Manganese Deficiency in Chlamydomonas Results in Loss of Photosystem II and MnSOD Function, Sensitivity to Peroxides, and Secondary Phosphorus and Iron Deficiency1[W][OA]

Michael D. Allen, Janette Kropat, Stephen Tottey2, José A. Del Campo3, and Sabeeha S. Merchant*
Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095–1569

For photoheterotrophic growth, a Chlamydomonas reinhardtii cell requires at least $1.7 \times 10^7$ manganese ions in the medium. At lower manganese ion concentrations (typically <0.5 μM), cells divide more slowly, accumulate less chlorophyll, and the culture reaches stationary phase at lower cell density. Below 0.1 μM supplemental manganese ion in the medium, the cells are photosynthetically defective. This is accompanied by decreased abundance of D1, which binds the MnCa cluster, and release of the OEE proteins from the membrane. Assay of Mn superoxide dismutase (MnSOD) indicates loss of activity of two isoforms in proportion to the Mn deficiency. The expression of MSD3 through MSD5, encoding various isoforms of the MnSODs, is up-regulated severalfold in Mn-deficient cells, but neither expression nor activity of the plastid Fe-containing superoxide dismutase is changed, which contrasts with the dramatically increased MSD3 expression and plastid MnSOD activity in Fe-deficient cells. Mn-deficient cells are selectively sensitive to peroxide but not methyl viologen or Rose Bengal, and GPXs, APX, and MSRA2 genes (encoding glutathione peroxidase, ascorbate peroxidase, and methionine sulfoxide reductase 2) are slightly up-regulated. Elemental analysis indicates that the Mn, Fe, and P contents of cells in the Mn-deficient cultures were reduced in proportion to the deficiency. A natural resistance-associated macrophage protein homolog and one of five metal tolerance proteins were induced in Mn-deficient cells but not in Fe-deficient cells, suggesting that the corresponding gene products may be components of a Mn$^{2+}$-selective assimilation pathway.

Manganese is nutritionally essential for growth and survival of all living organisms because of its function as a redox cofactor in some enzymes or as an activator at a metal binding site of other enzymes (Frieden, 1985; Marschner, 1995; Christianson, 1997; Yocum and Pecoraro, 1999; Keen et al., 2000; Jakubovics and Jenkinson, 2001; Kehres and Maguire, 2003). For example, redox active manganese is present in manganese superoxide dismutase (MnSOD), which is the principal antioxidant enzyme of mitochondria, whereas in arginase, the catalytic Mn$^{2+}$ activates bound water to generate the nucleophile for hydrolysis of the guanidinium group of Arg (van Loon et al., 1986; Lebovitz et al., 1996). In photosynthetic organisms, manganese is also present as a polynuclear cluster in PSII where it catalyzes the water-splitting reaction (for review, see Merchant and Sawaya, 2005).

The MnSODs and PSII are expected to be the prime targets of Mn deficiency in plants (Yu and Rengel, 1999), and indeed the importance of manganese in the photochemical reactions of photosynthesis was recognized half a century ago because of the impact of deficiency on oxygen evolution and phototrophic growth (Pirson, 1955; Teichler-Zallen, 1969).

Three types of Mn$^{2+}$ transporting systems are known in bacteria: the MntABC-type proteins that were originally discovered by Pakrasi and coworkers (Bartsevich and Pakrasi, 1995) as being necessary for PSII function in cyanobacteria, the MntH-type proteins that are related to the eukaryotic divalent metal transporters called natural resistance-associated macrophage proteins (Nramps; for review, see Kehres and Maguire, 2003), and a P-type ATPase identified in Lactobacillus plantarum (Hao et al., 1999). The expression of the transporters is regulated by manganese nutrition status and involves specific sensor-regulator signal transduction pathways (e.g. Ogawa et al., 2002; Yamaguchi et al., 2002: Chandler et al., 2003; Guedon et al., 2003). The expression of the MntH-type proteins is also
determined by iron status, because these transporters, although more selective for Mn$^{2+}$, do include Fe$^{2+}$ among the substrates they handle (Kehres et al., 2002).

Mn assimilation in eukaryotes is attributed to members of the widely distributed Nramp family related to MntH mentioned above. The founding member, Nramp1, was discovered in mouse as a host resistance factor, and its function as a H$^+$-divalent cation symporter, especially for Mn$^{2+}$, became apparent when a related protein in yeast (Saccharomyces cerevisiae) was shown to be involved in Mn$^{2+}$ uptake and when transport activity was eventually established for Nramp2 (also called DCT1 or DMT1) by functional assay in the Xenopus oocyte system (Supek et al., 1996; Gunshin et al., 1997; Jabado et al., 2000). Nramp2 is induced by Fe deficiency in animals and probably functions largely in iron assimilation in vivo, indicating that individual Nramp family members may play distinct roles in micronutrient homeostasis.

In plants as well, the Nramps form a family of related proteins but with functionally distinct roles based on subcellular location, organ specific pattern of expression, metal specificity, and pH sensitivity (Belouchi et al., 1997; Curie et al., 2000; Thomine et al., 2003). The Arabidopsis (Arabidopsis thaliana) proteins are capable of transporting both Mn$^{2+}$ and Fe$^{2+}$ based on their ability to rescue smf1 or fet3 fet4 yeast strains, which are defective in high affinity Mn$^{2+}$ and Fe$^{2+}$ uptake, respectively, and at least two members are induced in vivo by Fe deficiency (Curie et al., 2000; Thomine et al., 2000, 2003). Likewise, in tomato (Lycopersicon esculentum), individual members show a different pattern of expression in response to Fe deficiency (Bereczky et al., 2003). The regulation of plant NRAMP gene expression by manganese has received less attention.

