Fe Sparing and Fe Recycling Contribute to Increased Superoxide Dismutase Capacity in Iron-Starved Chlamydomonas reinhardtii


INTRODUCTION

Fe deficiency is one of several abiotic stresses that impacts plant metabolism because of the loss of function of Fe-containing enzymes in chloroplasts and mitochondria, including cytochromes, FeS proteins, and Fe superoxide dismutase (FeSOD). Two pathways increase the capacity of the Chlamydomonas reinhardtii chloroplast to detoxify superoxide during Fe limitation stress. In one pathway, MSD3 is upregulated at the transcriptional level up to 10²-fold in response to Fe limitation, leading to synthesis of a previously undiscovered plastid-specific MnSOD whose identity we validated immunochemically. In a second pathway, the plastid FeSOD is preferentially retained over other abundant Fe proteins, heme-containing cytochrome f, diiron magnesium protoporphyrin monomethyl ester cyclase, and Fe₂S₇-containing ferredoxin, demonstrating prioritized allocation of Fe within the chloroplast. Maintenance of FeSOD occurs, after an initial phase of degradation, by de novo resynthesis in the absence of extracellular Fe, suggesting the operation of salvage mechanisms for intracellular recycling and reallocation.

Fe is an important micronutrient for photosynthetic organisms due to its extensive use as a cofactor in the photosynthetic apparatus (Raven et al., 1999), and it is commonly a limiting nutrient for photosynthetic organisms in both marine and terrestrial environments (Marschner, 1995; Morel and Price, 2003). As a result, photosynthetic organisms in particular have evolved sophisticated mechanisms for Fe assimilation and homeostasis, and these are well documented through studies in several reference organisms, such as Arabidopsis thaliana and Chlamydomonas reinhardtii in the laboratory or diatoms in the field (reviewed in Guerinot and Yi, 1994; Marschner and Römheld, 1994; Walker and Connolly, 2008, Jeong and Guerinot, 2009; Armbrust, 2009, Marchetti and Cassar, 2009; Blaby-Haas and Merchant, 2012). The mechanisms underlying metabolic acclimation in Fe-deficient cells are, by comparison, less investigated.

1 Current address: Life Technologies, 5791 Van Allen Way, Carlsbad, CA 92008-7321.
2 Current address: Department of Chemistry and Biochemistry, Eastern Oregon University, One University Blvd., La Grande, OR 97850.
3 Address correspondence to sabeeha@chem.ucla.edu.
4 The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Sabeeha S. Merchant (sabeeha@chem.ucla.edu).
5 Online version contains Web-only data.

References

Merchant and Helmann, 2012. Examples include replacement of metal cofactors in enzymes, Cd- or Co-containing carbonic anhydrases in place of the usual Zn enzymes, or use of different chemical species, sulfolipids in place of phospholipids in P-deficient bacteria and plants (Benning et al., 1995; Güler et al., 1996; Lane and Morel, 2000a, 2000b; Xu et al., 2007; Yu et al., 2002). Mechanisms of Cu sparing in the chloroplast, involving substitution of plastocyanin with cytochrome c₅₅ and Cu/Zn superoxide dismutase (Cu/ZnSOD) with Fe superoxide dismutase (FeSOD), have been well documented in Cu-deficient algae and plants, respectively (Merchant et al., 2006; Burkhead et al., 2009; Pilon et al., 2011), but the sparing phenomenon has not been as well studied for chloroplast Fe.

This can be accomplished by replacing one element with another (usually less effective or more “expensive”) element (Merchant and Helmann, 2012). Examples include replacement of metal cofactors in enzymes, Cd- or Co-containing carbonic anhydrases in place of the usual Zn enzymes, or use of different chemical species, sulfolipids in place of phospholipids in P-deficient bacteria and plants (Benning et al., 1995; Güler et al., 1996; Lane and Morel, 2000a, 2000b; Xu et al., 2007; Yu et al., 2002). Mechanisms of Cu sparing in the chloroplast, involving substitution of plastocyanin with cytochrome c₅₅ and Cu/Zn superoxide dismutase (Cu/ZnSOD) with Fe superoxide dismutase (FeSOD), have been well documented in Cu-deficient algae and plants, respectively (Merchant et al., 2006; Burkhead et al., 2009; Pilon et al., 2011), but the sparing phenomenon has not been as well studied for chloroplast Fe.

Fe is an important micronutrient for photosynthetic organisms due to its extensive use as a cofactor in the photosynthetic apparatus (Raven et al., 1999), and it is commonly a limiting nutrient for photosynthetic organisms in both marine and terrestrial environments (Marschner, 1995; Morel and Price, 2003). As a result, photosynthetic organisms in particular have evolved sophisticated mechanisms for Fe assimilation and homeostasis, and these are well documented through studies in several reference organisms, such as Arabidopsis thaliana and Chlamydomonas reinhardtii in the laboratory or diatoms in the field (reviewed in Guerinot and Yi, 1994; Marschner and Römheld, 1994; Walker and Connolly, 2008, Jeong and Guerinot, 2009; Armbrust, 2009, Marchetti and Cassar, 2009; Blaby-Haas and Merchant, 2012). The mechanisms underlying metabolic acclimation in Fe-deficient cells are, by comparison, less investigated.

The Plant Cell Preview, www.aspb.org © 2012 American Society of Plant Biologists. All rights reserved.
One of the advantages of the green alga *C. reinhardtii* as a reference organism is the ease of manipulation of its Fe status (Merchant et al., 2006). We previously defined four stages of Fe nutrition in photoheterotrophic *C. reinhardtii*. Fe replete (18 μM), Fe deficient (1 to 3 μM, depending on the strain), Fe limited (0.5 μM or less), and Fe excess (200 μM or more), based on growth rate, the appearance of chlorosis, the expression of genes encoding Fe assimilation components, and acquired sensitivity to high light (La Fontaine et al., 2002, Moseley et al., 2002a; Long and Merchant, 2008). An immediate response to Fe deficiency is upregulation of Fe assimilatory components (Merchant et al., 2006). *C. reinhardtii* appears to have two pathways for Fe acquisition. One consists of a plasma membrane ferrooxidase, FOX1, coupled to a trivalent cation transporter, FTR1 (La Fontaine et al., 2002; Herbik et al., 2002; Kosman, 2003), and another consists of two ZIP family transporters, IRT1 and IRT2, which function in an alternate assimilation route (Allen et al., 2007b; Chen et al., 2008). Other components that facilitate Fe uptake are a ferrireductase, FRE1, and periplasmic proteins FEA1/2 (Allen et al., 2007b). The upregulation of the assimilation pathway is an indication that cells are experiencing Fe deficiency, and FOX1 has served as a useful marker of intracellular Fe status.

In Fe-poor photoheterotrophic growth conditions, *C. reinhardtii* initiates a program to degrade various Fe-containing chloroplast proteins, including photosystem I (PSI), the cytochrome b₆f complex, and ferredoxin. In addition, the PSI-associated light-harvesting complex is remodeled to ameliorate photooxidative stress resulting from compromised function of its Fe₄S₄ clusters (Moseley et al., 2002a; Naumann et al., 2005). The signal for the remodeling program may be the state of occupancy of individual chlorophyll proteins (like PsaK), which depend on the activity of the di-Fe-containing magnesium protoporphyrin monomethyl ester cyclase in the chlorophyll biosynthetic pathway whose activity is reduced in Fe deficiency (Spiller et al., 1982; Tönnies et al., 2003). By contrast, mitochondrial respiratory complex proteins (also Fe-containing) are maintained, suggesting a hierarchy of Fe distribution to respiration in the mitochondria versus photosynthesis in the chloroplast in photoheterotrophically growing cells (Naumann et al., 2007; Terauchi et al., 2010). Plastid ferritin may buffer the Fe during the transition (Busch et al., 2008).

