabidopsis (Col0) cDNA using the following primers: msd1-attB, 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGATTCGTTGTGTAGC-3’ and, 5’-GGGGACCACTTTGTACAAGAAAGCTGGGT15GTTCATCTCCTTATGTCATCG-3’ (attB site is underlined), and cloned into the pK2WG7 vector for antisense expression in plants (Karimi et al., 2001) 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 (Col0) plants (Clough and Bent, 1998). Transformants were selected by germination of seeds on MS-agar plates containing kanamycin (50 µg/ml). Resistant plants were transferred to soil and propagated.

Plant Material and Growth Conditions

Arabidopsis seeds were surface sterilised and layered onto a sterile liquid culture media (4.4 g/l MS, 10 g/l sucrose, 0.4 g/ MES, 0.12 g/l agar, pH 5.8). Seedlings were grown for 10d under contious 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 4-5 weeks on compost supplemented with Vermiculite at 22°C and a photoperiod of 16 h and a light intensity of 150-200 µE·m⁻²·s⁻¹. For root-growth assays seeds were surface sterilised and grown on vertical 1 % agar plates supplemented with 0.5x MS media and effectors as stated.

Isolation of Mitochondria
Mitochondria were isolated from 50 g fresh weight of 10-day-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 sucrose, 25 mM tetrasodiumpyrophosphate, 1 % (w/v) bovine serum albumin, 1 % (w/v) PVP-40, 2 mM Na₂EDTA, 10 mM KH₂PO₄, 20 mM ascorbate, 5 mM cysteine pH 7.5). The filtered cell extract was separated by differential centrifugation and mitochondria were purified on a PVP-Percoll gradient. The isolated mitochondria were washed twice and resuspended in 0.3 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, aconitase, IDH, 2-oxoglutarate dehydrogenase, NAD-malic enzyme and pyruvate dehydrogenase were assayed as described in Jenner et al. (2001). ICDH activity assay were performed after Igamberdiev and Gardeström (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, 2 mM NAD, 0.2 mM TPP, 0.12 mM CoA. The reaction was initiated with 1 mM 2-oxoglutarate. Ascorbate peroxidase was assayed according to Janda et al. (1999), monodehydroascorbate reductase according to Miyake and Asada (1992), and glutathione peroxidase according to Yoshimura et al. (2004). Nitroblue tetrazolium stains of excised leaves were performed as described in Dutilleul et al. (2003).

**1D-, 2D-Gel Electrophoresis, Immunodetection, and MALDI-TOF-MS**

Proteins were either extracted from frozen leaf tissue in a buffer containing 100 mM Tris, 1mM 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, with a 1:1,000 dilution of anti-FeSOD (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-rabbit antibodies raised against the recombinant tobacco proteins in 1:500 dilutions (Lancien et al. 1998). Detection was performed with a 1:10,000 dilution of horseradish
peroxidase-linked secondary antibody using a chemiluminescence detection kit (Perbio) on X-ray films exposed to the Western Blot membranes for the exact same length of time. Proteins were identified by analysis of peptide mass fingerprints following digestion with Trypsin according to Sweetlove et al., (2002). Mass spectra were acquired by MALDI-ToF using a Shimadzu Axima CFR+ (Shimadzu Biotech, Manchester, UK) in positive ion reflectron mode and mass lists matched against a translation of the NCBI gene database using an in-house Mascot server (Matrix Science Ltd, London, UK).

