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A Metal-Accumulator Mutant of *Arabidopsis thaliana*

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A mutation designated *man1* (for manganese accumulator) was found to cause *Arabidopsis thaliana* seedlings to accumulate a range of metals. The *man1* mutation segregated as a single recessive locus located on chromosome 3. When grown on soil, mutant seedlings accumulated Mn (7.5 times greater than wild type), Cu (4.6 times greater than wild type), Zn (2.8 times greater than wild type), and Mg (1.8 times greater than wild type) in leaves. In addition to these metals, the *man1* mutant accumulated 2.7-fold more S in leaves, primarily in the oxidized form, than wild-type seedlings. Analysis of seedlings grown by hydroponic culture showed a similar accumulation of metals in leaves of *man1* mutants. Roots of *man1* mutants also accumulated metals, but unlike leaves they accumulated 10-fold more total Fe (symplasmic and apoplasmic combined) than wild-type roots. Roots of *man1* mutants possessed greater (from 1.8- to 20-fold) ferric-chelate reductase activity than wild-type seedlings, and this activity was not responsive to changes of Mn nutrition in either genotype. Taken together, these results suggest that the *man1* mutation disrupts the regulation of metal-ion uptake or homeostasis in Arabidopsis.

Ferric-chelate reductase activity is widespread among plant species and has been proposed to play a central role in regulating the uptake of Fe and other metals into plants (Bienfait and Lüttge, 1988; Welch et al., 1993). In soil, Fe exists predominantly in the oxidized Fe(III) state but is transported across root membranes as the Fe(II) ion (Chaney et al., 1972). Reduction of Fe(III) to Fe(II) is necessary for plant uptake and can be mediated by the ferric-chelate reductase present in plasma membranes of root cells. Many plant species increase the activity of ferric-chelate reductase in their roots when Fe is deficient as part of the so-called strategy I mechanism for increasing the availability of Fe (Bienfait et al., 1983; Buckhout et al., 1989; Welch et al., 1993; Guerinot and Yi, 1994). In pea (*Pisum sativum*) seedlings, the rate of Fe uptake by roots was found to be directly related to ferric-chelate reductase activity of root plasma membranes and did not appear to be limited by the transport of Fe(II) across membranes of root cells (Grusak et al., 1990b). These observations suggest that ferric-chelate reductase activity is the rate-limiting step in Fe acquisition and that its activity regulates the amount of Fe taken up by plants.

Although metals such as Cu, Zn, and Mn do not usually need to be reduced prior to uptake by roots, their accumulation in leaves is correlated with ferric-chelate reductase activity of roots. For instance, the pea mutant E107 has constitutively high ferric-chelate reductase activity in root plasma membranes and, in addition to excessive Fe concentrations, accumulates Mn, Ca, Mg, and K in shoots to higher concentrations than wild-type seedlings (Grusak et al., 1990a; Welch and LaRue, 1990). Wild-type pea seedlings suffering from Fe deficiency increase ferric-chelate reductase activity in roots and accumulate a range of cations in leaves (Welch et al., 1993). Coincident with increased ferric-chelate reductase activity in pea roots, the ability of roots to reduce Cu(II) and Mn(III) also increases (Norvell et al., 1993), with recent evidence indicating that the same enzyme of tomato (*Lycopersicon esculentum*) roots is responsible for both the reduction of ferric chelates and Cu(II) (Holden et al., 1995). Furthermore, Holden et al. (1995) suggested that the reduction of Cu(II) by tomato roots has little or no physiological relevance in plants growing in soil. Welch et al. (1993) speculated that ferric-chelate reductase has a general role in regulating cation uptake by plant roots. They suggested that in addition to reducing Fe(III), the reductase may regulate the activity of transporters, possibly ion channels, for other cations. In their model, Welch et al. (1993) suggested that ferric-chelate reductase reduces disulfide groups of the transporters, resulting in increased cation transport, whereas oxidation of the disulfide groups inactivates the transporters. By acting on transporters of cations, the ferric-chelate reductase activity might influence the uptake rate of cations without being directly involved in their reduction.

