Differential Inhibition of *Arabidopsis* Superoxide Dismutases by Peroxynitrite-mediated Tyrosine Nitration

Christian Holzmeister[1,§], Frank Gaupels[1,§], Arie Geerlof[2], Hakan Sarioglu[3], Michael Sattler[2,4], Jörg Durner[1,5], Christian Lindermayr[1,] *

From the [1]Institute of Biochemical Plant Pathology and [2]Institute of Structural Biology
Helmholtz Zentrum München – German Research Center for Environmental Health, 85764 München/Neuherberg, Germany
[3]Department of Protein Science, Helmholtz Zentrum München-German Research Center for Environmental Health, 85764 München/ Neuherberg, Germany
[4]Munich Center for Integrated Protein Science at Chair of Biomolecular NMR, Department Chemie, Technische Universität München, 85747 Garching, Germany
[5]Chair of Biochemical Plant Pathology, Technische Universität München, 85354 Freising, Germany.

[§]Both authors contributed equally to this work.
ch.holzmeister@gmx.de
frank.gaupels@helmholtz-muenchen.de
arie.geerlof@helmholtz-muenchen.de
hakan.sarioglu@helmholtz-muenchen.de
sattler@helmholtz-muenchen.de
durner@helmholtz-muenchen.de

*To whom correspondence should be addressed:
Christian Lindermayr
Tel. +49 89 3187 2285
Fax +49 89 3187 3383
Email: Lindermayr@helmholtz-muenchen.de

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Summary: Superoxide dismutases (SODs) are differential inhibited by peroxynitrite-mediated tyrosine nitration. Tyr63 is the main target responsible for inactivation of MnSOD1. This mechanism seems to be evolutionarily conserved in multicellular organisms.

Abstract

Despite the importance of superoxide dismutases (SODs) in the plant antioxidant defense system little is known about their regulation by post-translational modifications. Here, we investigated the *in vitro* effects of nitric oxide derivatives on the seven SOD isoforms of *Arabidopsis thaliana*. S-nitrosoglutathione, which causes S-nitrosylation of cysteine residues, did not influence SOD activities. By contrast, peroxynitrite inhibited the mitochondrial manganese SOD1 (MSD1), peroxisomal copper/zinc SOD3 (CSD3) and chloroplastic iron SOD3 (FSD3) but no other SODs. MSD1 was inhibited by up to 90 % but CSD3 and FSD3 only by a maximum of 30 %. Down-regulation of these SOD isoforms correlated with tyrosine (Tyr) nitration and both could be prevented by the peroxynitrite scavenger urate. Site-directed mutagenesis revealed that – amongst the 10 Tyr residues present in MSD1 – Tyr63 was the main target responsible for nitration and inactivation of the enzyme. Tyr63 is located nearby the active center at a distance of only 5.26 Å indicating that nitration could affect accessibility of the substrate binding pocket. Interestingly, the corresponding Tyr34 of human manganese SOD is also nitrated, suggesting that this might be an evolutionarily conserved mechanism for regulation of manganese SODs.

Introduction

In plant cells the reactive oxygen species (ROS) superoxide (O_2^-) arises as a potentially harmful by-product of photosynthetic and respiratory electron transport chains. It can also be enzymatically produced by various oxidases for serving as a signal or intermediate in general metabolism, development and stress responses (Mittler *et al.*, 2011). Independent of origin and function O_2^- levels are carefully controlled by the antioxidant system (Foyer and Noctor, 2009). O_2^- is either scavenged by antioxidants such as reduced ascorbate and glutathione or is efficiently converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD; $O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$). H_2O_2 in turn is subsequently degraded to water by catalase and peroxidases. Thus, by controlling O_2^- (and indirectly H_2O_2) levels SODs are important regulators of cellular redox homeostasis and signaling.

Plant SODs are commonly classified according to their active site cofactors into manganese SOD (MnSOD), iron SOD (FeSOD) and copper/zinc SOD (CuZnSOD). *Arabidopsis*

possesses of 7 SOD isoforms namely one MnSOD (MSD1), three FeSODs (FSD1-3) and three CuZnSODs (CSD1-3) (Kliebenstein *et al.*, 1998). While MSD1 has a mitochondrial targeting sequence, FSD2, FSD3 and CSD2 were localized in chloroplasts, CSD1 and FSD1 in the cytosol and CSD3 in peroxisomes (Huang *et al.*, 2012; Kliebenstein *et al.*, 1998; Myouga *et al.*, 2008). Gene expression of the SOD isoforms was differentially regulated in response to stress treatments known to promote the accumulation of ROS. For instance, ozone fumigation strongly induced CSD1 but repressed CSD3 and FSD1 expression (Kliebenstein *et al.*, 1998). These results suggest that the different SOD isoforms have specific functions under stress conditions. Moreover, SOD transcript levels did not always correlate with protein abundance and enzyme activity indicating that SODs are controlled on multiple levels including post-transcriptional and post-translational mechanisms (Kliebenstein *et al.*, 1998; Madamanchi *et al.*, 1994). In this context it is interesting that recent publications hint at a role of nitric oxide (NO) dependent protein modifications in the regulation of mammalian SODs (Radi 2013).