Fe-containing SOD, which is located in the chloroplast (Sakurai et al., 1993; Chen et al., 1996; Kitayama et al., 1999) is another potential target of Fe deficiency in *C. reinhardtii*. The SODs are a polyphyletic family of metalloproteins, the most common of which contain Fe (FeSODs), Mn (MnSODs), Cu plus Zn (Cu/ZnSODs), or Ni (NiSODs) as active site catalysts. The FeSODs and MnSODs exhibit very similar sequence and structure, reflecting a common ancestry, while the Cu/ZnSODs and NiSODs are distinct from the FeSODs and MnSODs and from each other (Fink and Scandalios, 2002; Priya et al., 2007). The FeSODs, MnSODs, and Cu/ZnSODs are widely distributed, and organisms typically possess enzymes of more than one class; many cyanobacteria have both FeSODs and MnSODs, metazoa and fungi produce both MnSODs and Cu/ZnSODs, and embryophytes synthesize all three forms (Fink and Scandalios, 2002; reviewed in Grace, 1990; Bowler et al., 1992; Pilon et al., 2011). Charophyte green algae also have all three forms, but chlorophyte green algae, including *C. reinhardtii*, lack Cu/ZnSODs, perhaps as a means of reducing their Cu quota (Asada et al., 1977; de Jesus et al., 1989; Egashira et al., 1989; Merchant and Helmann, 2012).

SODs represent the first line of antioxidant defense (reviewed in Imlay, 2008), and their importance can be seen in the severe oxygen-dependent phenotypic defects exhibited by mutants of *Escherichia coli* and *Saccharomyces cerevisiae* that lack one or more of these enzymes (Bilir et al., 1985; Carliz and Touati, 1986; Farr et al., 1986; van Loon et al., 1986). Plant mutants lacking chloroplast FeSODs also show strong phenotypes: They are pale and grow poorly in the light because of damage to the plastome, emphasizing their importance in photoprotection (Myouga et al., 2008).

The degradation of Fe-containing proteins during the onset of Fe deficiency may release free Fe, which is known to contribute to oxidative stress by catalyzing the conversion of superoxide and hydrogen peroxide (H₂O₂) to the highly reactive hydroxyl radical via Fenton chemistry (reviewed in Halliwell and Gutteridge, 1984; Liochev and Fridovich, 1999). Indeed, symptoms of oxidative stress are evident in Fe-deficient cyanobacteria (Latifi et al., 2005). Therefore, any reduction in FeSOD activity during Fe deficiency in *C. reinhardtii* could be particularly deleterious, especially under illumination. Here, we show that two mechanisms serve to not only maintain but increase SOD activity in the chloroplast during Fe deficiency: First, a hierarchy of Fe allocation acting at the level of de novo synthesis favors FeSOD over other plastid proteins; second, a previously undiscovered chloroplast MnSOD is induced at the transcriptional level to augment the FeSOD activity.

RESULTS

Hierarchy of Fe Allocation to Various Chloroplast Proteins

To distinguish the impact of Fe deficiency on FeSOD versus other chloroplast Fe proteins, we monitored the abundance of various Fe-containing chloroplast proteins by immunoblotting of extracts from cells nourished continuously in batch culture with different amounts of Fe. In keeping with the sacrifice of PSI and photosynthesis in photoheterotrophic *C. reinhardtii*, cytochrome f abundance was drastically reduced in Fe-limited cells (Figure 1). Similarly, the abundance of the magnesium protoporphyrin monomethyl ester cyclase (cyclase) was also markedly reduced. The cyclase has a diiron center and may be a key target of Fe deficiency (Spiller et al., 1982). The abundance of the major classic leaf-type ferredoxin (corresponding to the petF gene product) was also very sensitive to Fe nutrition; even mild Fe deficiency resulted in a substantial decrease in its abundance (cf. 1 μM to 3 μM Fe), but, perhaps because of its function in multiple essential metabolic pathways in the chloroplast, a low amount was maintained even in severely Fe-limited cells. The abundances of two other ferredoxins, Fdx3 and Fdx6, are also progressively reduced in Fe-deficient cells (Terauchi et al., 2009). By contrast, the abundance of FeSOD in Fe-limited cells...
Wild-type (CC125) *C. reinhardtii* cultures (shown in the top row) were grown in TAP medium containing the indicated concentration of supplemental Fe supplied as an Fe-EDTA chelate. Cultures were grown at 24°C for 4 d, with 50 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) of constant light, and the abundance of FeSOD and various Fe-containing proteins in *C. reinhardtii* was determined at the different stages of Fe nutrition. Total proteins (20 \(\mu\)g) were separated by denaturing gel electrophoresis and transferred to PVDF or nitrocellulose for immunoblot analysis. Soluble proteins (60 \(\mu\)g) were separated by nondenaturing gel electrophoresis and FeSOD visualized by activity staining (FeSOD, bottom panel).

(0.5 and 0.2 \(\mu\)M Fe) was not markedly reduced compared with that in Fe-replete cells as assessed by immunoblotting. This also contrasts with the substantial reduction in the major MnSOD that is observed in Mn-limited cells (Allen et al., 2007a). To assess whether the immunoreactive material is active, we analyzed the samples on nondenaturing gels for SOD activity and confirmed that the activity was retained as well (Figure 1).

We conclude that FeSOD is preferentially retained against a background of loss of other chloroplast Fe proteins in photoheterotrophic cells acclimated to Fe limitation, demonstrating a selective impact of Fe deficiency on Fe proteins within a single compartment.

**De Novo Synthesis of FeSOD during Fe Limitation**

To address the question of how individual proteins might be targeted during Fe deficiency, we monitored the temporal transition from Fe-replete to Fe-starvation. Cells were grown Fe-replete (i.e., 20 \(\mu\)M added Fe) to a density of \(\sim\)6 to 9 \(\times\) 10\(^5\) cells/mL and transferred to fresh Fe-free medium (Figure 2; labeled –Fe) or as a control to fresh replete medium (20 \(\mu\)M Fe, labeled +Fe). The cultures were sampled at time 0 or at 24-h intervals for 5 d to measure cell density, chlorophyll content, Fe content of cells and media, and the activity and abundance of various Fe-containing chloroplast proteins and SODs. Cell growth was highly reproducible between three independent experiments. Fe-starved cells reached a density of \(\sim\)5 to 6 \(\times\) 10\(^6\) cells/mL (indicative of growth limitation), while Fe-replete cultures reached 1.2 \(\times\) 10\(^7\) cells/mL (Figure 2A). The chlorophyll content in the –Fe cultures was reduced already after 1 d corresponding to the decrease in intracellular Fe content and eventually reached a new steady state, as noted previously (Figure 2B). In the first 24 h of Fe starvation, there was no impact on cell division; there was a nearly fourfold increase in cell density resulting in a fourfold decrease in Fe content per cell (Figure 2C), and the cells were entering Fe limitation by the end of this period as evidenced by the markedly reduced growth rate after this point (Figure 2A). The Fe content of spent –Fe medium was <1.5 nM (corresponding to the detection limit), 4.2 nM, and 42 nM for three individual experiments (Figure 2D; averages of technical triplicates). The concentration of Fe in +Fe medium fell by around 4 \(\mu\)M by the end of the experiment as the cultures reached stationary phase, in line with the amount of Fe taken up to meet the Fe quota of replete cells. Interestingly, the Fe-replete cultures experience reproducible transient Fe deficiency (accompanied by slightly reduced chlorophyll content) during the period of rapid exponential growth even in fully Fe replete medium because the assimilation pathway cannot keep up with intracellular demand (Figures 2B and 2C).