In yeast, there are three Nramp-type transporters: Smf1p, Smf2p, and Smf3p (for review, see Culotta et al., 2005). Smf1p and Smf2p are especially involved in...
manganese homeostasis and the biosynthesis of Mn-containing enzymes, although they do show broad substrate specificity, while Smf3p function is related to iron mobilization from the vacuole in an Fe-deficient situation. The PHO84-type phosphate transporters have also been shown to participate in low affinity Mn\(^{2+}\) uptake in yeast, perhaps because of a substrate preference of the phosphate transporter for a neutral M(II)HPO\(_4\) substrate (Fristedt et al., 1999; Jensen et al., 2003). Because the phosphate transporters are not regulated by manganese nutrition status, this activity can lead to accumulation of toxic amounts of manganese in yeast.

In plants, proteins of the cation diffusion facilitator family (named MTP for metal tolerance protein) have been shown to confer Mn tolerance, implicating them in Mn\(^{2+}\) efflux or sequestration into the vacuole (for review, see Hall and Williams, 2003; Kochian et al., 2004; Hanikenne et al., 2005).

Mn deficiency in plants, especially problematic in alkaline soils, is noted by leaf discoloration and impacts freezing tolerance, reproductive fitness, and carbohydrate metabolism (Marschner, 1995). In some regions in Australia, manganese is recognized as one among several micronutrient deficiencies that limits productivity in agricultural zones, and at a reforested site in Europe, Mn deficiency was implicated in needle chlorosis and hence perhaps is a contributing factor to forest decline (Donald and Prescott, 1975; Hiltbrunner and Flückiger, 1996). Yet the molecular details of manganese metabolism and biochemical consequences of deficiency are underinvestigated relative to the metabolism of iron.

Studies of molecular responses to nutrition are facilitated with a microorganism model system because of the ease with which nutrient supply can be manipulated. *Chlamydomonas reinhardtii* is a widely used model for understanding mechanisms underlying adaptive responses to both macronutrients, like nitrogen, sulfur, and phosphorus, and micronutrients, like the trace transition elements, as they relate to plant metabolism (Grossman, 2000; Merchant et al., 2006). Here, we present the molecular phenotype of Mn deficiency in wild-type *Chlamydomonas*.

**RESULTS**

**Growth in Mn-Deficient Medium**

The standard *Chlamydomonas* Tris-acetate-phosphate (TAP) medium with Hutner’s trace elements contains 25 \(\mu M\) Mn ions. To test the manganese requirement for growth, cells were transferred twice sequentially into Mn\(^{2+}\)-free medium (see “Materials and Methods”) and then tested after a third transfer into medium containing the indicated amounts of Mn ions. *Chlamydomonas* will take up more manganese than required when provided with 25 \(\mu M\) in the medium; sequential transfer is therefore required to deplete the excess stores of manganese (Supplemental Fig. S1). As noted previously for Chlorella, growth in the dark under heterotrophic conditions showed a very slight effect of manganese removal (Eyster et al., 1958). Under phototrophic conditions, a growth phenotype (longer doubling time and stationary phase at lower cell density) was evident when the manganese in the medium was reduced to 0.25 \(\mu M\) and became more severe as the manganese supplementation was further reduced (Fig. 1, A and B).

**PSII Function Is Compromised**

It is likely that loss of photosynthetic electron transfer function contributes to the growth phenotype,
because there is a strict requirement of manganese for photosynthesis (Fig. 1C, orange trace). The rise phase of the kinetic trace is indicative of PSII function (reduction of primary electron-accepting plastoquinone of PSII \( Q_A \)), while the decay phase reports on downstream events through the Cyt \( b_6f \) complex and PSI (reoxidation of \( Q_A \)). The position of the peak reflects the balance between PSII and PSI function. The shape of the curve in Mn deficiency is therefore consistent with a specific loss of PSII function upstream of \( Q_A \).

Indeed, under phototrophic conditions, Chlamydomonas cells do not grow when the manganese supplementation in the medium falls below 0.1 mM (Merchant et al., 2006). When manganese was added back to the phototrophic cultures containing no supplemental manganese, photosynthetic electron transfer function was quickly (by 1 h), albeit not immediately (by 0.5 h), restored and increased with time (Fig. 1D). The peak of the chlorophyll fluorescence kinetic curve shifted progressively leftward, indicative of a restoration of PSII function. Measurement of the Mn content of cells indicated an increase within 1 h of supplementation but reached the level maintained in fully replete cells only after 4 h, suggesting that manganese metabolism, including assimilation, compartmentation, and cluster formation, may be the time-dependent step rather than the synthesis of PSII components (see below). The addition of protein synthesis inhibitors cycloheximide and chloramphenicol did not block recovery of PSII, indicating that the polypeptide components are pre-existing (data not shown).

We noted a complete loss of phototrophic growth in cells grown in medium containing <0.1 \( \mu \text{M} \) manganese (Merchant et al., 2006). When we monitored the abundance of D1, we noticed an incomplete loss (50%–75% decrease; Fig. 2A). When we monitored the OEE proteins, a fraction of the OEE1 and OEE2 polypeptides and almost all of the OEE3 protein were released from the membrane and recovered instead in the soluble fraction (Fig. 2B). We conclude that manganese is not absolutely required for the accumulation of PSII polypeptides, although it does stabilize the complex on the membrane.

Reduced MnSOD Activity in Deficient Cells

SODs are found with different metal cofactors used for catalysis: CuZnSOD, FeSOD, MnSOD, and NiSOD (Wolfe-Simon et al., 2005). Biochemical analysis identified at least one FeSOD and at least two other MnSODs in Chlamydomonas organelles (Sakurai et al., 1993; Kitayama et al., 1999). This corresponds well with the occurrence of a single \( \text{FSD1} \) gene encoding an FeSOD and five genes, \( \text{MSD1} \) through \( \text{MSD5} \), encoding MnSODs in the version 3.0 draft of the Chlamydomonas genome (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). We identified two major activities in soluble protein extracts of Chlamydomonas as an FeSOD and a MnSOD (Fig. 3). Mn deficiency had a marked impact on the activity of the major MnSOD; reduced activity was noted when the medium Mn\(^{2+} \) supplement was reduced to 0.5 \( \mu \text{M} \) and the loss was proportionally greater as manganese nutrition was reduced.