NO is an important messenger in many physiological processes (Gaupels *et al.*, 2011a; Leitner *et al.*, 2009; Mur *et al.*, 2013; Yun *et al.*, 2011). During stress responses NO often interacts with ROS and antioxidants thereby forming reactive nitrogen species (RNS) (Gross *et al.*, 2013; Hill *et al.*, 2010; Scheler *et al.*, 2013). Such NO derivatives can cause post-translational modifications of proteins by S-nitrosylation ($^{\cdot}$NO adduct) of cysteine (Cys) residues and metal groups or nitration (-NO adduct) of tyrosine (Tyr) and tryptophan residues (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011; Astier and Lindermayr, 2012; Gaupels *et al.*, 2011a; Hill *et al.*, 2010; Kovacs and Lindermayr, 2013). S-nitrosoglutathione (GSNO), nitrosonium ion (NO^+) and dinitrogentrioxide (N_2O_3) represent major RNS promoting S-nitrosylation while peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2) mediate protein nitration (Hill *et al.*, 2010). NO-dependent protein modifications have an effect on the activity of antioxidant enzymes. One prominent example is mammalian MnSOD, which can be Tyr nitrated (MacMillan-Crow *et al.*, 1996; Radi, 2013). *In vitro* and *in vivo* under inflammatory conditions MnSOD was site-specifically nitrated at Tyr34, which caused inhibition of SOD activity and consequently disturbance of mitochondrial redox homeostasis (Radi, 2013; Yamakura *et al.*, 1998). Less is known about regulation of plant SODs by NO. Occasionally, SODs of various plant species were listed amongst candidate S-nitrosylated and Tyr nitrated proteins (Lin *et al.*, 2012; Sehrawat *et al.*, 2013; Tanou *et al.*, 2009). However, NO-modifications were not confirmed *in vitro* nor was the effect of RNS on SOD activity investigated in any detail.

Here, we report the differential inhibition of *Arabidopsis* SODs by Tyr nitration. We observed that overall SOD activity was decreased in leaf extracts from GSNO/NO accumulating GSNO reductase-deficient mutants as compared to WT although the

expression of SOD-coding genes was nearly unchanged. From these results we concluded that SOD isoforms might be inhibited by NO-dependent post-translational modifications. This prompted us to undertake a systematic candidate approach for defining the role of RNS in regulation of all seven *Arabidopsis* SOD isoforms. *In vitro* tests demonstrated that SOD activities were not altered upon GSNO treatment but MSD1, FSD3 and CSD3 were inhibited to different degrees by $ONOO^-$. Inhibition of the enzymes correlated with increased Tyr nitration. Site-directed mutagenesis revealed that nitration of Tyr63 caused most of the almost complete inactivation of MSD1 by $ONOO^-$. In sum, nitration of MSD1 is a good model for post-translational regulation of plant enzymes as a whole and SOD isoforms in particular. Putative physiological effects of SOD inhibition by nitration under stress conditions are discussed.

Materials and Methods

Plant Material

Arabidopsis thaliana seeds (ecotype Col-0) were sown on soil:sand mixture (4:1). After vernalization for 2 days (4°C dark), plants were cultivated in a climate chamber at 60% relative humidity under long-day conditions (16 h light / 8 h dark cycle, 20°C day / 18°C night regime, 70 µmol m^{-2} s^{-1} photon flux density).

Cloning and heterologous expression of Arabidopsis SODs

For cloning the cDNAs of the different SOD isoforms the lambda phage-based site-specific recombination (Stratagene) was used (Landy, 1989). The isolation of the cDNAs of the different SODs was achieved by RT-PCR using gene-specific oligonucleotides (Supplemental Table S1). Briefly, total RNA extractions were performed from 100 mg leaf tissue using the TRIzol reagent according to the supplier's instructions (Invitrogen). QuantiTect Reverse Transcription Kit (Qiagen) was used to synthesize cDNA according to the protocol of the supplier. The introduction of the DNA recombination sequence (att) at the 5´- and 3´-end of the coding sequence of each isoform was achieved by PCR using the isoform-specific att-primers (Supplementary Table S1) and the amplified cDNAs as template. The resulting PCR products were introduced into pDONR221 by recombination using BP Clonase enzyme mixture according to the instructions of the manufacturer. After verifying the sequences of the different SODs they were transferred into the expression vectors pDEST17 and pDEST42 by recombination using LP Clonase enzyme mixture. pDEST17 and pDEST42 allows production of N-terminal or C-terminal His_6-tag fusion proteins, respectively. For optimal production different bacterial expression strains were

tested (BL21 DE3, Rosetta DE3 and Rosetta DE3 pLysS) and the most productive strain for each SOD was selected.

E. coli strains harbouring the different plasmids for production of recombinant SODs were grown in 50 ml Luria-Bertani medium at 37°C overnight. These cultures were used to inoculate 2 l auto-induction medium (Studier, 2005). The bacteria were grown overnight at 37°C until OD_{600nm} 2 was reached. Afterwards bacterial cells were harvested by centrifugation.