When we monitored the abundance of various Fe-containing proteins, we noted that expression of the Fe deficiency biomarkers ferredoxin and ferritin were dramatically increased over days 0 to 5 of Fe starvation (Figure 2E). The continuous increase in levels of ferritin and ferredoxin throughout this time course indicates clearly that the cells were still fully capable of de novo protein synthesis and that they became progressively more Fe limited. By contrast, there was a progressive decrease in ferredoxin, cytochrome \(f\), and the diiron-containing cyclase in the –Fe samples. Cytochrome \(f\) and ferredoxin showed a similar pattern on day 1 with a rapid decrease, but while the former continued to decrease to nearly undetectable levels, the latter was subsequently maintained at a new steady state level, consistent with its essential role in plastid metabolism. The abundance of the cyclase increased over the first 24 h, while the cells continued to divide (although not to the same extent as in the +Fe controls), but then decreased markedly relative to the day 0 level by day 5 of the experiment like ferredoxin and cytochrome \(f\) did.

Surprisingly, FeSOD abundance also decreased dramatically in response to Fe starvation in the first 24 h, but in contrast with cytochrome \(f\) and ferredoxin, the response was not sustained (Figure 2E); protein abundance starts to recover on day 2 until by day 5 it was restored to the day 0 level. We monitored the activity of FeSOD in nondenaturating gels and noted that the activity exactly paralleled polypeptide abundance. These results indicate that the mechanism of selective maintenance of FeSOD involves selective resynthesis after a period of nonselective degradation of multiple Fe-containing proteins. We hypothesize that the resynthesis relies on Fe that is recycled from expendable proteins, such as cytochrome \(f\), ferredoxin, and the cyclase, since there is very little if any exogenous Fe available (Figure 2D). In the case of the cyclase, the impact of degradation was offset by increased synthesis in response to cell division (Figure 2E, +Fe, right panel). The virtually identical pattern of activity and immunoblot analyses
indicate de novo synthesis of FeSOD in days 2 through 5 as opposed to remetallation of the apoprotein. It is possible that the Fe that populates the FeSOD active site may come from plastid ferritin, which serves as the buffer or depot for Fe released from degradation of PSI and other proteins (Busch et al., 2008).

Figure 2. Fe Reallocation to FeSOD during Transition to Fe Starvation.
Strain CC125 was grown in TAP medium with 18 μM Fe, washed, inoculated into TAP medium with 0 μM or 18 μM supplemental Fe (labeled −Fe and +Fe, respectively), and allowed to grow for 5 d.

(A) to (D) Comparison of cell density, measured by counting on a hemocytometer (A), chlorophyll content, measured spectrophotometrically (B), Fe content of cells (C), and spent media, measured by inductively coupled plasma-mass spectrometry (D). Data from three individual experiments are shown, represented by circles, triangles, and squares; chlorophyll content was calculated for a single experiment (the data series represented by square symbols in the other three panels).

(E) Abundance of MnSOD3 and hierarchy of Fe allocation. Soluble proteins (60 μg) were separated by nondenaturing gel electrophoresis and SODs visualized by activity staining (top panels for MnSOD3 and FeSOD). Total proteins (20 μg) were separated by denaturing gel electrophoresis and transferred to PVDF or nitrocellulose for immunoblot analysis.

A Previously Undiscovered SOD Expressed under Fe Limitation
A previously uncharacterized SOD activity was apparent in the activity staining gels. This activity was distinct, based on its migration in nondenaturing gels (Figure 3, the low mobility band
activity was evident only in cells acclimated to Fe limitation (Asada et al., 1975; incubation of the gel with 3 mM H$_2$O$_2$ prior to staining almost abolished the FeSOD activity previously reported but did not significantly diminish the novel, Fe limitation-inducible activity, indicating that the enzyme responsible for this activity is not an FeSOD (see Supplemental Figure 1 online).

**Six Candidate SOD Genes in C. reinhardtii**

We surveyed the C. reinhardtii genome to identify the gene responsible for the newly discovered activity. Genes coding for SODs were identified by querying the version 3.0 draft genome using BLAST to distinguish gene models corresponding to homologs of SODs of other organisms, including fungi, plants, and animals. We identified six genes encoding candidate Fe/MnSODs, corresponding to protein IDs 182933, 53941, 193511, 196649, 114882, and 115390 in the version 3.0 genome. All six residues identified as perfectly conserved in Fe/MnSODs are present in the predicted proteins, and they were assigned as putative Fe- versus MnSODs on the basis of distinguishing sequence characteristics (Figure 4; Wintjens et al., 2004). The genes were named FSD1, MSD1, MSD2, MSD3, MSD4, and MSD5, respectively. The major MnSOD (H$_2$O$_2$ insensitive) detected by activity staining was identified by sequencing of tryptic peptides as the MSD1 gene product and named MnSod1. The FeSOD (H$_2$O$_2$ sensitive) was confirmed as the FSD1 gene product by the same method (see Supplemental Figure 2 and Supplemental Tables 1 and 2 online). We did not identify any candidate Cu/ZnSOD-encoding genes nor any copper chaperone for superoxide dismutase–type copper chaperone involved in their assembly (Culotta et al., 1997), consistent with previous reports that C. reinhardtii lacks this form of SOD, nor any gene potentially encoding a nickel SOD. The assignment of FeSOD1 as the FSD1 gene product was also validated in protein similarity network analysis (Figure 5), which grouped FeSOD1 with other FeSODs.

Direct sequencing of cDNAs confirmed that all six genes are expressed (Table 1). In nutrient-replete Tris-acetate medium, FSD1, MSD1, and MSD2 were expressed at high levels, and MSD3, MSD4, and MSD5 at much lower levels (Figure 6). Transcript abundance has been shown to predict cognate protein abundance for 70 to 75% of transcripts in S. cerevisiae and mouse (Kislinger et al., 2006; Lu et al., 2007). Therefore, MSD3, MSD4, and MSD5 were thought to be candidates for encoding the new Fe deficiency–inducible SOD.

Improved gene models, shown schematically in Supplemental Figure 3 online (see also Supplemental Methods 1 online), have been incorporated into the version 4.0 assembly of the C. reinhardtii genome at the Joint Genome Institute and the sequence data submitted to the GenBank/EMBL/DDBJ databases under accession numbers GQ413964 (FSD1), GQ413965, GQ413966, GQ415402, GU134344, and GU134345 (MSD1–5, respectively). Sequence and protein similarity network analyses (Figure 5) supported the assignment of MnSod2 as a typical mitochondrial MnSod, consistent with the detection of this protein in the C. reinhardtii mitochondrial proteome (Atteia et al., 2009), but the separation of MnSod1 and MnSod3 through MnSods in the network is suggestive of specialized function for these algal isoforms.