![Figure 4](https://example.com/figure4.png) Selective sensitivity of Mn-deficient cells to \( \text{H}_2\text{O}_2 \). Cells were grown in 0.1-\( \mu \text{M} \) supplemental Mn or 25-\( \mu \text{M} \) Mn conditions for 2 d. The growth rate of cells in 0.1 \( \mu \text{M} \) supplemental Mn is similar to that of cells grown with 25 \( \mu \text{M} \) Mn. Cultures were diluted to 1 \( \times \) 10\(^6 \) cells/mL before the addition of the indicated concentrations of \( \text{H}_2\text{O}_2 \) (A) or methyl viologen (MV; B). Growth was monitored by counting cells in a hemocytometer (bottom). Cultures were photographed 24 (H\(_2\)O\(_2\)) and 48 (MV) h after exposure to the chemical (top). A duplicate experiment is shown in Supplemental Figure S3.
Table 1. Primers used for real-time PCR

The primer pairs for each gene model (corresponding to the Version 3.0 draft genome) are shown. The upper row of each set corresponds to the forward direction for the gene model and the lower row to the reverse direction. All primer sequences are written 5' to 3'. The percent efficiency for each primer pair is calculated based on the theoretical doubling of product at each cycle. For 100% efficient primers, the amount of product doubles at each cycle.

| Name | Protein ID | Percent Efficiency | Primer Pair |
|------|------------|--------------------|-------------|
| APX1 | 186597     | 91                 | TCAAGGAGTAGACAGGCAAGGG | CCGCTTACAGCACGGTAAAC |
| CAT1 | 150104     | 86                 | CAGAGGACCTTACTGAAACTTTG | CTGGCCGATACCATCTTCTT |
| COPT1| 196102     | 106                | TCTGTTTCTGCTGGGTTCCTCC | GTACACGGCCAGCTTCCCTG |
| COT1 | 116945     | 101                | CGCCGGACACCACTGTATC   | AGACGAGCTGATTGGCGAGCA |
| CTP1 | 121438     | 92                 | GGTGTTGACCTGGCTACGCTTG | GACACCACGGCCAGAGAAG |
| CTR1 | 196101     | 81                 | CCTTCACATCCTGCTTCCTTC | CCCTGCTGCTGGGTTCCTC |
| CTR2 | 196096     | 87                 | GCGCTGTACAGCTCCTCTCT | GCGTGTGGCTTGAGCGAAGAG |
| CBLP | 105734     | 108                | GCCACACAGAGTGGGCTTGCG | CCTTGCGATGATGATGCTGT |
| FSA1 | 129929     | 93                 | TGCGTTGCTCAGCCAGGGTGC | ACGCCGAGGGCCTTAAGGTC |
| FSD1 | 182933     | 95                 | CCCTGGGGCTTGGAGGCTGGT | ACGGGCCGGAGGGGCTTGA |
| GPX3 | 137012     | 102                | GACACGGCTTCTGCGTAGCTG | ACGGCAAGCTGACCACAGCA |
| GPX4 | 138373     | 96                 | CCAAGGACCTTACTGGAAC   | TATAGATCCGGCCGGCAACA |
| GPX5 | 143122     | 87                 | GCTCGTGTTATGGGTTTCCTG | GCTGTGGCTTGAGCGAAGAG |
| HMA1 | 195998     | 97                 | ACGTTGGACGACAGAGACAGA | CTCACAGCCCCCTCACCA |
| IRT1 | 196644     | 104                | GGGCTTCTGCGTGCTGCTTC | AGGGGACAGGAAGAAGAAA |
| IRT2 | 174212     | 97                 | GCGGCTTCTGCGTGCTGCTTC | GTAGATGGGTGCGGGCTTCG |
| MSD1 | 53941      | 102                | TATAGATGGGTGCGGGCTTCG | GCCGCGGTGGAGGGCCGGCTG |
| MSD2 | 193511     | 101                | AACAGTTCGAGGGCTGACGCTG | TCAGAGCGGAGGCTGACG |
| MSD3 | 196645     | 109                | ACCAGACCCACCAGAAGAG  | CAGCCGCCAACAGGATAAC |
| MSD4 | 114882     | 88                 | CCATGAGGTGGTTGTCCTG   | GAACTGGTAAAGCACAGATG |
| MSD5 | 115390     | 90                 | CAGACGCAACAGGACAACC  | GCAGCCACCAGCTTACAGT |
| MSRA2| 196100     | 100                | ACCCGGTGCTGGGCTGGGCA | GAGCCACCGGCCATCAGT |
| MTP1 | 119301     | 95                 | CTTGTGCTGTGCTGCTGCTG | GAGCCGGCTTGCGTGATACCA |
| MTP2 | 150298     | 97                 | ATAGAGGTGGGTGCTGCTGCA | GCAGGCTTCTGTCCTACAGTC |
| MTP3 | 195957     | 96                 | ATCCAGGCTTCTGTACGACTG | GTAAGGGGCTTCTACGGGCTC |
| MTP4 | 150298     | 101                | ATCACTGTTGGGCGGAGATCG | GAGCGGCTTGCTGCTGCTG |
| MTP5 | 149470     | 96                 | CTTGTGCTGTGCTGCTGCTG | GAGCGGCTTGCTGCTGCTG |

Table 1. (Continued.)