Extraction, purification and treatments of SODs with GSNO and peroxynitrite

For protein extraction the cells were resuspended in 160 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole, 10 mM $MgCl_2$, 1 mM protease-inhibitor AEBSF, 0,02% 1-thioglycerol, 0,2 µg/ml DNaseI, 1 mg/ml lysozyme) and disrupted by high pressure homogenization and sonification. Cellular debris was removed by centrifugation (25,000 g, 1 h, 4°C). The recombinant proteins were purified by affinity chromatography using 1,0 ml Ni-NTA agarose in Econo-Pac columns (Biorad, Munich, Germany). The protein extracts were applied onto the columns two-times and washed with 30 ml of washing buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0,02% L-glycerol). Adsorbed proteins were eluted from the matrix in three 5 ml fractions with 300 mM imidazole in washing buffer. Eluates were frozen in liquid nitrogen and stored at -20 °C until analysis.

The purified enzymes were re-buffered in potassium phosphate buffer (pH 8.0) using Zeba spin columns (Thermo Scientific, Rockford, USA). Afterwards, the enzymes were treated with 250 µM and 500 µM GSNO for 20 min (RT, in dark). Control treatment was done with 500 µM GSNO in presence of 5 mM DTT. Alternatively, purified SODs were treated for 20 min with different concentrations of $ONOO^-$ (RT, in dark). $ONOO^-$ was purchased from Calbiochem (Darmstadt, Germany) in 4.7% NaOH at 160 – 200 mM. The exact concentration was determined according to the manufacturer´s instructions. Control treatment was done with 500 µM $ONOO^-$ in presence of 100 µM urate. Excess GSNO, DTT, $ONOO^-$ and urate were removed with Zeba spin columns before determination of SOD activities.

SOD activity assay - The activity of the purified, recombinant SODs was determined using the nitroblue tetrazolium (NBT) – formazan method (McCord and Fridovich, 1969) or the cytochrome c-based assay (McCord, 2001).

Detection of SOD nitration by anti-nitrotyrosine western blot

Proteins were separated by SDS-PAGE on 12% polyacrylamide gels (Laemmli, 1970), were transferred onto PVDF membranes and blocked with 1% nonfat milk powder and 1% bovine serum albumin. The blots were incubated with goat anti-nitrotyrosine antibody (1:2000) at 4

°C overnight, followed by incubation with rabbit anti-goat IgG conjugated with horseradish peroxidase (1:3000) (Invitrogen, Darmstadt, Germany) for 1 h at RT. Cross-reacting protein bands were visualized via chemiluminescence using the West Pico Chemiluminescence Detection Kit (Thermo Scientific, Rockford, USA).

Site-directed mutagenesis

The modification of single nucleotide residues was performed as previously described (Lindermayr *et al.*, 2003). Briefly, for mutation, a pair of oligonucleotides was synthesized harbouring the desired alterations (Supplemental Table S1). For amplification, 60 ng plasmid DNA was used in a total volume of 10 µl, including 1 µM each primer, 200 µM dNTPs, and 1 U of iProof DNA polymerase. After denaturation (1 min at 98°C) 20 cycles were conducted, consisting of 25 s at 98°C, 55 s at 55°C, and 6 min at 72°C, followed by a final extension step at 72°C for 10 min. Subsequently, the parental and hemi-parental template DNA was digested with *Dpn*I and the amplified plasmids were transformed into *E. coli* DH5α. The mutation was verified by sequencing.

Modelling of the 3D structure of MSD1

Amino acid sequences were aligned and modelled using SWISS-Model (www.expasy.ch). The crystal structure of *Caenorhabditis elegans* MnSOD (PDBcode: PDB 3DC6) was used as template for the prediction of the putative conformation of *Arabidopsis* MSD1. Pymol software (DeLano Scientific, Portland, USA) was used for model visualisation.

Nano-HPLC-MS$^{2/3}$ and Data Analysis

For mass spectrometric analyses proteins were digested with trypsin at 37°C for 16h in 50 mM NH_4HCO_3, pH 8.0. The used trypsin/protein ratio was 1/20. All nano-HPLC-MS$^{2/3}$-experiments were performed on a Ultimate 3000 HPLC nanoflow system (Dionex) connected to a linear ion trap-Fourier transform mass spectrometer (LTQ-Orbitrap, Thermo Fisher Scientific, San Jose, CA, USA). For LTQ-Orbitarp mass spectrometry, the digested peptides were first separated by reversed-phase chromatography (PepMap, 15cm_75 mm id, 3 mm/100 Å pore size, LC Packings) operated on a nano-HPLC (Ultimate 3000, Dionex) with a nonlinear 170 min gradient using 2% ACN in 0.1% formic acid in water (A) and 0.1% formic acid in 98% ACN (B) as eluents with a flow rate of 250 nL/min. The nano-LC was connected to a linear quadrupole ion trap-Orbitrap (LTQ Orbitrap XL) mass spectrometer (Thermo-Fisher, Bremen, Germany) equipped with a nano-ESI source. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Survey full scan MS spectra (from m/z 300 to 1500) were acquired in the Orbitrap with resolution R560 000 at m/z 400 (after accumulation

to a target value of 1 000 000 charges in the LTQ). The method used allowed sequential isolation of the most intense ions, up to ten, depending on signal intensity, for fragmentation on the linear ion trap using collisionally induced dissociation at a target value of 100 000 ions. High-resolution MS scans in the orbitrap and MS/MS scans in the linear ion trap were performed in parallel. Target peptides already selected for MS MS/MS were dynamically excluded for 30 s. General conditions were as follows: electrospray voltage, 1.25–1.4 kV; no sheath and auxiliary gas flow. The following modifications were set to be variable: nitration of Tyr residues.