**NMR analysis of [3-13C]pyruvate metabolism**

Coupled mitochondria from WT and AS-5 seedlings (500 µg mitochondrial protein in 1 ml wash buffer) were diluted into 4 ml of buffer containing 0.2 M mannitol, 0.1 M Mops, 5 mM MgCl₂, 0.1 % w/v BSA, 20 mM KH₂PO₄, 20 mM glucose, 2.1 mM citrate, 1.3 mM succinate, 0.6 mM malate, 0.3 mM NAD⁺, 0.1 mM ADP, 0.1 mM TPP, 0.02 mM fumarate, 0.02 mM isocitrate and 10 mM [3-13C]pyruvate (Aldrich Chemical Company, Milwaukee, WI, USA) in 10% D₂O, pH 7.2. Hexokinase (Roche, Lewes, East Sussex, UK) was also included to give a concentration of 0.15 U mL⁻¹ in the diluted suspension. These conditions were similar to those described elsewhere (Smith et al., 2004; Nunes-Nesi et al., 2005) and they allowed the metabolism of the labelled pyruvate to be monitored continuously under conditions of state 3 respiration. The mitochondrial suspension was oxygenated in a 10 mm diameter NMR tube using an air-lift system (Fox et al., 1989) and proton-decoupled ¹³C NMR spectra were recorded at 150.9 MHz on a Varian Unity Inova 600 spectrometer (Palo Alto, CA) using a broadband probehead. Twenty four spectra were recorded in 15 min blocks over a period of 6 h using a 90º pulse angle, a 1.016 s acquisition time and a 6 s relaxation delay. Low power frequency modulated decoupling was applied during the relaxation delay to maintain the nuclear Overhauser effect and this was switched to higher power Waltz decoupling during the acquisition time to remove the proton couplings. Chemical shifts are quoted relative to the mannitol CH₂OH signal at 63.90 ppm.

**Measurement of photosynthetic parameters**

Either 1g of seedlings or leaf discs of 10-mm diameter were incubated in 10 mM MES-KOH, pH 6.5, containing 2.32 KBq ml⁻¹ of [1-¹⁴C]-, [2-¹⁴C]-, [3:4-¹⁴C]-, or [6-¹⁴C]Glc in a leaf-disc oxygen electrode chamber (Hansatech, Kings Lynn, Norfolk, UK) as described in Nunes-Nesi et al. (2005). ¹⁴CO₂ evolved was trapped in KOH in hourly intervals and quantified by liquid
scintillation counting. Gas-exchange measurements were performed as described by Nunes-
Nesi et al. (2005).

Determination of Metabolite Levels
Ascorbate, dehydroascorbate, and glutathione (GSH) levels were determined as described in
Baier et al., (2000). Chlorophyll measurements were performed as described by Nunes-Nesi
et al. (2005). The levels of all other metabolites were quantified by GC-MS as described in
Roessner et al. (2001).

In vivo roGFP measurements
Homozygous AS-5 plants were crossed with heterozygous lines expressing roGFP1 in the
cytosol and in the mitochondria (Schwarzländer et al., submitted). The progeny were screened
for kanamycin resistance (indicating presence of the MSD antisense construct) and for
expression of the roGFP in the appropriate sub-cellular compartment by confocal laser
scanning microscopy. Confocal laser scanning microscopy and data processing were carried
out as described in Schwarzländer et al. (submitted). Whole leaves of seedlings were placed in
a closed perfusion chamber RC-21BR (Warner Instruments LLC, Hamden, CT, USA).
Images were collected with a 25× lens (Zeiss 25× 0.8 N.A. Plan-NEOFLUAR multi-
immersion lens) in multi-track mode of a Zeiss confocal microscope LSM510META
equipped with lasers for 405 nm and 488 nm excitation. The 405/488 nm laser power was
kept constant at 1:4. Leaf samples from seedlings were perfused with ½-strength MS-
medium, pH 5.8 for ~2.5 min. Each experiment included an internal calibration at the end of
the experiment by perfusion with 10 mM DTT for ~10 min, washing with ½-strength MS-
medium for ~1.5 min and perfusion with 100 mM H₂O₂ for ~10 min for in situ calibration to
drive the roGFP to a fully reduced and fully oxidised form respectively. The ratiometric
analysis of the image time series was performed with a custom MatLab analysis suite (The
MathWorks, Nantick, MA) available on request from M.D. Fricker.