| Name | Protein ID | Percent Efficiency | Primer Pair |
|------|------------|--------------------|-------------|
| NIK1 | 173008     | 102                | CCCACCTAGCAGGCGAGCAC | GCCGGCATGACGAGTAGGAG |
| NRAMP1| 128400     | 109                | GGCGGGGATTTACCCCATGC | CACGGCGTGGCGGAAGTGAG |
| NRAMP2| 113049     | 87                 | CCGGAGGCTTACAGGCTGCC | ACTTTGATGGGCAAGAAGC |
| ZIP1 | 122719     | 100                | GCCGCTTTCAAGCCTTACAC | AGCAACCTGCCCTGTCCTC |
| ZIP3 | 100805     | 92                 | GCGGTATCTGCTGGTCGTC | CCTCTTGAGCTGTGCTGCT |
| ZIP6 | 183171     | 86                 | GGGAAGAAGCCTGGCTGAAA | CCTCTTGAGCTGTGCTGCT |
| ZRT1 | 146152     | 90                 | TGCTCTGCTGCTTCCCTCC | CCGGCGGTGTCCTCCCTT |
| ZRT2 | 107206     | 97                 | GCGGTATCTGTCCCTCCTTC | CCTGAGTTGTTCCTTCCT |
| ZRT3 | 168584     | 96                 | CCTTCCCACACAACACAGGA | TGGCGATGATGTGTGTGTC |
| ZRT4 | 196642     | 92                 | GCCGCTTCTGCTGCTGCTG | GACAGTACAGGCGGGTTCT |
| ZRT5 | 196756     | 100                | TGCCGTTTCCACAGGCTTAC | ACCACAGCAGCCAGCAGAG |

Manganese Deficiency in Chlamydomonas

Because MnSOD has antioxidant functions, we tested the impact of manganese nutrition on the ability of Chlamydomonas cells to resist oxidative stress (Fig. 4). We found that there was no impact of manganese nutrition on sensitivity to methyl viologen (Fig. 4B) or metronidazole, which promote the generation of superoxide through PSII activity, nor to Rose Bengal and Neutral Red, which promote singlet oxygen (Supplemental Fig. S2; Ledford and Niyogi, 2005). On the other hand, Mn-deficient cells (grown in 0.1 M Mn) were more susceptible to hydrogen peroxide ($\text{H}_2\text{O}_2$) relative to Mn-replete cultures (Fig. 4A) as well as to cumene hydroperoxide or t-butyl hydroperoxide (Supplemental Fig. S2). It is possible that plastid...
FeSOD, whose activity is not greatly reduced at 0.1 μM manganese, is an adequate defense against plastid-generated oxidative stress.

When we tested the expression of antioxidant enzymes (Table I), we noted that of the six SOD-encoding genes, three were induced by Mn deficiency: MSD3, MSD4, and MSD5. Increased expression was evident only upon severe Mn deficiency: for MSD3 at a supplement of 0.1 μM Mn2+ or less, and for the other two only in the zero-supplement growth medium (Fig. 5A). The increase of MSD mRNAs lagged behind the loss of MnSOD activity (Fig. 3). This suggests that increased abundance of MSD3, MSD4, and MSD5 mRNAs is not directly responsive to manganese nutrition and loss of MnSOD activity but rather to a downstream consequence (see “Discussion”). When Mn2+ was resupplied to Mn-deficient cells, MSD3 mRNAs were reduced in abundance with a time course that paralleled the recovery of PSII activity (compare Fig. 1 with Fig. 8, discussed below). The genes encoding glutathione peroxidases and ascorbate peroxidase were only slightly induced in Mn-deficient versus Mn-replete cells (Fig. 5B), which is comparable to the increase in peroxide- or methyl viologen-treated cells and much less than the 102-fold increase in GPX gene expression noted in cells treated with singlet oxygen generating photosensitizers (Leisinger et al., 2001). The expression of antioxidant selenoenzymes was likewise not increased in Mn-deficient cultures.

**Plastid MnSOD Activity and MSD3 Expression Increase in Fe Deficiency**

Because there is no increase in the accumulation of FSD1 mRNA nor any increase in FeSOD activity, it is evident that FeSOD cannot cover the loss of MnSOD function in Mn-deficient cells. On the other hand, in Fe-deficient cells, where FeSOD activity is reduced, the activity of the major MnSOD isoform was increased to compensate for the deficiency (Fig. 6, inset). This isoform is likely the product of the MSD3 gene, because its expression is dramatically (103-fold) increased in Fe deficiency (Fig. 6; Supplemental Fig. S3). We suggest that the MSD3 gene is directly responsive to iron nutrition rather than secondarily to reactive oxygen species, because we cannot mimic this pattern of expression by imposition of various oxidative stress conditions (high light, peroxide, methyl viologen, or Rose Bengal treatment; J. Long and S. Merchant, unpublished data). Because the major MnSOD activity was found in the chloroplast fraction, the increased expression of the MSD3 gene may compensate for loss of chloroplast FeSOD in Fe deficiency by MnSOD (Fig. 6B).

**Candidate Manganese Transporters**

When cells are starved for a nutrient, the first line of defense is the activation of assimilatory transporters. The identity of the manganese uptake transporter(s) is not known, but an NRAMP homolog is an excellent candidate (see introduction). RNAs corresponding to both NRAMP genes of Chlamydomonas increased in Mn-deficient relative to Mn-replete cells, with NRAMP1 showing a pattern of expression typical for an assimilatory transporter (Fig. 7). Specifically, the RNA was increased in abundance already at 1 μM manganese supplementation, prior to the appearance of symptoms (Figs. 1–4), but at a concentration that is just barely adequate to support the accumulation of manganese to the level maintained in a replete culture (1.3–1.5 × 107 Mn atoms/cell) and continued to increase (up to 8-fold relative to Mn-replete cells) as the Mn2+ content of the medium was further reduced. NRAMP2 expression also increased in cells grown in 1 μM Mn2+ but did not increase further with reduced Mn2+ supplementation, and the magnitude of the change was less than that for NRAMP1 (Fig. 7). For MTP4, the increase in expression was not evident until the cells were severely Mn deficient, which is not typical of an assimilatory transporter.