![Figure 3](image-url)
MSD3 Is Induced under Fe Limitation, Mn Deficiency, and H₂O₂ Stress

When we tested the expression of the SOD-encoding genes in response to Fe limitation by real-time PCR, we noted that MSD3 was dramatically upregulated under conditions of Fe limitation, with its mRNA increasing up to 103-fold (Figures 3B and 6). By contrast, expression of the other SOD-encoding genes was either little changed (MnSOD1 and MSD2) or increased only slightly (≤10-fold) (MSD4 and MSD5). When the mRNAs were quantified in RNA-sequencing experiments, we noted that MSD3 mRNA became the most abundant of the SOD-encoding transcripts in Fe-deficient cells (Figure 7). We observed a similar pattern of expression in cells grown under conditions of Mn deficiency, with levels of MSD3 mRNA increasing up to 200-fold over those measured in Mn-replete cells (Figure 6). This counterintuitive response results from secondary Fe deficiency in Mn-deficient C. reinhardtii (Allen et al., 2007a, 2007b). When RNAs were sampled from cells acclimated to Fe deficiency and Fe-limited conditions, the MSD3 transcript abundance paralleled the activity of the novel MnSOD (Figure 3). Expression of the other two highly expressed MnSOD-encoding genes, MSD1 and MSD2, changed little (less than or equal to twofold) across the range of Fe concentrations tested, and mRNAs for MSD4 and MSD5 were not detected in this experiment. The MSD3 gene was thus considered a likely candidate for encoding the newly discovered inducible MnSOD activity.

To determine this unambiguously, we generated mono-specific antibodies against the recombinant protein (see Supplemental Figure 4 online). The antibodies recognized a protein with an
apparent molecular mass of ~35 kD only in Fe-limited cells (0.5 or 0.2 µM added Fe) (i.e., samples exhibiting the novel MnSOD activity) but not in cells grown with higher concentrations of added Fe (Figure 3). To relate the novel activity to the immunoreactive band, we subjected the native gel to immunoblotting and demonstrated that the low mobility activity and the immune-reactive band, we subjected the native gel to immunoblotting and demonstrated that the low mobility activity and the immune-reactive band are the same. The MSD3 gene responds also to H₂O₂ stress (Figure 6). MSD3 RNA was increased in abundance up to 20- to 60-fold in response to H₂O₂, while expression of the other five SOD-encoding genes was either little changed (MSD1, MSD2, and MSD5) or increased only two- to sixfold (FSD1 and MSD4).

Table 1. Genes Encoding SODs in C. reinhardtii

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein ID</th>
<th>RPKM³</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSD1</td>
<td>Cre10.g436050</td>
<td>481.0</td>
</tr>
<tr>
<td>MSD1</td>
<td>Cre02.g096150</td>
<td>281.0</td>
</tr>
<tr>
<td>MSD2</td>
<td>Cre13.g605150</td>
<td>82.8</td>
</tr>
<tr>
<td>MSD3</td>
<td>Cre16.g676150</td>
<td>4.6</td>
</tr>
<tr>
<td>MSD4</td>
<td>Cre12.g490300</td>
<td>0.2</td>
</tr>
<tr>
<td>MSD5</td>
<td>Cre17.g703150</td>
<td>0.2</td>
</tr>
</tbody>
</table>

SOD transcript abundance determined by RNA-sequencing in C. reinhardtii strain 2137 (data from the National Center for Biotechnology Information Gene Expression Omnibus, accession number GSE25124; Castruita et al., 2011). Cells were grown phototrophically in nutrient-replete TAP medium (18 µM Fe).

³Corresponding to the Au10.2 models in the version 4.0 genome assembly.

MSD3 Is Transcriptionally Regulated by Fe and H₂O₂

To distinguish the mechanism of regulation of the MSD3 gene by Fe and H₂O₂, reporter constructs (Figure 9A) were introduced into the Arg auxotrophic C. reinhardtii strain CC425 (cw15 arg7) by cotransformation with pARG7.8 (carrying the complementing gene). Arg prototrophs were tested for arylsulfatase expression on plates containing either high (18 µM), medium (3 µM), or low (1 µM) concentrations of added Fe using the chromogenic substrate X-sulfate (de Hostos et al., 1988). Cotransformants were screened by PCR to check cotransformation frequencies and for the presence of full-length reporter construct DNAs. Approximately 50% of pJD100GW-5'MSD3 cotransformants carried full-length reporter DNA, and ~35% of these were arylsulfatase expressers (see Supplemental Table 3 online). A single representative reporter-expressing colony from the transformation is shown in Figure 9B (blue halo indicates arylsulfatase activity). All cotransformants exhibiting Fe-responsive arylsulfatase expression carried full-length reporter construct DNA. By contrast, no colony arising from the pJD100 control transformation showed significant arylsulfatase activity. Quantitation of arylsulfatase expression by enzyme assay showed clearly that the 5’ flanking DNA from MSD3 carries Fe-responsive expression on the reporter gene (Table 2). The average increases in arylsulfatase expression in Fe-deficient and Fe-limited over Fe-replete cells were ~15-, ~26-, ~79-, and ~297-fold for cells grown with 3, 1, 0.5, and 0.2 µM added Fe, respectively. Arylsulfatase expression from the pJD100GW-5'MSD3 reporter construct was also increased when transformants were grown under conditions of Mn deficiency (which generates secondary Fe deficiency). The increase in arylsulfatase expression in deficient over replete cells was ~14-fold (Table 2), similar to that observed for the endogenous MSD3 gene, and consistent with the observation that the amount of Fe in Mn-deficient cells is similar to the Fe content of cells grown in medium containing 3 µM added Fe (Allen et al., 2007a). When transformants carrying the pJD100GW-5'MSD3 construct were challenged with H₂O₂, expression of the ARS2 mRNA was strongly upregulated (Figure 9C) with kinetics similar to those observed for the endogenous MSD3 gene.

We conclude that Fe deficiency and H₂O₂ act at the transcriptional level on the MSD3 gene. To investigate whether posttranscriptional mechanisms contribute to changes in MSD3
RNA from Fe-deficient (closed circles) and Mn-deficient (open circles) cells and from cells treated with 1 mM H2O2 (triangles) was analyzed by quantitative real-time PCR for the abundance of transcripts encoding FSD1 and MSD1 through MSD5. Primers used are listed in Supplemental Table 5 online. C. reinhardtii strains 2137, CC125, and CC425 were grown in TAP medium with either 0.5 or 18 µM added Fe and either 0 or 25 µM added Mn to ~3 × 10^6 cells/mL. For H2O2 treatment, cells were grown to ~2 × 10^6 cells/mL, H2O2 added to 1 mM final concentration, and cells harvested 2 h later. This concentration of H2O2 was chosen based on previous studies (Leisinger et al., 2001). The fold difference in abundance of each mRNA is shown after normalization to CBLP. Each data point is the average of technical triplicates and represents an individual experiment.

mRNA abundance, we monitored the decay of MSD3 transcript levels after blocking transcription with Actinomycin D. We observed no difference in persistence of the MSD3 transcript between cells resupplied with Fe and those left without (see Supplemental Figure 5 online) and conclude that regulation of MSD3 transcript abundance by Fe nutrition occurs primarily at the transcriptional level.