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) after the
manufactures protocol. cDNA products were standardized for semiquantitative RT-PCR using
ubq10 (At4g05320) primers as reference. Cycle numbers were optimised for each template
using cDNA from wild type plants to assure that the amplification reaction was tested in the exponential phase. Primers:

citrate synthase (At2g44350) fwd: 5’-GGGATATGGTCAACGTTGC-3’, rev: 5’-CTCTTTGGCCTCTAAGTGC-3’; aconitase (At2g05710) fwd: 5’-CAAGCAAAACATGGAGCTTG-3’, rev: 5’-AAGCATTTGCCCTCAGCTT-3’; idhI (At4g35260) fwd: 5’-AATTACGTGTCCCCCCCTG-3’, rev: 5’-TGCATATCCACGAAAGTGC-3’; idhII (At2g17130) fwd: 5’-AAGCTCTGTTGCTCT-3’; idhIII (At4g35650) fwd: 5’-GCTTCTCTGCTCTGCTTG-3’, rev: 5’-CATTGCTTCTGCTGCC-3’; idhIV (At5g03290) fwd: 5’-CATCAGAAACACGGAAGG-3’, rev: 5’-AAGGGCTACACCTTCTTCC-3’; idhVI (At3g09810) fwd: 5’-CAAGCAAACATGGAGCTTG-3’, rev: 5’-AAGCATCAGTCACACGTCGG-3’; NADPH-idh (org., At5g14590) fwd: 5’-GCTTCTCTGCTCTGCTTG-3’, rev: 5’-CATTGCTTCTGCTGCC-3’; NADPH-idh (cyt., At1g65930) fwd: 5’-AAGCTCTGTTGCTCT-3’; ferritin 1 (At5g01600) fwd: 5’-ATGGGCTCAACGACTCTC-3’, rev: 5’-CTAGTCCCTCAAGTCAACG-3’; prxIIF(At3g06050) fwd: 5’-ACACGGGAGTTTGGTTCAG-3’, rev: 5’-GTCTTAAAGATGACTTCTGC-3’; mdhar (at1g63940) fwd: 5’-CAGCTGTGCTGGGGAATCTA-3’, rev: 5’-CTGCGACATCTCAATAGCA-3’.

Statistical Analysis

Students t-tests (p<0.05, significant; p<0.1, marginally significant) were performed using Microsoft Excel (Microsoft, USA). Significant differences (p<0.05) are highlighted with an asterisk. Multifactorial analysis of variance was carried out using ANOVA with post-hoc LSD (p<0.05) with STATISTICA for WINDOWS software (version 5.5, Stat Soft, USA). Different letters in the figures indicate significant differences (p<0.05).

Supplemental Data

Supplemental Table S1. GC-MS metabolite data.

Supplemental Figure 1. Representative diagrams of ratiometric analysis of roGFP1 oxidation in cytosol and mitochondria of WT and AS-5 seedlings followed by in vivo calibration with 10 mM DTT and 100 mM H$_2$O$_2$. 

www.plantphysiol.org on October 1, 2017 - Published by Downloaded from Copyright © 2008 American Society of Plant Biologists. All rights reserved.
ACKNOWLEDGMENTS

The authors would like to thank Dan Kliebenstein (University of California, Davis) for the anti-MnSOD and anti-FeSOD antibodies and Michael Hodges (Université Paris, Orsay Cedex) for the anti-IDH and anti-ICDH antibodies.
LITERATURE CITED


Igamberdiev AU, Gardestrom P (2003) Regulation of NAD- and NADP-dependent isocitrate dehydrogenases by reduction levels of pyridine nucleotides in mitochondria and


cytosol of pea leaves. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1606: 117-125


consequence of decreasing mitochondrial malate dehydrogenase activity in transgenic tomato plants. Plant Physiol 137: 611-622


Van Loon APGM, Pesoldhurt B, Schatz G (1986) A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen. Proc Natl Acad Sci (USA) 83: 3820-3824


LEGENDS TO FIGURES

Figure 1: Screening of MnSOD-antisense plants. (A) RT-PCR analysis for altered expression of msd1 transcript. Transcripts were amplified by gene-specific RT-PCR, and EtBr-stained gels were scanned and densitometrically analysed. (B) Western blots of wild type, AS-5 and AS-7 mitochondrial protein extracts, using a specific antiserum against MnSOD protein. Mitochondria were isolated from 10d-old seedlings grown in liquid culture. Western-blots were scanned and densitometrically analysed.