When we analyzed the expression of NRAMP1 upon transfer of Mn-replete cells to Mn-deficient medium,
a 4-fold increase in expression was evident already upon the first round of transfer, and this correlates nicely with a reduced manganese content of cells after the first transfer (Fig. 8). Maximum expression was achieved after the second sequential transfer to Mn-deficient medium, again correlated with maximally reduced total manganese content of cells. When Mn\(^{2+}\) was added back to the deficient cells, \(\text{NRAMP1}\) expression did not decrease immediately and remained high even 2 h later (by which time the manganese content of cells had reached the level maintained in a replete situation) but returned to basal levels within 24 h (Fig. 8). This contrasts with the immediate change in expression of the \(\text{MTP4}\) gene (within 1 h) upon replenishment of Mn\(^{2+}\) to the deficient culture and suggests that regulatory mechanisms that affect the \(\text{NRAMP1}\) protein directly may be operational. In plants, members of the cation diffusion facilitator family or cation efflux family (called MTPs) have been implicated in manganese homeostasis, particularly in a situation of manganese excess (Mäser et al., 2001). We tested five members of this family and noted that one, \(\text{MTP4}\), showed significantly (9-fold) increased accumulation but only when the manganese content of the medium was severely reduced (Fig. 8). Unlike \(\text{NRAMP1}\), whose expression was increased upon the first transfer to medium lacking manganese supplementation, \(\text{MTP}\) expression was actually depressed in this situation and increased only after the second transfer to deficient medium (Fig. 8). Given the role of the MTPs in cation efflux, this pattern of expression would be more consistent with an intracellular distributive transporter that could function to prioritize manganese distribution from one organelle to another in a situation of limiting intracellular manganese. This may account for the loss of MnSOD but not PSII at 0.5 \(\mu\text{M}\) manganese in the medium (Fig. 1 versus Fig. 3).

We tested also the expression of genes encoding candidate copper transporters (assimilatory molecules of the CTR family, distributive molecules of the HMA family), zinc and iron transporters of the ZIP family and components of the high affinity iron uptake pathway, ferroxidase, ferric transporter, and ferrireductase (Merchant et al., 2006). None of the genes showed a pattern of expression consistent with a function in manganese assimilation (Fig. 7; Supplemental Fig. S4). The mRNA for one Zn-deficiency-responsive transporter, \(\text{ZRT2}\), was consistently reduced in expression in Mn deficiency, and the pattern was evident already at 1 \(\mu\text{M}\) manganese in the medium (where no symptoms are evident), suggesting a direct response to manganese nutrition rather than an indirect response to stress.

**Secondary Fe Deficiency Results from Mn Deficiency**

When we tested the expression of components of the iron assimilation pathway (La Fontaine et al., 2002), we noted that the \(\text{FOX1}, \text{FTR1}, \text{FEA1}, \text{and FRE1}\)
genes were up-regulated (Table II). The increase in
gene expression was only about 10% of that noted in
Fe-deficient (1 µM iron) cells. The loss of FeSOD acti-
vity and the appearance of mild chlorosis in the
Mn-deficient culture together suggest the cells are Fe
deficient. Analysis of the metal content of Chlamydo-
monas cells indicated a reduction in the Fe content
correlated with a progressive decrease from 0.25 µM
manganese nutrition in the medium (Fig. 9). Without
manganese supplementation, the cells contained
about one-half the iron of a Mn-replete culture. The
effect is specific for iron, because the Cu and Zn
content of cells are not affected by manganese nutri-
tion (Fig. 9). The amount of iron in the cells is compar-
able to the iron content of cells grown in Fe-deficient
conditions (1–3 µM iron, 25 µM Mn²⁺), but, as noted
above, the increase in gene expression was only a frac-
tion of that observed in Fe deficiency. Because the
iron assimilation pathway relies on proteins that are
processed through the secretory pathway and pre-
sumably glycosylated, we wondered whether the
phenotype might be explained by loss of function of
Mn-dependent protein-modifying glycosyl transfer-
ases. Nevertheless, when we monitored the synthesis
of plasma membrane ferroxidase, periplasmic arylsul-
fatase, and carbonic anhydrase, we saw no evidence
for reduced secretion of any of these proteins (data not
shown). In fact, the ferroxidase was increased in abun-
dance in Mn deficiency, as expected from the increased
abundance of the mRNA (Table II). Some phenotypes of
Mn deficiency could be partially restored by provision
of extra iron (50 µM) in the medium, and reduction of
iron from the standard 18 to 1 µM exacerbated those
phenotypes, suggesting that a component of the
Mn-deficiency phenotype is related to iron nutrition;
yet, the iron content of the Mn-deficient cells could not
be restored by excess iron supplementation to the level
maintained by nutrient-replete Chlamydomonas, and
the expression of genes involved in iron assimilation
remained slightly elevated.

Figure 7. Relative expression of genes encoding
candidate manganese transporters. RNA was isolated
from cells grown in TAP medium containing the
indicated amounts of manganese supplement plus
excess Fe (50 µM). Gene expression was assessed by
real-time PCR as described in the legend to Figure 5.
Genes encoding transporters of the CTR family
(COPT1, CTR1, and CTR2), HMA family (CTP2 and
HMA1), cation diffusion facilitator family (MTP1-
MTP5), Nramp family (NRAMP1 and NRAMP2), and
ZIP family (IRT1, IRT2, ZIP1, ZIP3, ZIP6, and ZRT1-
ZRT5) were identified in the Chlamydomonas draft
genome by sequence homology. All experiments
were performed in experimental duplicate in 18
and 50 µM Fe.
Figure 8. (Legend appears on following page.)
Phosphorus Content Is Reduced

The total P content of Mn-deficient cells was also reduced relative to the Mn-replete condition in proportion to the deficiency and approached but did not reach the P content of cells starved for P for 24 h (Fig. 10). One of the four genes, PTA3, encoding a PHO84-type phosphate transporter, was 25-fold up-regulated, and a second gene, PTA4, was 5-fold up-regulated by Mn deficiency. The PHO84 transporters use a divalent cation as the counterion (Fristedt et al., 1994; Persson et al., 1999): the up-regulation of a subset suggests that Mn2+ may be the preferred counterion for these transporters. In yeast, up-regulation of PHO84 can suppress Δsmf2, indicative of Mn2+ co-transport (Jensen et al., 2003).