Results

Cloning, heterologous expression and purification of Arabidopsis SODs

SODs are important enzymes of the antioxidant system and several enzyme activities of this system are affected by NO. Mammalian MnSOD, for instance, is a target for Tyr nitratation (MacMillan-Crow *et al.*, 1996; Radi, 2013). Under inflammatory conditions human MnSOD is site-specifically nitrated at Tyr34, which results in inhibition of SOD activity and consequently disturbance of mitochondrial redox homeostasis (Radi, 2013; Yamakura *et al.*, 1998). Less is known about regulation of plant SODs by NO. Although, SODs of various plant species were identified as candidates for S-nitrosylation and Tyr nitration (Lin *et al.*, 2012; Sehrawat *et al.*, 2013; Tanou *et al.*, 2009), NO-dependent modifications were not confirmed until now. In *Arabidopsis* seven different SODs are described, including one MSD, three FSDs and three CSDs. The deduced amino acid sequences of the different isoenzymes show very different homology among each other (44-46% within the FSDs, 45-57% within the CSDs) (Table 1). Moreover, the identity of the amino acid sequences between MSD1 and FSDs is higher (29-31%) than the identity between MSD1 and CSDs (18-21%), concluding that MSD1 is closer related to FSDs (Table 1). The corresponding amino acid sequence alignments are provided in the Supplement (Fig. S1 – S4).

We heterologously produced and purified all seven *Arabidopsis* SOD proteins for *in vitro* analyses of their regulation by S-nitrosylation of cysteine residues or nitration of Tyr residues. First, we isolate the coding sequence of all seven *Arabidopsis* SOD proteins. The isolation of the cDNAs of the different SODs was achieved by RT-PCR using gene-specific oligonucleotides and the amplified coding sequences were expressed in *Escherichia coli* as fusion proteins containing either N-terminal or C-terminal His_6-tags. For optimal production different bacterial expression strains were tested (BL21 DE3, Rosetta DE3 and Rosetta DE3 pLysS) and the most productive strain for each SOD was selected. After affinity

chromatography on Ni-NTA-agarose, the seven proteins showed the expected relative molecular masses in SDS-polyacrylamide gels and on the immunoblot (Fig. 1).

The activity of the purified, recombinant SODs was determined using the nitroblue tetrazolium (NBT) – formazan method (Fig. 2). In this assay, O_2^- ions are generated from the conversion of xanthine and O_2 to uric acid and H_2O_2 by xanthine oxidase. The O_2^- anion then converts a NBT into a formazan dye. Addition of SOD to this reaction reduces O_2^- ion levels, thereby lowering the rate of formazan dye formation. SOD activity is monitored at a wavelength of 570 nm and determined as the percent inhibition of the rate of formazan dye formation. The different types of SODs were verified using specific inhibitors (H_2O_2 for FSDs and NaCN for CSDs). MSD1 is insensitive to both inhibitors (Fig. 2).

MSD1, FSD3 and CSD3 are inhibited by $ONOO^-$.

Interestingly, the total SOD activity in *atgsnor* plants is lower than in WT plants (Fig. S5), which is probably related to the higher levels of NO-derivatives in the mutant (Feechan *et al.*, 2005). Since the decreased SOD activity in *atgsnor* cannot be explained by transcriptional regulation (Fig. S6) we hypothesized that it is regulated on protein level. The two most important NO-dependent post-translational modifications are S-nitrosylation of Cys residues and nitration of Tyr residues. Assuming that SOD activity might be inhibited by S-nitrosylation of critical Cys residues, MSD1, FSD3 and all three CSDs, were treated with the S-nitrosylating agent GSNO, since these isoform have at least one cysteine residue. However, none of these SODs was inhibited by GSNO (Fig. 3). Next, we tested the effect of $ONOO^-$ on SOD activity. To this end, all SODs, which have at least one Tyr residue (MSD1, all three FSDs and CSD3) were treated with different concentrations of $ONOO^-$. A concentration-dependent inhibition of MSD1, FSD3 and CSD3 could be observed, whereas the activity of the other two tested FSD isoforms was not affected by this treatment (Fig. 4). Especially MSD1 seems to be very sensitive to this treatment. Its activity decreased to about 10% with 500 µM $ONOO^-$, while the activity of FSD3 and CSD3 was reduced to 65%. However, it has to be mentioned that the observed differences in the efficiency of $ONOO^-$-dependent inhibition of the different SODs could be caused by different ratio of applied protein and $ONOO^-$. For a better comparison we calculated the ratio of applied protein per nmol $ONOO^-$ for the highest $ONOO^-$ concentration used (500 µM) (Fig. 4).

Inhibition of enzyme activity by $ONOO^-$ correlated with increased protein nitration as detected by immunoblot analyses using an anti-nitrotyrosine antibody (Fig. 5). Notably, western blot signals were stronger for MSD1 than FSD3 and CSD3. Because of the high sensitivity of MSD1 to $ONOO^-$ this isoform has been analysed in more detail.