Figure 2: Growth performance of MnSOD-antisense and wild type seedlings. (A) Growth of wild type, AS-5 and AS-7 seedlings on vertical MS-Agar plates. (B) Effects of abiotic stressors on root growth. Root length of 7d-old wild type, AS-5, and AS-7 seedlings were determined in 3 independent experiments from at least 10 plants per line for each treatment.
(mean ± SE). (C) Dry weight of 10d-old seedlings grown in liquid culture (n=5, mean ± SE).

Different letters indicate significant differences between the different lines (ANOVA post-hoc LSD, p<0.05)

**Figure 3:** RoGFP1 redox status in mitochondria and cytosol of WT and AS-5 seedlings.
(A-D) Expression of roGFP1 in mitochondria (A, C) and cytosol (B, D) in leaf epidermis cells of WT and AS-5 seedlings, respectively. (E, 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 DTT and 100 mM H\textsubscript{2}O\textsubscript{2} as described by Schwarzländer et al., (in preparation) in detail (n=5, mean ± SE). Asterisks indicate significant differences between WT and AS-5 (*t*-test, p<0.05).

**Figure 4:** Effects of MnSOD suppression on antioxidant status from 10d-old seedlings grown in liquid culture.
(A) Ascorbate content and redox status in whole seedlings (n= 3, mean ± SE). (B) Enzyme activities of APX and MDHAR in purified mitochondria from WT and AS-5 (n=4, mean ± SE). (C) Western blot analysis of FeSOD and MnSOD protein content in whole seedlings (D) Semiquantitative RT-PCR analysis of antioxidant gene transcript abundance in WT and MnSOD-antisense lines. Transcripts were amplified by gene-specific RT-PCR, and EtBr-stained gels were scanned and densitometrically analysed. Numbers are given as average induction factors from three independent experiments. (E) *In situ* detection of superoxide levels by NBT-staining of seedlings grown under control conditions and MS-media supplemented with 50 µM and 100 µM Fe, respectively. Different letters indicate significant differences between the different lines (ANOVA post-hoc LSD, p<0.05).

**Figure 5:** Changes in protein abundance and carbonylation in mitochondrial proteins from 10d-old AS-5 and WT seedlings. Proteins were separated by isoelectric focussing on 3–10 non-linear immobilized pH gradients, subsequently separated by SDS-PAGE, Coomassie-blue stained (A, C) or transferred onto nitrocellulose membranes and subjected to Western blot analysis with an anti-DNP antibody to detect carbonylated proteins (B, D); wild type (A, B) and AS-5 (C, D). Scanned images from 3 independent experiments were compared by PDQuest analysis software (Bio-Rad). Labelled spots were analyzed for changes in protein and carbonylation level and identified by MALDI-TOF-MS analysis (see table 1).

**Figure 6:** Respiratory activity and TCA cycle flux in intact coupled mitochondria.
(A) Activities of respiratory complexes and TCA cycle enzymes measured in isolated mitochondria from 10d-old wild type and AS-5 seedlings isolated from three independent experiments (mean ± SE). (B) \textsuperscript{13}C NMR spectra recorded from dilute suspensions of WT and
AS-5 mitochondria metabolising [3-13C]pyruvate, showing the [4-13C]citrate and [4-13C]2-oxoglutarate signals at 44.85 and 31.2 ppm respectively during the sixth hour of the experiment. (C) Average rate of [4-13C]citrate and [4-13C]2-oxoglutarate production in isolated mitochondria from 10 d-old WT and AS-5 seedlings calculated from the peak area per min measured over a 6 h time period (n=3, mean ± SE). WT, black bars; AS-5, white bars. * indicates significant differences to wild type (t-test, p<0.05), †indicates marginally significant differences to wild type (t-test, p<0.1).