The pattern of PTA gene expression in Mn-deficient Chlamydomonas is different from that in P deficiency, where PTA3 is unaffected while PTA4 is 30- to 50-fold up-regulated. In addition, PTA1 is unresponsive in Mn deficiency, while it is 103-fold repressed by P deficiency (Moseley et al., 2006). These results indicate independent regulation of PTA genes by manganese and phosphorus nutrient status. Note that PTA2, which encodes a different type of phosphate transporter, and PHOX, which encodes a phosphatase, were not up-regulated in Mn-deficient cells, suggesting that the P-deficiency response was not turned on.

DISCUSSION

Assimilation and Transport Mechanisms

During acclimation to a metal cofactor deficiency, an organism activates assimilation mechanisms to acquire that metal or mechanisms that conserve utilization of the nutrient. These mechanisms are induced early in the transition from sufficiency to deficiency. When cofactor supply is exceeded by metabolic demand, symptoms of deficiency ensue (Merchant et al., 2006). We therefore expect that changes in the expression of assimilatory transporters would precede the establishment of deficiency phenotypes. Accordingly, we surveyed the Chlamydomonas genome for the pattern of expression of genes encoding candidate transition metal transporters and noted that only one, NRAMP1, showed a pattern consistent with a function as a manganese assimilation component. Its expression was increased already in the first transfer from cells grown in Mn excess (25 μM) to a medium without manganese supplementation (Fig. 8) and in cells grown with supplementation in the medium to 1 μM (Fig. 7), which is just barely enough to support the accumulation of PSI1 and MnSODs.

This analysis relies on the assumption that the transporters are regulated, at least in part, by supply-and-demand-dependent changes in the abundance of the mRNAs. The assumption is validated by the known transcriptional responses in Chlamydomonas to Cu, Fe, and Zn deficiency (for review, see Merchant et al., 2006), but we certainly cannot rule out mechanisms that act directly at the level of the protein. In fact, because of the toxicity of most of the essential transition metals, the transporters do seem to be subject to nutrient-excess-dependent posttranslational modifications/degradation (e.g. Gitan et al., 1998; Guo et al., 2004; Wang et al., 2004). Therefore, we conclude that NRAMP1 is likely to be one but perhaps not the only manganese assimilation transporter. Chlamydomonas NRAMP1 is a member of the MntH group C family, which includes many proteins from prokaryotes (Cellier et al., 2001). The possibility that NRAMP1 functions in organellar manganese uptake should therefore be considered.

The expression of MTP4 was also increased but only in symptomatic Mn deficiency (when expression of MSD3 and FEA1 was also increased), suggesting that it is either a high affinity assimilatory transporter or that it might play a role in preferential intracellular compartmentation of manganese, for example by catalyzing efflux from the mitochondrion (see below). The latter function would be consistent with the function of the MTPs in plants where they sequester metal ions in the vacuole and hence confer metal tolerance. Subcellular localization of the gene product in Mn-replete versus -deficient medium would be informative in terms of distinguishing these models.

In yeast, manganese is also assimilated at low affinity by a PHO84 type of transporter, which moves phosphate with a divalent counterion (Fristedt et al.,

Figure 8. Time course of changes in gene expression in response to Mn deficiency and resupply. RNA from cells subject to serial transfer to Mn-deficient TAP medium was analyzed (left column). The expression is presented relative to Mn-replete conditions. MnCl2 (25 μM) was added to Mn-deficient cultures and RNA was extracted from the cells at the indicated times and assayed for accumulation of specific mRNAs by real-time PCR (right column) as described in the legend to Figure 5. The pattern noted for MSD3 is typical also of MSD1 and MSD2, and the pattern noted for FEA is typical of the expression of other genes encoding iron assimilation components.

Table II. Fe-responsive gene expression during Mn deficiency

| Gene   | Relative Gene Expression | 1 μM Iron | 0 μM Supplemental Mn2+ |
|--------|--------------------------|-----------|------------------------|
| FEA1   | 2.0 × 10^2               | 2.4 × 10^1 |
| FOX1   | 3.5 × 10^1               | 4.1 × 10^0 |
| FRE1   | 3.3 × 10^2               | 1.9 × 10^2 |
| FTR1   | 5.3 × 10^1               | 4.7 × 10^0 |

Gene expression was assessed by real-time PCR. Cells were made Fe or Mn deficient and harvested as described in “Materials and Methods.” Relative expression was normalized to CBLP expression, and average Cq value was calculated from technical triplicates and compared to expression in replete conditions. Expression was calculated by the 2^ΔΔCq method (Livak and Schmittgen, 2001).
This pathway is not regulated by manganese overload in yeast and therefore can lead to overaccumulation of intracellular manganese when cells are grown in Mn-excess conditions. The connection between manganese and phosphorus assimilation may be obligatory for Chlamydomonas, because Mn-deficient cells showed reduced phosphorus content and two of the four PHO84-type transporters of Chlamydomonas were induced in Mn deficiency.

To estimate how much manganese is required in the growth medium for Chlamydomonas and to establish conditions for the study of Mn deficiency, we measured the metal content of cells grown with various levels of manganese supplementation (Fig. 9). With 25 μM manganese in the medium, the cells overaccumulate manganese beyond what is required to maintain manganese enzymes. The excess manganese may constitute a storage pool as described in cyanobacteria (Keren et al., 2002). As the supplementation is reduced to the submicromolar range (from 0.5 to 0), the manganese content of the cells is reduced in proportion to the supply. Based on assay of MnSOD and PSII function, we conclude that 1 μM manganese is just barely sufficient, and hence we recommend an optimum supply of about 3 to 5 μM for phototrophically grown Chlamydomonas.