**MnSOD3 Is Localized to the Chloroplast**

The specific transcriptional upregulation of a previously undiscovered MnSOD in Fe deficiency raised a question concerning its localization because MnSOD is found in the mitochondrial matrix in most eukaryotes. If MnSOD3 functions as a backup for FeSOD, as suggested by its increased expression in Fe-limited cells, it must be localized in the same compartment as FeSOD because the superoxide anion cannot freely cross biological membranes (Takahashi and Asada, 1983; Missirlis et al., 2003; Lynch and Fridovich, 1978). Therefore, we localized MnSOD3 by biochemical fractionation. Chloroplasts and mitochondria were isolated from strain CC425 cells grown under conditions of Fe limitation and purified by density gradient centrifugation. The efficiency of this fractionation was assessed by immunoblotting with antibodies to alternative oxidase 1, a protein of the inner mitochondrial membrane, and the chloroplast-localized ketol-acid reductoisomerase and FeSOD (Vanlerberghe and McIntosh, 1997; Dumas et al., 1989; Kitaya ma et al., 1999). This analysis showed that the mitochondrial fraction was free of chloroplast material and the chloroplast fraction only slightly contaminated with mitochondria. When we probed the fractions by immunoblotting with antibodies against MnSOD3, we found that it was present in the chloroplast fraction (Figure 10).

Based on the prevailing view of Fe sparing, the simplest interpretation would be that a new chloroplast-localized MnSOD compensates for the potential loss of a FeSOD in Fe-deficient cells. However, as shown above, the FeSOD activity appears to be maintained. And in fact, MnSOD3 abundance and activity does not mirror the decrease in FeSOD. Instead, the activity and abundance increase as the cells begin to experience Fe limitation and continue to increase up to day 5 even though FeSOD activity is restored (Figure 2E). This suggests that the function of MnSOD3 may be to increase the capacity of the chloroplast to detoxify superoxide rather than to compensate for reduced FeSOD activity.

We measured total SOD activity during the transition to Fe starvation in an independent set of experiments involving several different wild-type strains. Total SOD activity per cell increased 1.5- to twofold after 4 d of Fe starvation in all four strains (Figure 11A). More strikingly, the specific activity of total SOD in soluble extracts increased threefold to 3.5-fold over the same period (Figure 11B), due to a reduction in the amount of soluble protein per cell, which declined to about half its initial level within the first 24 h of Fe starvation and then stayed stable over the remaining 4 d of the experiment (Figure 11C). FeSOD abundance exhibited a similar pattern of loss and recovery as observed previously (Figure 11D).

**DISCUSSION**

**Maintenance of FeSOD**

When C. reinhardtii cells get less Fe than they need to maintain the cellular Fe quota, the precious nutrient is selectively distributed. The mitochondria are prioritized over the chloroplast,
and then refolded in the presence of Fe²⁺ (Yamakura and FeSOD does not incorporate Fe unless the protein is unfolded resynthesis of the polypeptide and de novo metallation. Apo-f

Figure 8. Identification of MnSOD3 as the MSD3 Gene Product.

Soluble proteins (60 and 20 µg) isolated from cells grown under conditions of Fe repletion (18 µM Fe) or starvation (0 µM added Fe, 5 d) were separated by nondenaturing gel electrophoresis and the gel divided in three. For one part of the gel (60 µg protein), SODs were visualized by activity staining; for the other parts (20 µg protein), proteins were transferred to nitrocellulose for immunoblotting with anti-MnSOD3 (1:1000) or anti-FeSOD (1:2000).

evenly when a reduced carbon source is available (Naumann et al., 2007; Terauchi et al., 2010). In acetate-grown cells, a program of proteolysis, particularly of the photosynthetic apparatus, is initiated in the chloroplasts (Moseley et al., 2002a). Fe released from the degradation of these proteins is most likely used for maintenance of the mitochondrial respiratory chain (Naumann et al., 2007). Here, we show that prioritization of Fe use occurs even within an organellar compartment, with FeSOD being maintained at its usual abundance in Fe-deficient cells while Fd abundance decreases to a new steady state (Figure 2E). How is this achieved? The steady state level of any macromolecule is determined by a balance between rates of synthesis and degradation. FeSOD could be maintained simply by being protected from degradation, but the time course experiment (Figure 2) argues against this model. At day 1, FeSOD activity and abundance are greatly reduced, comparable to the reduction in ferredoxin and cytochrome f abundances. Nevertheless, while cytochrome f and ferredoxin either continue to decrease or are simply maintained at a new lower steady state level, respectively, FeSOD abundance actually increases. The virtually identical pattern of the activity staining and the immunoblotting indicates that the increase is attributed to specific resynthesis of the polypeptide and de novo metallation. Apo-FeSOD does not incorporate Fe unless the protein is unfolded and then refolded in the presence of Fe³⁺ (Yamakura and Suzuki, 1976), suggesting that in vivo, Fe incorporation occurs during folding of the nascent polypeptide, as demonstrated for Mn incorporation into yeast mitochondrial MnSOD (Luk et al., 2005). Because the activity of FeSOD is H₂O₂ sensitive, we know that the increase can be attributed to metallation by Fe rather than mismetallation by Mn ion.

This raises the question of how FeSOD is selectively targeted for resynthesis. One mechanism might be through increased synthesis of the apoprotein. This could occur at the level of gene expression generating more template for synthesis of the apoprotein; however, FSD1 mRNA levels are not impacted by Fe deficiency. Translational regulation of the FSD1 mRNA is another model that could account for increased synthesis. A second mechanism, which we favor, is by selective Fe allocation to the FeSOD assembly pathway. Increased expression of a specific assembly chaperone would increase FeSOD synthesis and balance the ongoing degradation. In the absence of a nutritional supply of Fe, increased accumulation would result only when there is an adequate supply of Fe salvaged from the degradation of PSI, ferredoxin, cytochrome f, and other Fe-containing proteins. Indeed, accumulation of FeSOD is evident only at day 2, after there is an opportunity for Fe recycling from the degradation of other proteins. Thus, maintenance of a particular protein (FeSOD) might occur by relying on continuous resynthesis, dependent on a pool of cofactor that is salvaged from degradation of other abundant Fe proteins and selectively allocated to FeSOD. The upregulation of plastid ferritins that serve as an Fe depot in Fe-deficient C. reinhardtii is consistent with this idea (Busch et al., 2008; Long et al., 2008).

Figure 9. Transcriptional Regulation of MSD3 by Poor Fe Nutrition or by H₂O₂ Stress.

(A) Schematic of reporter gene construct. White segments represent the ARS reporter gene, black the minimal TUB2 promoter, red the MSD3 5' flanking DNA. The numbers beneath the MSD3 5' flanking segment indicate positions relative to the transcript start site determined by 5'rapid amplification of cDNA ends.

(B) Arylsulfatase activity on plates. Single representative transformants growing on agar-solidified TAP medium with the indicated Fe supplement were stained for arylsulfatase activity (visualized as a blue halo around colonies) by spraying with a solution of the chromogenic substrate 5-bromo-4-chloro-3-indoxyl sulfate.