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

**Figure 8: Transcript levels of TCA cycle enzymes, IDH and ICDH protein content, and ICDH activity in 10d-old WT and MnSOD-antisense seedlings** (A) Semiquantitative RT-PCR analysis of nuclear-encoded gene products from the TCA-cycle enzymes citrate synthase, aconitase and NAD(P)-isocitrate dehydrogenase. Transcripts were amplified by gene-specific RT-PCR, and EtBr-stained gels were scanned and densitometrically analysed. 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 post-hoc LSD, p<0.05).

**Figure 9: Photosynthesis and other primary physiological parameters in rosette leaves of WT and MnSOD-antisense plants.** (A) Assimilation rates under varying light intensities. (B) Chlorophyll a and chlorophyll b contents. (C) 14CO2 evolution from leaf discs in the light. Leaf discs were taken from 4-week old plants and were incubated in 10 mM MES-KOH, pH 6.5, supplemented with [3:4-14C]-, or [6-14C]-labelled glucose. The experiment was performed in triplicates with leaf discs taken from 10 individual plants each (mean ± SE). (D) Relative organic acid contents. Metabolites were determined by GC-MS analysis. Data from each metabolite are normalized to the respective mean value from wild type (to allow statistical assessment, individual samples from this sets of plants were normalized in the same way; n = 6, mean ± SE). WT, black bars; AS-5, white bars; AS-7, grey bars. Different letters indicate significant differences between the different lines (ANOVA post-hoc LSD, p<0.05).

**Figure 10: Effects of decreased MnSOD levels on leaf antioxidant defence systems from 5week old plants.** (A) Ascorbate peroxidase activity, (B) glutathione peroxidase activity, (C) glutathione content, (D) ascorbate content. (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 ± SE, ANOVA post-hoc LSD,
p<0.05).
Table 1: Identification of mitochondrial protein spots fractionated by IEF-SDS-PAGE with altered abundance or protein carbonyl content in AS-5 line compared to WT. Peptide masses after digestion with trypsin were matched against a translated NCBI database using an in-house MASCOT server.

<table>
<thead>
<tr>
<th>Spot I.D.</th>
<th>Entry Details of Match</th>
<th>Average increase in carbonylation relative to WT</th>
<th>Average change in abundance relative to WT</th>
<th>Gel MM/pI</th>
<th>Match MM/pI</th>
<th>No. MP</th>
<th>% protein coverage</th>
<th>Error ppm</th>
<th>TP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 At3g07770</td>
<td>heat shock protein 89-1 (HSP90 related)</td>
<td>2.4</td>
<td>0.84</td>
<td>90000/4.65</td>
<td>90537/5.26</td>
<td>9</td>
<td>12</td>
<td>41</td>
<td>M</td>
</tr>
<tr>
<td>2 At4g37910</td>
<td>heat shock protein 70 like protein</td>
<td>2.4</td>
<td>0.79</td>
<td>70000/4.8</td>
<td>73030/5.51</td>
<td>14</td>
<td>26</td>
<td>84</td>
<td>M</td>
</tr>
<tr>
<td>3 At1g63940</td>
<td>monodehydroascorbate reductase, putative</td>
<td>1.7</td>
<td>1.38</td>
<td>48000/5.9</td>
<td>52469/7.05</td>
<td>12</td>
<td>32</td>
<td>68</td>
<td>C/M</td>
</tr>
<tr>
<td>4 At4g27585</td>
<td>stomatin band 7 family protein, similar to stomatin-like protein</td>
<td>2.3</td>
<td>1.45</td>
<td>46000/5.95</td>
<td>44992/6.35</td>
<td>9</td>
<td>22</td>
<td>134</td>
<td>M</td>
</tr>
<tr>
<td>5 At3g10920</td>
<td>MSD1 (manganese superoxide dismutase 1)</td>
<td>-</td>
<td>0.36</td>
<td>25500/6.3</td>
<td>25428/8.47</td>
<td>6</td>
<td>41</td>
<td>52</td>
<td>M</td>
</tr>
<tr>
<td>6 At2g33150</td>
<td>acetyl-CoA C-acyltransferase</td>
<td>-</td>
<td>1.55</td>
<td>44500/9.0</td>
<td>48548/8.62</td>
<td>10</td>
<td>37</td>
<td>60</td>
<td>M</td>
</tr>
<tr>
<td>7 At2g30970</td>
<td>aspartate aminotransferase (AAT1)</td>
<td>-</td>
<td>2.3</td>
<td>42000/6.95</td>
<td>47727/8.36</td>
<td>8</td>
<td>23</td>
<td>79</td>
<td>M</td>
</tr>
<tr>
<td>8 At2g30970</td>
<td>aspartate aminotransferase (AAT1)</td>
<td>-</td>
<td>2.3</td>
<td>36100/5.55</td>
<td>47727/8.36</td>
<td>10</td>
<td>30</td>
<td>85</td>
<td>M</td>
</tr>
<tr>
<td>9 At1g222450</td>
<td>COX6B (Cytochrome c oxidase 6B)</td>
<td>-</td>
<td>1.6</td>
<td>39000/4.25</td>
<td>21182/4.29</td>
<td>7</td>
<td>32</td>
<td>129</td>
<td>O</td>
</tr>
</tbody>
</table>