We noted also that Mn-deficient cells had reduced Fe content, and this correlated well with the appearance of chlorosis (data not shown). In fact, chlorosis could be relieved by provision of extra iron in the medium, suggesting a connection between manganese and iron homeostasis. A simple explanation might be that manganese nutrition reduced the expression of the high affinity iron transport pathway consisting of FOX1, FTR1, and FEA1 (Merchant et al., 2006). But this was clearly not the case. In fact, the expression of these components was actually increased, although by only 10% of the increase expected based on the iron content of cells (50% of a replete cell). We suspect that the iron nutrition phenotype is a complex balance between the need to reduce the intracellular ferrous availability in response to peroxide stress resulting from Mn deficiency and the compensatory need to assimilate more iron because of the reduced intracellular iron availability.

**Figure 9.** Intracellular metal content. Cells were grown in medium supplemented with Mn²⁺ to the indicated concentration, and total Mn (A), Fe (B), Cu (C), and Zn (D) content was measured by inductively coupled plasma-mass spectrometry (see “Materials and Methods”). All experiments were performed in biological duplicate.
Hierarchy of Mn Distribution

Two of the more abundant manganese enzymes in a photosynthetic cell are PSII and the MnSODs (Raven, 1990), and the impact of Mn deficiency on the activity of these two enzymes (Figs. 1 and 3) is well known and therefore predictable. What is interesting, however, is that the loss of activity of the major MnSOD precedes loss of PSII activity, indicative of regulated manganese supply to intracellular or intraorganellar compartments.

Oxidative Stress

The loss of MnSOD activity, which is not compensated by an increase in FeSOD, should result in increased sensitivity to superoxide. Nevertheless, this was not the case. This might be explained by the finding that methyl viologen-induced superoxide stress acts predominantly in the chloroplast in light grown cells (Bowler et al., 1991), where FeSOD activity may be an adequate protectant.

On the other hand, the cells did appear to be more sensitive to H₂O₂ (Fig. 4) or organic peroxides (Supplemental Fig. S2) relative to Mn-replete cultures, and they also showed an increase in the expression of genes encoding antioxidant enzymes (Fig. 5B). We suspect that these increases, including that of the MSD3 through MSD5 genes, occur in response to oxidative stress rather than directly to manganese nutrition, because they are noted only coincident with the appearance of symptoms and when the manganese content is severely depleted, and they parallel the pattern of expression of the MSD genes in response to H₂O₂ treatment (J. Long and S. Merchant, unpublished data).

Iron toxicity is exacerbated in the presence of H₂O₂, because ferrous ion promotes the production of hydroxyl radical from H₂O₂, and the decreased cellular content of iron (Fig. 9) may be a protective mechanism (Storz and Imlay, 1999). A connection between manganese nutrition and peroxide and iron metabolism has been noted already in various bacteria (Horsburgh et al., 2002). An increase in lipid peroxidation in Mn-deficient rats was correlated with damage to mitochondrial membranes, suggesting that Mn deficiency in Chlamydomonas may result in localized oxidative stress (Zidenberg-Cherr et al., 1983).

Replacement of FeSOD by MnSOD

Although FSD1 expression was not increased to compensate for the loss of MnSOD in Mn deficiency, MnSOD activity was increased in Fe-deficient cells. One of the five MSD genes was dramatically up-regulated in Fe-deficient cells (Fig. 6A). This response is quite distinct in terms of magnitude as well as specificity from the response of the MSD genes to oxidative stress (Fig. 5), suggesting that the MSD3 gene, but not the MSD4 and MSD5 genes, also responds separately to iron nutrition. Interestingly, cell fractionation experiments suggest that the major MnSOD isoform shows dual localization to both the mitochondria and chloroplast, indicating that this isoform may cover the loss of the chloroplast FeSOD. The replacement of an FeSOD by a MnSOD has been observed in bacteria and suggested as well in diatoms that show an increased requirement for manganese in an Fe-deficient growth environment (Privalle and Fridovich, 1993; Campbell and Laudenbach, 1995; Peers and Price, 2004).

CONCLUSION

The individual MSD genes show unique patterns of expression in response to Fe deficiency or oxidative stress. It is likely that the major plastid-localized
Manganese Deficiency in Chlamydomonas

Immunoblot Analysis

For immunoblot analysis, proteins were separated on an SDS-containing polyacrylamide gel (10% monomer for CF1, 11% for D1, or 15% monomer for OE1, OE2, and OE3) and transferred onto polyvinylidene difluoride (0.45 μm, Millipore) for 1 h at 4°C under constant voltage (100 V) in 25 mM Tris, 192 mM Gly, 0.01% SDS, and 20% methanol. Membranes were blocked with 5% dry milk in Tris-buffered saline (10 mM Tris-Cl, 150 mM NaCl, pH 7.5) + Tween 20 (0.05% [v/v]). Primary antibodies were used at 1:1,000 (D1), 1:2,000 (OE1), or 1:20,000 (OE2, OE3, and CF1), and a 1:5,000 dilution of goat anti-rabbit horseradish peroxidase (Pierce Biotechnology) was used as the secondary antibody. Signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology).

SOD Activity Gels

Total soluble proteins were separated on a polyacrylamide gel (10% monomer) and analyzed in gel for SOD activity as described by Beauchamp and Fridovich (1971). FeSOD activity was identified by sensitivity to 20 μM H2O2 treatment prior to activity staining.

Nucleic Acids Analysis

Total Chlamydomonas RNA was prepared as described by Quinn and Merchant (1998).