Mass spectrometric identification of nitrated Tyr residues in MSD1.

To identify the modified Tyr residues in MSD1, peroxynitrite-treated MSD1 was analysed by mass spectrometry. In total, MSD1 has ten Tyr residues. Modelling of the three-dimensional structure of MSD11 revealed that especially Tyr63, Tyr198 and Tyr209 were located close to active site manganese ion in a distance lower than 10 Å (5.3 Å, 9.1 Å, 9.3 Å, respectively) (Fig. 6). MSD1 was treated with 500 µM peroxynitrite and digested with trypsin. This protease generated analyzable peptides containing the different Tyr residues mentioned above. For each nitrated Tyr residue an increase in mass by 45 Da was expected. All identified nitrated Tyr residues are summarized in Table 2. Tyr residues 209, 221 and 226 are not accessible to nitration, since they were only found in their unmodified form. Especially nitration of Tyr63, which is closest to the active site manganese, could be of special importance for the inhibitory effect of peroxynitrite on MSD1, since it corresponds to Tyr34 in human MnSOD.

Nitration of Tyr63 is responsible for inhibition of MSD1 activity.

To test if nitration of Tyr63 inhibits MSD1 activity this residue was changed by site-directed mutagenesis to phenylalanine. This amino acid is structurally related to Tyr but cannot be nitrated. Wild type and mutated MSD1 (MSD1/Y63F) were treated with different concentrations of $ONOO^-$ and their activities were determined. Both wild type and modified MSD1 showed similar specific activity upon addition of decomposed $ONOO^-$ (control). However, treatment with 100 and 250 µM $ONOO^-$ resulted in no and 500 µM $ONOO^-$ in only 30 % inhibition of MSD1/Y63F, whereas wild type MSD1 was inhibited by about 30, 50 and 90 %, respectively (Fig. 7A and B). Immunoblot analyses with anti-nitrotyrosine antibodies demonstrated that overall Tyr nitration of MSD1/Y63F was much lower than that of wild type MSD1 (Fig. 7C).

Discussion

ROS are produced in unstressed and stressed cells as a by-product of aerobic metabolism. Plants have a well-developed antioxidant defence, involving both limiting the formation of ROS as well as instituting their removal. SODs are enzymes that catalyze the dismutation of O_2^- into oxygen and H_2O_2. In *Arabidopsis* seven different SODs are described, which differ in their metal-cofactor and subcellular location. Here we present MSD1, FSD3 and CSD3 as new candidates for NO-dependent post-translational regulation. GSNO, which can S-nitrosylate Cys residues, did not affect activity of MSD1, FSD3 and CSD3. However, incubation with the Tyr nitrating agent $ONOO^-$ significantly reduced the activity of all three enzymes with MSD1 being the most sensitive isoform. Because of the different purification efficiency of the different SOD isoforms we had to use different amounts of total protein.

This might probably affect the inhibition efficiency of $ONOO^-$. Therefore, we calculated the ratio of applied protein per nmol $ONOO^-$ for the highest $ONOO^-$ concentration used (500 µM). The highest protein amount was used in the FSD2 and MSD1 inhibition assays. Since 500 µM $ONOO^-$ resulted in nearly total loss of MSD1 activity this enzyme seems to be the most $ONOO^-$-sensitive SOD-isoform. FSD2 activity is only slightly affected by $ONOO^-$ (10% with 500 µM $ONOO^-$), but a stronger inhibition cannot be excluded, if lower protein amounts are used.

Similar to the plant MSD1, human and bacterial MnSODs are also very sensitive to $ONOO^-$ (MacMillan-Crow *et al.*, 1998; Surmeli *et al.*, 2010). An inhibition of 30% with 100 µM $ONOO^-$ might occur under physiological conditions assuming that $ONOO^-$ levels in plants are similar to that in the animal system. Here the rate of $ONOO^-$ production can reach 50 – 100 µM per min in certain cellular compartments including mitochondria (Szabo *et al.*, 2007). However, since NO production in plants is lower than in the animal system, $ONOO^-$ levels might be also lower. The concentration-dependent inhibition of MSD1 positively correlated with the level of Tyr nitration (Fig. 4 and Fig. 5). Inhibition of activity as well as protein nitration was prevented by the $ONOO^-$ scavenger urate.

Primarily nitration of Tyr63 was responsible for the $ONOO^-$ sensitivity of MSD1, as inferred by the finding that the $ONOO^-$-dependent inhibition was strongly reduced in a MSD1 mutant with Tyr63 replaced by phenylalanine, which cannot be nitrated. Tyr63 is located very close to the active center of the enzyme (5.26 Å distance) in an amino acid sequence, which is also conserved in human MnSOD (Fig. 8A). Accordingly, the corresponding Tyr34 of human MnSOD is nitrated by $ONOO^-$ resulting in down-regulation of the enzymatic activity (MacMillan-Crow *et al.*, 1998; Yamakura *et al.*, 1998). It was proposed that a $-NO_2$ group at ortho-position of the aromatic ring further reduces the distance to the manganese-ion in the active center (Fig. 8B), thereby affecting access and ligation of O_2^- to the substrate binding pocket. Moreover, crystal structure analyses of human MnSOD revealed a network of hydrogen bonds in the direct environment of the active center (Perry *et al.*, 2010). Tyr34 is part of this network which probably promotes the proton transfer onto a bond O_2^- anion. Nitration of the Tyr residue followed by a decrease of its pKa-value would probably deprotonate the phenol ring system causing a decrease or disruption of the hydrogen bond network. Other possible consequences of Tyr34 nitration include electrostatic interference between the nitro group and the negatively charged substrate O_2^- and a shift in the redox potential of the enzyme (Edwards *et al.*, 2001). The observed inactivation of *Arabidopsis* MSD1 by $ONOO^-$-mediated nitration of Tyr63 is probably based on similar mechanism like described above for Tyr34 nitration of human MnSOD. However, it has to be mentioned that the activity of the MSD1 mutant (MSD1/Y63F) is still slightly inhibited by $ONOO^-$ (Fig.