(C) Coordinate accumulation of mRNAs for MSD3 (closed symbols) and the ARS2 reporter gene driven by the MSD3 promoter (open symbols) in cells challenged with H₂O₂. Transformants carrying either the MSD3 5' flanking DNA fused to the ARS2 reporter gene (circles), or the ARS2 reporter gene alone (squares), were grown to early log phase (2 x 10⁶ cells/mL) in TAP medium, and H₂O₂ was added to 1 mM. Cells were collected at the time points shown and RNA analyzed by real-time PCR for the abundance of transcripts encoding MSD3 and ARS2. Data shown are for representative individual transformants.
The balancing of synthesis and degradation is nicely evident from comparing the pattern of accumulation of the diiron-containing cyclase (Figure 2E). CTH1 mRNA (encoding the cyclase) increases transiently upon transfer of cells to fresh medium, most likely to accommodate the rapid growth rate. The amplitude of the increase in Fe-limited cells is damped relative to the +Fe control, presumably because of ongoing degradation in the former situation, and eventually the cyclase abundance is also decreased substantially like ferredoxin and cytochrome f.

Why is FeSOD prioritized? We expect an increased propensity for O$_2^-$ generation by the Mehler reaction in Fe-deficient chloroplasts because its high Fe content renders PSI particularly susceptible to Fe deficiency stress (Sandmann and Malkin, 1983). Reduced energy input via remodeling of the PSI antenna is one mechanism to circumvent the problem (i.e., by reducing the production of O$_2^-$) (Moseley et al., 2002a; Naumann et al., 2005). Increased dissipation of the superoxide is another mechanism. Many studies have established the importance of chloroplast SOD activity for plant survival during oxidative stress (Tsang et al., 1991; Bowler et al., 1991; Herbert et al., 1992; Van Camp et al., 1996; Phee et al., 2004; Sunkar et al., 2004; Myouga et al., 2008). For example, when FeSOD was overexpressed in Arabidopsis chloroplasts, the transgenic plants were more tolerant of methyl viologen–induced stress, whereas loss of the chloroplast FeSODs resulted in pale or albino plants, depending on the severity of the loss (Myouga et al., 2008). Since the phenotype of the FeSOD-deficient plants is dependent on light intensity, it suggests that plastid SODs are important for handling O$_2^-$ generated by the light reactions.

How are Fe proteins recognized for degradation? In the case of those with labile FeS clusters, their steady state abundance relies on the operation of cluster repair mechanisms, and these are sensitive to Fe availability (Garland et al., 1999; Jensen and Culotta, 2000; Djaman et al., 2004). In Fe deficiency, the apoproteins would not be remetallated, making them susceptible to proteases (Reyda et al., 2008). For other Fe proteins, a recognition mechanism is not obvious, but their degradation might be a consequence of oxidative stress damage from reaction of the active site Fe with H$_2$O$_2$ (Halliwell and Gutteridge, 1984; reviewed in Imlay, 2008). That there is some level of selectivity for Fe-containing proteins (although not the type of Fe center) is evident from the observation that many other proteins like CF$_1$ and plastocyanin are retained.

### Table 2. Fe Nutrition Status Responsive Arylsulfatase Expression from an MSD3-ARS2 Reporter Gene Construct

<table>
<thead>
<tr>
<th>Construct</th>
<th>µM Added Fe</th>
<th>µM Added Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJD100GW</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>pJD100GW –5’MSD3</td>
<td>11 ± 2</td>
<td>0 ± 0.2</td>
</tr>
</tbody>
</table>

Transformants carrying the MSD3 5' flanking DNA fused to the ARS2 reporter gene (n = 3) or a control construct (n = 3) were grown to mid to late log phase (2 × 10$^6$ to 2 × 10$^7$ cells/mL) in TAP media with various amounts of added Fe (as indicated), or with (25 µM) or without (0 µM) added Mn, and culture supernatants assayed for arylsulfatase activity using p-nitrophenyl sulfate. Activities are expressed as picomoles of p-nitrophenol liberated per 10$^6$ cells per minute and are given as mean ± SE.

#### Increased Capacity for Detoxifying Superoxide

Even though FeSOD is retained in Fe-limited cells, a new SOD activity is induced in chloroplasts (Figures 2, 3, and 10). In previous work, the product of the MSD3 gene could not be unambiguously related to a particular isomerase on the activity gel. In this work, we used isomerase-specific antibodies to relate the inducible chloroplast-localized activity with low electrophoretic mobility in nondenaturing gels to the MSD3 gene. Upregulation of MnSOD by Fe limitation has been observed in many microorganisms and is viewed as a Fe-sparing mechanism (i.e., replacement of an abundant Fe-containing enzyme with an Fe-independent one) (Campbell and Laudenbach, 1995; Kim et al., 1999). In this work, we show that the new activity is an addition to the existing activity rather than a replacement, resulting in a net increase in the capacity to detoxify superoxide (Figure 11). In fact, its activity continues to increase at day 3 even when FeSOD activity is already recovering. When we compare the total count of SOD-encoding mRNAs, the increased capacity for

![Figure 10. MnSOD3 Is Localized to the Chloroplast.](image)

Strain CC425 was grown in TAP medium containing 0.5 µM Fe. Soluble proteins (60 µg) from whole cells, and from isolated chloroplasts (CP) and mitochondria, were separated by nondenaturing gel electrophoresis and SODs visualized by activity staining (top panels). Total proteins (20 µg) were separated by denaturing gel electrophoresis and transferred to PVDF or nitrocellulose for immunoblot analysis with anti-MnSOD3, anti-FeSOD (chloroplast), and anti-KARI (chloroplast stroma), and anti-alternative oxidase (mitochondrial inner membrane).
The use of a MnSOD to provide this extra capacity is sensible in this situation because Fe is in limiting supply. Another possibility is that the MnSOD has a different function. In cyanobacteria, which have both Fe- and MnSODs, the two isoforms have different localizations suggestive of distinct, and probably complementary, functions (Hopkin et al., 1992; Thomas et al., 1998; Li et al., 2002; Regelsberger et al., 2004). In this context, we note that the predicted isoelectric points of C. reinhardtii FeSOD and MnSOD3 are very different (6.3 and 9.5, respectively), which might well lead to differential localization within the chloroplast, although clearly both are soluble enzymes. Therefore, we suggest that upregulation of MnSOD3 in Fe-deficient C. reinhardtii may occur to provide additional antioxidant defense rather than only as a replacement for FeSOD. This is suggested by the pattern of MnSOD3 expression, which is high even when FeSOD abundance is unaffected or only marginally affected (Figures 2 and 3; see Supplemental Figure 6 online). Could the inducible Mn-containing form be a safer option for protection of nucleic acids in the Fe-limited cell? The fact that the MSD3 gene is regulated by peroxide as well is consistent with this idea.

Chloroplast MnSOD

A diatom, Thalassiosira pseudonana, with two genes for MnSODs and two for FeSODs (but none for Cu/ZnSOD; Armburst et al., 2004), was also found to house a chloroplast-located MnSOD, and Fe deficiency promoted the assembly of more MnSOD (Peers and Price, 2004; Wolfe-Simon et al., 2006). Given the typical low level of Fe in 30% of the world’s oceans and their habitation by a variety of diatom species, the use of a MnSOD during Fe limitation stress might be widespread in nature.