*Chew et al. 2003*
Table headings: No. MP, number of peptides matching to predicted protein sequence (P<0.05); GEL MM/pI, observed molecular mass (MM) and pI of the sample from the gel in Fig. 4; MATCH MM/pI predicted molecular mass (MM) and pI of matched sequence; TP, predicted localization of sequence by TargetP, M: mitochondrial, C: chloroplast, S: secretory pathway, O: other.
Figure 1: Screening of MnSOD-antisense plants.
(A) RT-PCR analysis for altered expression of msd1 transcript. Transcripts were amplified by gene-specific RT-PCR, and EtBr-stained gels were scanned and densitometrically analysed. (B) Western blots of wild type, AS-5 and AS-7 mitochondrial protein extracts, using a specific antiserum against MnSOD protein. Mitochondria were isolated from 10d-old seedlings grown in liquid culture. Western-blots were scanned and densitometrically analysed.
Figure 2: Growth performance of MnSOD-antisense and wild type seedlings. (A) Growth of wild type, AS-5 and AS-7 seedlings on vertical MS-Agar plates. (B) Effects of abiotic stressors on root growth. Root length of 7d-old wild type, AS-5, and AS-7 seedlings were determined in 3 independent experiments from at least 10 plants per line for each treatment (mean ± SE). (C) Dry weight of 10d-old seedlings grown in liquid culture (n=5, mean ± SE). Different letters indicate significant differences between the different lines (ANOVA post-hoc LSD, p<0.05)
Figure 3: RoGFP1 redox status in mitochondria and cytosol of WT and AS-5 seedlings. (A-D) Expression of roGFP1 in mitochondria (A, C) and cytosol (B, D) in leaf epidermis cells of WT and AS-5 seedlings, respectively. (E, 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 DTT and 100 mM H2O2 as described by Schwarzländer et al., (in preparation) in detail (n=5, mean ± SE). Asterisks indicate significant differences between WT and AS-5 (t-test, p<0.05).
Figure 4: Effects of MnSOD suppression on antioxidant status from 10d-old seedlings grown in liquid culture. (A) Ascorbate content and redox status in whole seedlings (n= 3, mean ± SE). (B) Enzyme activities of APX and MDHAR in purified mitochondria from WT and AS-5 (n=4, mean ± SE). (C) Western blot analysis of FeSOD and MnSOD protein content in whole seedlings (D) Semiquantitative RT-PCR analysis of antioxidant gene transcript abundance in WT and MnSOD-antisense lines. Transcripts were amplified by gene-specific RT-PCR, and EtBr-stained gels were scanned and densitometrically analysed. Numbers are given as average induction factors from three independent experiments. (E) In situ detection of superoxide levels by NBT-staining of seedlings grown under control conditions and MS-media supplemented with 50 µM and 100 µM Fe, respectively. Different letters indicate significant differences between the different lines (ANOVA post-hoc LSD, p<0.05).
Figure 5: Changes in protein abundance and carbonylation in mitochondrial proteins from 10d-old AS-5 and WT seedlings. Proteins were separated by isoelectric focussing on 3–10 non-linear immobilized pH gradients, subsequently separated by SDS-PAGE, Coomassie-blue stained (A, C) or transferred onto nitrocellulose membranes and subjected to Western blot analysis with an anti-DNP antibody to detect carbonylated proteins (B, D); wild type (A, B) and AS-5 (C, D). Scanned images from 3 independent experiments were compared by PDQuest analysis software (Bio-Rad). Labelled spots were analyzed for changes in protein and carbonylation level and identified by MALDI-TOF-MS analysis (see table 1).
Figure 6: Respiratory activity and TCA cycle flux in intact coupled mitochondria. (A) Activities of respiratory complexes and TCA cycle enzymes measured in isolated mitochondria from 10d-old wild type and AS-5 seedlings isolated from three independent experiments (mean ± SE). (B) $^{13}$C NMR spectra recorded from dilute suspensions of WT and AS-5 mitochondria metabolising [3-$^{13}$C]pyruvate, showing the [4-$^{13}$C]citrate and [4-$^{13}$C]2-oxoglutarate signals at 44.85 and 31.2 ppm respectively during the sixth hour of the experiment. (C) Average rate of [4-$^{13}$C]citrate and [4-$^{13}$C]2-oxoglutarate production in isolated mitochondria from 10 d-old WT and AS-5 seedlings calculated from the peak area per min measured over a 6 h time period (n=3, mean ± SE). WT, black bars; AS-5, white bars. * indicates significant differences to wild type (t-test, p<0.05), †indicates marginally significant differences to wild type (t-test, p<0.1).
Figure 7: $^{14}$CO2 evolution from seedlings in the light.