7B), concluding that probably also nitration of other tyrosine residues affect MSD1 activity, even though to a much smaller extent than nitration of Tyr63.

Previously, MnSODs of rice and potato were identified as targets for phosphorylation and oxidation, but an effect on the enzyme activity was not analyzed (Bykova *et al.*, 2003; Kristensen *et al.*, 2004). It will be interesting to investigate, if Tyr nitration interferes with phosphorylation or oxidation events.

In comparison to MnSODs much less is known about the regulation of CSDs and FSDs by $ONOO^-$. *Arabidopsis* FSD3 shares 45 % identity and 54 % similarity in the amino acid sequence with MSD1 (Table 1). The structure is also similar between both SODs (Fig. 9). Moreover, Tyr82 of FSD3 is in the same conserved amino acid sequence like Tyr63 of MSD1 and Tyr34 of human MnSOD (Fig. 8A), all of which are located in a distance of only 5.25 – 5.40 Å from their active center ion (Fig. 9). According to these sequence comparisons Tyr82 would be a good candidate regulatory site for inhibition of FSD3 by nitration. However, FSD1 and FSD2 possess the same conserved Tyr residue (Fig. 9) without being $ONOO^-$ sensitive. Small variations in sequence and/or protein conformation might explain the differences in $ONOO^-$ sensitivity amongst FSD isoforms as well as between FSD3 and MSD1. Alternatively, Tyr nitration of FSD3 correlates with but is not causal for enzyme inhibition. CSDs are different from MSD1 and FSDs both in sequence as well as structure (Table 1 and Fig. 9). Amongst the three CSD isoforms of *Arabidopsis* only CSD3 has a Tyr residue. Our data demonstrate that Tyr115 is nitrated by $ONOO^-$ concomitant with a reduced enzyme activity. Notably, human recombinant CuZnSOD was shown to be inhibited by tryptophan rather than Tyr nitration (Yamakura *et al.*, 2001). The exact mechanism of differential inhibition of FSD3 and CSD3 but no other FSDs and CSDs remains to be deciphered in future studies using site-directed mutagenesis and structural analyses.

Our data imply that MSD1, CSD3 and FSD3 would be partially inhibited by Tyr nitration under stress conditions, which promote the formation of $ONOO^-$. Studies with *Arabidopsis* lines altered in the expression of SOD isoforms provide some hints on possible consequences of SOD inhibition. A detailed functional investigation of *Arabidopsis* FSDs revealed that chloroplastic FSD2 and FSD3 collaborate in ROS scavenging and chloroplast development (Myouga *et al.*, 2008). *fsd2-1 fsd3-1* double mutants showed an albino phenotype and were hyper-sensitive to oxidative stress induced by methyl viologen (Myouga *et al.*, 2008). By comparison, antisense lines of MSD1 displayed a disturbed redox homeostasis primarily in the mitochondria but to some extent also in the cytosol (Morgan *et al.*, 2008). Importantly, the mitochondrial tricarboxylic acid cycle (TCA) was interrupted through inhibition of aconitase and isocitrate dehydrogenase activity. The transgenic lines were able to adapt and did not show a decrease in down-stream respiratory CO_2 output (Morgan *et al.*, 2008). However, during short-term responses to stress down-regulation of MSD1 might have

transient but severe effects on mitochondrial TCA cycle, energy metabolism and redox homeostasis. For human kidney cells it was demonstrated that MnSOD inhibition by Tyr nitration induced irreversible oxidative injury of mitochondria during chronic rejection of human renal allografts (MacMillan-Crow *et al.*, 1996; MacMillan-Crow *et al.*, 1998).