The occurrence of structurally related Fe- and MnSODs in chloroplasts does raise the question of the mechanism of selective metal incorporation. In E. coli and S. cerevisiae mitochondria, the MnSOD can be isolated as a mixture of correctly and incorrectly metallated enzyme; the degree of mismetallation depends on the status of Fe and Mn homeostasis in the assembly compartment (Beyer and Fridovich, 1991; Culotta et al., 2006; Yang et al., 2006; Naranuntarat et al., 2009). Fe incorporation into the MnSOD appears to be more of a problem than Mn incorporation into FeSOD. Whereas this might appear to be a non-issue in Fe-depleted chloroplasts, we note that both proteins are actually being synthesized simultaneously (day 3 in Figure 2), and correct allocation of a limited resource (i.e., Fe) might be quite important. The mechanism by which this occurs awaits further investigation.

The SODs are among the most important antioxidant defenses in plants (Alscher et al., 2002). Genome sequences show that there are multiple SOD isoforms in most plants and algae. The chlorophyte algae lack Cu/ZnSOD, but instead have several forms of the Fe and Mn type. This may be an adaptation to low-oxygen environments; species of the genus C. reinhardtii are found in naturally hypoxic or even anoxic habitats (Harris, 2009), in which the availability of copper is limited by the insolubility of Cu(II) salts (Osterberg, 1974), whereas Mn becomes more soluble and bioavailable [as Mn(II)] as oxygen tension decreases (Wollast et al., 1979). In C. reinhardtii, sequence analyses indicate that MnSOD2 is a typical mitochondria-localized protein, but the
divergence of MnSOD1 and MnSOD3 through MnSOD5 is suggestive of specialized functions for these isoforms, as demonstrated here for MnSOD3. The regulation of C. reinhardtii MSD3 by peroxide stress as well as Fe deficiency could indicate the existence of a peroxide-responsive element in C. reinhardtii, but the response might also be mediated via peroxide destruction of an Fe/S-containing Fe sensor (Kumànovics et al., 2008; Mühlenhoff et al., 2010) (Figures 6 and 9).

METHODS

Strains and Culture Conditions
Chlamydomonas reinhardtii strains CC125, CC425 (cw15 arg2), and 2137 were obtained from the C. reinhardtii culture collection (Duke University) and grown in Tris-acetate-phosphate (TAP) medium (Harris, 2009) at 24°C and a photon flux density of 50 to 100 μmol m⁻² s⁻¹ provided by a mixture of cool white fluorescent bulbs at 4100K and warm white fluorescent bulbs at 3000K used in the ratio of 2:1. For experiments involving Fe nutrition, strains were maintained in standard TAP medium (18 μM Fe) and transferred to –Fe TAP supplemented with Fe-EDTA at the concentrations indicated (Moseley et al., 2002a). Cells were made Mn deficient as described by Allen et al. (2007a); deficiency was confirmed by examination of chlorophyll fluorescence decay kinetics. For H₂O₂ treatment, cells were grown to 10⁷ or 2 × 10⁷ cells/mL and treated with 1 mM H₂O₂ for 2 h prior to protein or RNA isolation. In the case of CC425, media were supplemented with Arg (1.15 mM, added prior to autoclaving). Transformants of strain CC425 were grown as above or on TAP agar plates supplemented with Fe-EDTA at the concentrations indicated (22 to 25°C, ~100 μmol m⁻² s⁻¹ light intensity).

Metal and Chlorophyll Measurements
Total metal content of cells was measured for 2 × 10⁶ cells as described by Allen et al. (2007a), with the exception that nitric acid was used at a final concentration of 2.4%. Spent medium was transferred to acid-washed tubes, and any remaining cells removed by further centrifugation before nitric acid was added (to 2.4%). Total metal content was measured by the standard addition method. Chlorophyll was measured as described by Moseley et al. (2000).

Nucleic Acid Isolation and Analysis
Total C. reinhardtii RNA was prepared as described previously (Quinn and Merchant, 1998). C. reinhardtii DNA for colony PCR was isolated as described by Berthold et al. (1993). DNA sequencing was performed by Agencourt Biosciences. Plasmids intended for transformation into C. reinhardtii were isolated using the Marligen High-Purity Plasmid Purification System. Improved gene models were generated as described in Supplemental Methods 1 online, Supplemental Figures 7 and 8 online, and Supplemental Table 4 online.

Real-Time PCR
cDNA preparation and quantitative RT-PCR were performed as described by Allen et al. (2007a). Primers used are listed in Supplemental Table 5 online. Data are presented as the fold change in mRNA abundance, normalized to the endogenous reference gene CBLP. Abundance of the CBLP mRNA does not change under the conditions tested.

RNA Abundance
Sequence data from Gene Expression Omnibus accessions were analyzed as described previously (Castruita et al., 2011).

RNA Half-Life Determination
Strain CC125 was grown to a cell density of ~2 × 10⁶ cells/mL in TAP medium with either 20 or 0 μM supplemental Fe for 2 d. The 0 μM Fe culture was then divided between two flasks and to one of these Fe was added to 20 μM. Actinomycin D (40 μg/mL) was added to all cultures, and RNA isolated from cells harvested at various time points was analyzed by real-time PCR. A second experiment was performed identically except that Actinomycin D was not added to the cultures.

Organelle Isolation
Chloroplasts and mitochondria were isolated as described by Terauchi et al. (2009). Isolated chloroplasts and mitochondria were resuspended in 10 mM sodium phosphate, pH 7.0, containing 20% (v/v) glycerol. When required, organelles were lysed as described by Allen et al. (2008).

SOD Activity Gels and Activity Assays
Soluble proteins were quantitatively released from cells by two freeze-thaw cycles (Merchant et al., 1991) and separated by nondenaturing PAGE (7.5 to 9% acrylamide monomer) using the system of Davis (1964). Gels were photopolymerized as described by Lyubimova et al. (1993). SOD activity was visualized as described by Beauchamp and Fridovich (1971). Activity due to FeSOD was identified by sensitivity of the enzyme to pretreatment of the gel with 3 mM H₂O₂ prior to activity staining. Total SOD activity was measured by the xanthine oxidase/xanthine/cytochrome c method as described by McCoy (1999).

Antibody Production and Immunoblotting
A DNA segment encoding the N-terminal 60% (A1 to Q122) of the mature FeSOD was amplified from the cDNA clone LC046e10 using primers FeSOD-F1 plus FeSOD-R2, digested with BamHI plus KpnI, and cloned in pTrxA (Invitrogen) to create in-frame fusions to Escherichia coli TrxA. The DNA region coding for the fusion protein, plus upstream DNA incorporating the TrxA ribosome binding site, was amplified using primers TrxA-F1 plus FeSOD-R3, digested with BamHI plus XhoI, and cloned in pET23(+ (EMD Biosciences/Novagen) to create an in-frame fusion to the His₆-coding region of the vector. Similarly, a DNA segment encoding the region E93 to A183 of the predicted MnSOD3 precursor was amplified from the cDNA clone MX248H08 using primers MSD3F-ab plus MSD3R-ab, digested with BamHI plus PstI, and cloned in pTrxA. The DNA region coding for the fusion protein, plus upstream DNA, was amplified using primers TrxA-F1 plus MSD3-R1, digested with NdeI plus XhoI, and cloned in pET22b(+) to create an in-frame fusion to the His₆-coding region of the vector. All primers used are listed in Supplemental Table 6 online.