Quantitative Real-Time PCR

Genomic DNA was removed from the total RNA preparation by treatment with RQ1 DNase (Promega) according to the manufacturer’s instructions. Complementary DNA, primed with oligo(dT), was generated with reverse transcriptase (Invitrogen), also according to the manufacturer’s instructions, and used in the amplification reaction directly after dilution. The amplification reaction was carried out with reagents from the iQ SYBR Green Supermix qPCR kit (Bio-Rad Laboratories). Each reaction contained the vendor’s master mix, 0.3 μM of each primer, and cDNA corresponding to 20 ng input RNA in the reverse transcriptase reaction. The reaction conditions for the Opticon 2 from MJ Research were: 95°C for 5 min, followed by cycles of 95°C for 10 s, 65°C for 30 s, 72°C for 30 s, up to a total of 40 cycles. The fluorescence was measured at each cycle at 72°C and 83°C. The 2-ΔΔCT method was used to analyze the database on the fluorescence at 83°C (Livak and Schmittgen, 2001). Melting curves were performed after the PCR reaction to assess the presence of a unique final product, and the product was analyzed by gel electrophoresis and sequenced from one reaction to verify that it represented the gene of interest. The data are presented as the fold change in mRNA abundance, normalized to an endogenous reference gene (CRP2), relative to the RNA sample from cells grown in 25 μM Mn (considered Mn replete). The results presented are the averages of technical triplicates but are representative of at least two independent experiments.

Measurement of Metal and Phosphorus Content

Cells were grown in the indicated metal concentration to stationary phase (>1 × 10^7 cells/mL) so that we could establish the minimal manganese requirement for growth of a Chlamydomonas culture. Cells (5 × 10^7) were collected for each measurement by centrifugation at 1,700g for 5 min. The cell paste was washed twice with 1 mL EDTA and once with deionized water (MilliQ, Millipore). The washed cell paste was then overlaid with nitric acid (MilliQ, Millipore). The volume of the cell paste was replaced by deionized water and treated the same way as the cell paste. Digested cell paste and blank were diluted with 9 mL deionized water for at least 48 h. To obtain a corresponding blank, the volume of the cell paste was replaced by deionized water prior to measurement. Total metal content was measured at the Interdisciplinary Center for Plasma Mass Spectrometry (University of California, Davis) by the standard addition method. Total phosphorus content was measured by inductively coupled plasma-atomic emission spectroscopy (detected limit 100 ppb) at the University of California, Los Angeles Molecular Instrumentation Center with reference to a standard solution of esterified phosphate.

MATERIALS AND METHODS

Growth Conditions

Chlamydomonas reinhardtii wild-type strain CC425 (Chlamydomonas culture collection, Duke University, NC) was maintained in the light (60–80 μmol m^-2 s^-1) in TAP medium supplemented with 100 μg/mL Arg (Harris, 1989). For Mn-deficiency experiments, cells in TAP medium (25 μM MnCl2) were transferred through two rounds of TAP medium without manganese supplement (1:500 dilution). We estimated a residual manganese content of between 0.02 to 0.1 μM from: (1) the calculated manganese content of the medium based on the measured manganese abundance in the high purity chemicals used for preparing stocks; (2) direct measurement of Mn content of the medium by inductively coupled plasma-mass spectrometry; and (3) the extrapolated Mn content of the medium based on measurement of Mn in the cell paste collected from cells grown without Mn supplementation. Fresh cultures were inoculated with these Mn-deficient cells (1:500 dilution) into medium containing the indicated concentration of manganese provided as MnCl2 chelated with EDTA. For Fe-deficiency experiments, replete cells (18–25 mL) were used to isolate chloroplasts (Rolland et al., 1997). The chloroplast fraction was resuspended in 10 mM sodium phosphate, pH 7.0, and assayed for purity by immunoblot.

Fluorescence Rise and Decay Kinetics

Room temperature fluorescence rise and decay kinetics were analyzed using a FluorCam 700MF (Photon Systems Instruments). Twenty-five micro-liters of mid-log phase liquid culture (2–4 × 10^7 cells/mL) was spotted onto a solid plastic surface and dark adapted for 10 min prior to measurement of the Kautsky effect in continuous red light at 150 μmol m^-2 s^-1 (Moseley et al., 2002).

Chloroplast Isolation

Cells of strain CC400, cw15 mt- (2 L) from mid-log phase cultures were collected by centrifugation (1,000 g, 10 min) and resuspended in 50 mL 0.3 μM sorbitol, 50 mM HEPES-KOH, pH 7.8, 2 mM EDTA, 5 mM MgCl2, 0.1% bovine serum albumin, 0.5% polyvinylpyrrolidone-40. The cells were lysed by passage of the suspension through a Yeda press (4.5 bar, 30 s). The lysed cells (25 mL) were used to isolate chloroplasts (Rolland et al., 1997). The chloroplast fraction was resuspended in 10 mM sodium phosphate, pH 7.0, and assayed for purity by immunoblot.

Protein Isolation

Chlamydomonas cultures were collected by centrifugation (1,000g, 5 min) and washed twice with 10 mM sodium phosphate, pH 7.0. The total protein fraction was further fractionated into soluble and membrane components as described in Howe and Merchant (1992). Protein concentration was determined as described in Lowry et al. (1951) against a bovine serum albumin standard.

Measurement of Metal and Phosphorus Content

Growth Conditions

Chlamydomonas cultures were collected by centrifugation (1,000g, 5 min) and washed twice with 10 mM sodium phosphate, pH 7.0. The total protein fraction was further fractionated into soluble and membrane components as described in Howe and Merchant (1992). Protein concentration was determined as described in Lowry et al. (1951) against a bovine serum albumin standard.
Supplemental Data

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

Supplemental Figure S1. Intracellular metal content in response to manganese depletion.

Supplemental Figure S2. Selective sensitivity of Mn-deficient cells to peroxides.

Supplemental Figure S3. MnSOD is up-regulated by Fe deficiency.

Supplemental Figure S4. Time course of changes in gene expression in response to Mn deficiency and resupply.

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