In addition to their role in the antioxidant system SODs have relatively under-investigated functions in regulating the RNS composition and signalling. Interactions of free radicals such as O_2^- and NO are important under stress conditions (Gross *et al.*, 2013). Excessive levels of O_2^- during oxidative stress cause a limitation in NO bioavailability through formation of $ONOO^-$. SOD in turn competes with NO for O_2^- thereby preventing the formation of $ONOO^-$ while favoring the accumulation of NO. Peroxiredoxin II E (PrxIIE) is another emerging player in RNS homeostasis. This hydro-peroxidase reduces peroxides to H_2O and the corresponding alcohol using reducing equivalents from glutaredoxin or thioredoxin (Dietz, 2003). Recently it was found that PrxIIE degrades $ONOO^-$ under normal growth conditions. However, after infection by an avirulent strain of *Pseudomonas syringae* PrxIIE was inhibited by S-nitrosylation of Cys121 resulting in $ONOO^-$ accumulation and increased Tyr nitration during the hypersensitive defense response (Gaupels *et al.*, 2011b; Romero-Puertas *et al.*, 2007). Combining the above pieces of information would suggest that elevated levels of NO in stressed WT *Arabidopsis* cause an inhibition of PrxIIE, accumulation of $ONOO^-$ and subsequently nitration-mediated inhibition of MSD1, CSD3 and FSD3. Down-regulation of the SODs would then lead to accumulation of O_2^-, which would further react with NO giving rise to even more $ONOO^-$ in the course of a self-amplification loop. On the other side elevated levels of NO might also result in S-nitrosylation of NADPH oxidase (Yun *et al.*, 2011), inhibiting its activity and blunting the production of O_2^-. In this way the self-amplification loop would be slowed down. It is noteworthy, that MSD1, FSD3 and CSD3 are localized in mitochondria, chloroplasts and peroxisomes, respectively, which represent major sites of ROS and NO synthesis during stress responses (Gross *et al.*, 2013). In sum, the results of our *in vitro* study provide a biochemical framework for future research aimed at deciphering how the differential regulation of SODs is involved in stress signaling, defense or cytotoxicity.

Supplementary Data

Supplementary data are available at JXB online.

Figure S1: Alignment of amino acid sequences of *Arabidopsis* FSD isoforms.

Figure S2: Alignment of amino acid sequences of *Arabidopsis* CSD isoforms.

Figure S3: Alignment of amino acid sequences of *Arabidopsis* FSD isoforms and MSD1.

Figure S4: Alignment of amino acid sequences of *Arabidopsis* CSD isoforms and MSD1.

Figure S5: Total SOD activity in *Arabidopsis* WT and GSNOR knock-out plants.

Figure S6: Expression analysis of *Arabidopsis* SODs.

Table S1: Oligonucleotides for cloning of superoxide dismutase nucleotide sequences and site-directed mutagenesis.

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Table 1: Amino acid sequence identity and similarity between the different *Arabidopsis* SOD isoforms.

	AA sequence identity (%)	AA sequence similarity (%)
FSD1 – FSD2	46	57
FSD1 – FSD3	44	58
FSD2 – FSD3	45	59
CSD1 – CSD2	47	53
CSD1 – CSD3	57	67
CSD2 – CSD3	45	54
MSD1 – FSD1	47	53
MSD1 – FSD2	57	67
MSD1 – FSD3	45	54
MSD1 – CSD1	19	28
MSD1 – CSD2	21	30
MSD1 – CSD3	18	31

Table 2. Determination of Tyr nitration of MSD1 by mass spectrometry.

Purified, reduced, recombinant MSD1 was incubated with 500 µM peroxynitrite and digested with trypsin. Peptides containing at least one Tyr residue were analyzed by mass spectrometry to determine Tyr nitration. Expected (single charged) and observed (multiple charged) m/z values for the different peptides are shown.

Identified peptide	Mascot Score	m/z (expected)	m/z (observed)	charge	modification
KHHQAYVTNY^{67}NNALEQLDQAVNKG	76	1.307	1.308	2	Nitro (+45)
KHHQAY^{63}VTNYNNALEQLDQAVNKGDASTVVKL	70	0.843	0.844	4	Nitro (+45)
KGGSLVPLVGIDVWEHAY^{198}YLQYKN	46	1.276	1.277	2	Nitro (+45)
KGGSLVPLVGIDVWEHAYY^{199}LQYKN	45	1.276	1.277	2	Nitro (+45)
KGGSLVPLVGIDVWEHAYYLQY^{202}KN	42	1.276	1.277	2	Nitro (+45)
RGIQTFTLPDLPYDY^{40}GALEPAISGEIMQIHHQKH	39	1.209	1.210	3	Nitro (+45)
RGIQTFTLPDLPY^{38}DYGALEPAISGEIMQIHHQKH	36	0.907	0.908	4	Nitro (+45)

FIGURE LEGENDES

Figure 1: Production, purification and detection of recombinant *Arabidopsis* SODs.

A) The coding sequences of the different *Arabidopsis* SODs were cloned into pDEST17 (N-terminal His_6) or pDEST42 (C-terminal His_6) according to the Gateway Technology. Three different bacteria production strains (RS-strain = Rosetta DE3 pLysS; R-strain = Rosetta DE3; BL-strain = BL21 DE3) were tested and the most productive one for each isoform was used. His-taged SODs were purified by Ni-NTA affinity chromatography. Crude bacteria lysate (L), flow-through (F) and eluate (E) were separated by SDS-PAGE and visualized by Coomassie Blue staining. Arrows indicate the produced SOD isoforms. The relative mass of protein standards are shown on the left. B) Detection of purified, recombinant *Arabidopsis* SOD isoforms. Eluates containing recombinant SOD isoforms were separated by SDS-PAGE and blotted onto nitrocellulose membrane. Detection of His-tagged proteins was achieved using anit-His antibody. The relative mass of protein standards are given on the left.

Figure 2: Enzyme activities of purified, recombinant SODs.