The resulting plasmids were transformed into E. coli BL21(DE3) for isopropyl-β-D-1 thiogalactopyranoside–inducible expression. The major (>90%) of the TrxA-FeSOD-His₆ fusion protein was soluble, while the TrxA-MnSOD3-His₆ fusion protein formed inclusion bodies that could be solubilized with 5 M urea. Both fusion proteins were purified by immobilized metal affinity chromatography on a Ni²⁺ charged His-Trap column (GE Healthcare), the TrxA-MnSOD3-His₆ fusion protein under denaturing conditions (5 M urea). Details of the purifications are given in Supplemental Methods 1 online. The fusion proteins were further purified by preparative SDS-PAGE; the regions of the gels containing the fusion proteins (visualized by Zn-imidazole staining) were excised and used directly for antisera production in rabbits (service provided by Agrisera and Pacific Immunology). The antisera were shown to be specific for FeSOD and MnSOD3, respectively (see Supplemental Figure 4 online).

For immunoblot analysis, proteins were separated by denaturing PAGE (10 to 15% monomer) and transferred to polyvinylidene fluoride and detected as described by Allen et al. (2007a). Primary antibodies were
used at the following dilutions: antialternative oxidase, 1:5000 (Agrisera AB AS06 152); anticytochrome f, 1:2000 (Agrisera AS06 119); antiferreredoxin, 1:1000 (Terauchi et al., 2009); anti-FeSOD, 1:5000. Alternatively, proteins were transferred to nitrocellulose and detected as described by Page et al. (2009). In this case, primary antibodies were used at the following dilutions: anti-CF, α/β, 1:10,000 (Merchant and Selman, 1983); anticytochrome f, 1:2000; antifechin1, 1:1000 (Long et al., 2008); antiferreredoxase, 1:300 (La Fontaine et al., 2002); anti-FeSOD, 1:1500; anti-MnSOD3, 1:1000; anti-KARI, 1:1000; anti- magnesium protoporphyrin monomethyl ester cyclase, 1:1000 (Moseley et al., 2002b).

**Reporter Constructs and Their Analysis**

All primers are listed in Supplemental Table 7 online. The DNA region upstream of the MSD3 gene 5’ end was amplified from *C. reinhardtii* CC125 genomic DNA using primers M3PFI and M3PRI, cloned via recombination in the Gateway entry vector pDONR221 (Invitrogen), and verified by sequencing. The cloned segment was transferred via recombination into the reporter vector pJD100GW (constructed by introduction of the Gateway cassette into the Klenow-blunted KpnI site of pJD100; ttub2B3,3,2,1/ars; Davies and Grossman, 1994) to generate the construct pJD100GW-5’MSD3. Plasmids pJD100 and pJD100GW-5’MSD3 were linearized with PsiI and BsaI, respectively, and cotransformed with pArg7.8 into strain CC425 as described previously (Quinn and Merchant, 1995). Arg protoplasts were picked, streaked onto agar-solidified TAP containing 1, 3, and 18 μM added Fe, and screened for arylsulfatase expression using 5-bromo-4-chloro-3-indolyl sulfate (X-sulfate; Gold BioTechnology) as described by de Hostos et al. (1988). Cotransformation frequency and intactness of integrated reporter constructs were assessed by cotransfecting PCR using primers pJD100-tub-f plus ARS1 (for the TUB2-ARS2 region) and ARS-3’-F plus ARS3’-R (for the ARS2 3’ end).

Selected transformants were grown in liquid TAP medium containing various amounts of added Fe, or TAP containing no added Mn, and arylsulfatase in the culture medium determined after removal of the cells by centrifugation (de Hostos et al., 1988). In the case of pJD100GW-5’MSD3, the three strongest arylsulfatase expressers were analyzed. In the case of pJD100, three transformants carrying full-length reporter constructs were analyzed.

**Sequence Analysis**

Protein similarity networks were generated from an all-versus-all BLAST analysis (pairwise alignment between all pairs of proteins) of SOD sequences in a local sequence database. Protein sequences were trimmed to the 20% identity and Generation of Improved Gene Models.

**Supplemental Figure 5.** Time Course of mRNA Decay for MSD3 and Known Fe Homeostasis Genes.

**Supplemental Figure 6.** Accumulation of SOD mRNAs in Response to Fe Starvation.

**Supplemental Figure 7.** A Single MSD3 Transcript Is Detected by Hybridization and PCR Amplification.

**Supplemental Figure 8.** Determination of MSD5 Intron 2 Size.

**Supplemental Table 1.** Tryptic Peptides Recovered from the Major MnSOD Activity Band.

**Supplemental Table 2.** Tryptic Peptides Recovered from the FeSOD Activity Band.

**Supplemental Table 3.** Summary of Transformant Analyses.

**Supplemental Table 4.** Primers Used to Generate Improved Gene Models.

**Supplemental Table 5.** Primers Used for Real-Time PCR.

**Supplemental Table 6.** Primers Used to Generate Protein Expression Constructs.

**Supplemental Table 7.** Primers Used for Reporter Construct Assembly and Analysis.

**Supplemental Methods 1.** Assessment of Anti-FeSOD and Anti-MnSOD3 Antibody Specificity and Generation of Improved Gene Models for MSD3, MSD4, and MSD5.

**ACKNOWLEDGMENTS**

We thank Maryse Block for the gift of antibodies to the spinach chloroplast ketol-acid reductoisomerase (KAR1), Carla Koehler for antibodies to *E. coli* glutathione S-transferase, and Joanne Long for RNA samples. We are grateful to Arthur Grossman’s laboratory, the Kazusa DNA Research Institute for providing cDNA clones, and Crysten Blaby-Haas for help with the protein similarity network analysis. This work was supported by the Department of Energy (DE-FG02-04ER15529). M.D.A. was supported in part by Institutional and Individual Ruth L. Kirkstein National Research Service Awards (GM07185 and GM077066), and S.K. was supported in part by an Institutional Ruth L. Kirkstein National Research Service Award (GM07185).

**AUTHOR CONTRIBUTIONS**


Received April 10, 2012; revised May 16, 2012; accepted May 24, 2012; published June 8, 2012.

**REFERENCES**

Allen, M.D., del Campo, J.A., Kropat, J., and Merchant, S.S. (2007b). *FEA1, FEA2, and FRE1*, encoding two homologous secreted proteins and a candidate ferrireductase, are expressed

**Supplemental Figure 1.** The Newly Discovered Fe Limitation-Inducible SOD Is Not Sensitive to Hydrogen Peroxide.


Fe Sparing and Fe Recycling Contribute to Increased Superoxide Dismutase Capacity in Iron-Starved *Chlamydomonas reinhardtii*


*Plant Cell*; originally published online June 8, 2012;
DOI 10.1105/tpc.112.098962

This information is current as of December 15, 2017

<table>
<thead>
<tr>
<th>Supplemental Data</th>
<th>/content/suppl/2012/05/24/tpc.112.098962.DC1.html</th>
</tr>
</thead>
<tbody>
<tr>
<td>eTOCs</td>
<td>Sign up for eTOCs at: <a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a></td>
</tr>
<tr>
<td>CiteTrack Alerts</td>
<td>Sign up for CiteTrack Alerts at: <a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a></td>
</tr>
<tr>
<td>Subscription Information</td>
<td>Subscription Information for <em>The Plant Cell</em> and <em>Plant Physiology</em> is available at: <a href="http://www.aspb.org/publications/subscriptions.cfm">http://www.aspb.org/publications/subscriptions.cfm</a></td>
</tr>
</tbody>
</table>

© American Society of Plant Biologists
ADVANCING THE SCIENCE OF PLANT BIOLOGY