(A-D) 10d-old seedlings were incubated in 10 mM MES-KOH, pH 6.5, supplemented with [1-$^{14}$C], [2-$^{14}$C], [3:4-$^{14}$C], or [6-$^{14}$C]-labelled glucose (n=3, mean ± SE). Different letters indicate significant differences between the different lines at time point t=6h (ANOVA post-hoc LSD, p<0.05).
Figure 8: Transcript levels of TCA cycle enzymes, IDH and ICDH protein content, and ICDH activity in 10d-old WT and MnSOD-antisense seedlings. (A) Semiquantitative RT-PCR analysis of nuclear-encoded gene products from the TCA-cycle enzymes citrate synthase, aconitase and NAD(P)-isocitrate dehydrogenase. Transcripts were amplified by gene-specific RT-PCR, and EtBr-stained gels were scanned and densitometrically analysed. 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 post-hoc LSD, p<0.05).
Figure 9: Photosynthesis and other primary physiological parameters in rosette leaves of WT and MnSOD-antisense plants. (A) Assimilation rates under varying light intensities. (B) Chlorophyll a and chlorophyll b contents. (C) $^{14}$CO$_2$ evolution from leaf discs in the light. Leaf discs were taken from 4-week old plants and were incubated in 10 mM MES-KOH, pH 6.5, supplemented with [3:4-$^{14}$C] or [6-$^{14}$C]-labelled glucose. The experiment was performed in triplicates with leaf discs taken from 10 individual plants each (mean ± SE). (D) Relative organic acid contents. Metabolites were determined by GC-MS analysis. Data from each metabolite are normalized to the respective mean value from wild type (to allow statistical assessment, individual samples from this sets of plants were normalized in the same way; n = 6, mean ± SE). WT, black bars; AS-5, white bars; AS-7, grey bars. Different letters indicate significant differences between the different lines (ANOVA post-hoc LSD, p<0.05).
Figure 10: Effects of decreased MnSOD levels on leaf antioxidant defence systems from 5 week old plants. (A) Ascorbate peroxidase activity, (B) glutathione peroxidase activity, (C) glutathione content, (D) ascorbate content. (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 ± SE, ANOVA post-hoc LSD, p<0.05).

Copyright © 2008 American Society of Plant Biologists. All rights reserved.