Shown is the inhibition of formazan formation by MSD1 (A), FSDs (B) and CSDs (C). Farmazan-formation with heat-inactivated protein extracts was set to 100%. To distinguish between the different SOD types specific inhibitors (H_2O_2 for FSDs and NaCN for CSDs) were used. MSD1 is insensitive to both inhibitors.

Figure 3: Effect of GSNO on enzyme activity of cysteine containing SODs.

Recombinante MnSOD, FeSOD3, Cu/ZnSOD1, Cu/ZnSOD2 and Cu/ZnSOD3 were treated with 250 µM (light grey) and 500 µM (white) GSNO for 20 min (RT, in dark). Control treatment was done with 500 µM GSNO in presence of 5 mM DTT (dark grey). Afterwards the activity was determined. Treatment with light-inactivated GSNO was used as control. These activities were set to 100%. Values represent means ±SD of three independent experiments.

Figure 4: Effect of peroxynitrite on enzyme activity of Tyr containing SODs.

Recombinant MSD1 (A, 22 µg), FSD3 (B, 15 µg), Cu/ZnSOD3 (C, 3 µg), FSD1 (D, 13 µg) and FSD2 (E, 28 µg) were treated with peroxynitrite for 20 min (RT, in dark). Afterwards the activity was determined by monitoring reduction of cytochrome *c*. The given values indicate the ratio of applied protein per nmol $ONOO^-$ calculated for the highest $ONOO^-$ used (500 µM). Filled squares: peroxynitrite treatment; open squares: peroxynitrite treatment in presence of 100 µM ureate; crosses: treatment with light-inactivated peroxynitrite. The activities of urate-treated samples were set to 100%. Values represent means ±SD of three independent experiments. Asterisks (**) indicate significant differences between control and peroxynitrite-treated samples (t-test, $p \leq 0.01$).

Figure 5: Detection of nitrated Tyr residues.

Purified, recombinant MSD1, FSD3 and Cu/ZnSOD3 were treated with different concentrations of peroxynitrite, separated by SDS-PAGE and blotted onto nitrocellulose membrane. Detection of nitrated Tyr residues was achieved using anit-NO_2-Tyr antibody. Treatment with 500 µM peroxynitrite in presence of 100 µM urate was used as control.

Figure 6: Structural model of *Arabidopsis* MSD1.

The structural model of *Arabidopsis* MSD1 was generated using SWISS-MODEL with the crystal structure of *Caenorhabditis elegans* MnSOD as template (PDBcode: PDB 3DC6). The Tyr residues are marked in green. The distances between Tyr side chains and the active side manganese ion (yellow) is given in Ångström in brackets.

Figure 7: Effect of peroxynitrite on enzyme activity of MSD1/WT and MSD1/Y63F.

Recombinant MSD1/WT (A) and MSD1/Y63F (B) were treated with different concentrations of peroxynitrite in presence (grey bars) and absence (black bars) of 100 µM urate for 20 min (RT, in dark). Afterwards the activity was determined. Activities without peroxynitrite were set to 100%. Values represent means ±SD of three independent experiments. Asterisks (**) indicate significant differences treatment with and without urate (t-test, $p \leq 0.01$). Tyr nitration was detected by immunoblot analysis (C). Purified, recombinant MSD1 and MSD1/Y63 protein were separated by SDS-PAGE and blotted onto nitrocellulose membrane. Detection of nitrated Tyr residues was achieved using anit-NO_2-Tyr antibody. The relative mass of protein standards are given on the left.

Figure 8: Structural illustration of nitration of conserved Tyr63 of MSD1. (A) Alignment of amino acid sequences of *Arabidopsis* FSD isoforms, MSD1 and human MnSOD (Genbank accession number: CAA32502). Dashes: Introduced gaps to maximize sequence similarity. Tyr63 of MSD1 and the corresponding Tyr in FSD1 (Tyr43), FSD2 (Tyr85), FSD3 (Tyr82) and human MnSOD (Tyr34) are highlighted in red. (B) Part of the structural model of AtMSD1 showing the substrate binding pocket. The structural model of *Arabidopsis* MSD1 was generated using SWISS-MODEL with the crystal structure of *Caenorhabditis elegans* MnSOD as template (PDB code: 3DC6). (left) The substrate binding pocket is modelled with unmodified Tyr63. The position where peroxynitrite attacks the aromatic ring system of Tyr63 is indicated with a red arrow. (right) The modelled substrate binding site is shown with nitrated Tyr63. Histidine and aspartate side chains are shown in yellow; the side chain of Tyr63 in marked in green. The distances of each side chain to the manganese-ion within the active site is given.

Figure 9: Structural model of MSD1, CSD3, FSD1, FSD2, FSD3 and human MnSOD.

The structural model of Arabidopsis SODs was generated using SWISS-MODEL with the crystal structure of *Caenorhabditis elegans* MnSOD as template (PDBcode: PDB 3DC6). The active side ion is shown in

grey. All Tyr residues are highlighted in yellow. Tyr63 of MSD1 and the corresponding tyrosine residues in FSD1 (Tyr43), FSD2 (Tyr85), FSD3 (Tyr82) and human MnSOD (Tyr34) are marked with a red arrow. The distance to the active site ion is given in brackets. Tyr115 of CSD3 is indicated in yellow.