Analysis of mutants perturbed in cation uptake or ferric-chelate reductase activity can serve to clarify the putative roles of ferric-chelate reductase in plants. In this paper, I describe the characterization of an *Arabidopsis thaliana* mutant, *man1* (for manganese accumulator), which accumulates high concentrations of a number of cations and also S. In contrast to the E107 pea mutant described above, the *man1* mutant did not accumulate high Fe concentrations in leaves, even though it possessed high ferric-chelate reductase activity in roots.

MATERIALS AND METHODS

Isolation of Mutant

The mutant was produced in the ecotype Columbia using ethyl methanesulfonate as the mutagen. An M_2 population was screened for mineral nutrition mutants using x-ray fluorescence spectrometry as described previously

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Abbreviation: EDDHA, *N,N'*-ethylenebis[2-(2-hydroxyphenyl)-glycine].

(Delhaize et al., 1993). Single leaves were pressed onto paper discs and analyzed for a range of elements using x-ray fluorescence spectrometry. Several seedlings with abnormal count rates for Mn were identified and grown to produce seed. One seedling that showed an inherited abnormality was selected for further study. The mutant was backcrossed three times to wild-type Columbia before being used in physiological experiments.

Growth of Seedlings

Seedlings were grown on soil, on agar, by hydroponic culture, or fully immersed in nutrient solution using previously described methods (Delhaize and Randall, 1995), except that Fe was supplied as 20 μM Fe(III):EDDHA instead of 20 μM Fe(III):EDTA in some experiments. Solutions used for hydroponic culture contained 0.625 mM KNO_3, 0.25 mM $CaCl_2$, 0.25 mM $MgSO_4$, 0.25 mM KH_2PO_4, 20 μM $FeCl_3$, 20 μM Na_2EDTA (or 20 μM EDDHA), 22 μM H_3BO_3, 4 μM $MnCl_2$, 0.7 μM $ZnCl_2$, and 0.4 μM $CuCl_2$, all at a final pH of 6.0. To ensure that no nutrients unintentionally became limiting, solutions used in hydroponic cultures were replaced weekly during the initial 14 d of growth and then every 4 d thereafter. For seedlings grown fully immersed or on agar, nutrients other than Fe (including 2.9 mM Suc) were supplied 4-fold more concentrated than those used for hydroponic culture. Seedlings grown by hydroponic culture or on agar were grown under artificial lighting.

Concentrations of Elements in Plant Material

The concentrations of Mg, P, Cl, S, K, Ca, Mn, Fe, Cu, and Zn in bulked leaf tissue (minimum of 0.4 g required for complete elemental analysis) were determined by x-ray fluorescence spectrometry (Hutton and Norrish, 1977; Norrish and Hutton, 1977). Fully oxidized S (S^{6+}) and carbon-bonded S concentrations were analyzed in the same samples also by x-ray spectrometry (Pinkerton et al., 1989). In leaves and roots of plants grown by hydroponic culture, there was insufficient material for the analysis of all elements by x-ray fluorescence spectrometry. These samples were analyzed for Mg, P, K, Ca, Mn, Fe, and Zn after ashing and acid digestion. Root tissue was excised and washed in 0.2 mM $CaCl_2$ before analysis. The tissue was placed into preweighed disposable borosilicate tubes (10 × 75 mm) and dried overnight at 70°C. The tubes were reweighed to determine the dry weight, and then the samples were ashed at 530°C for 16 h. The ash was dissolved in a mixture consisting of 100 μL of concentrated HNO_3 and 100 μL of 30% (w/w) H_2O_2. The ash solution was heated for 2 h at 70°C, and then distilled water was added to a final volume of 3 mL. Samples were analyzed by flame atomic absorption spectroscopy (Perkin-Elmer Cetus) for Mg, K, Ca, Mn, Fe, and Zn, and P was analyzed using a colorimetric assay (Irving and McLaughlin, 1990). The digestion method could not be used for the analysis of S because of the loss of volatile forms, and Cu concentrations were too low to be analyzed. Mn concentrations in shoots of single plants were measured using an atomic absorption spectrophotometer equipped with a graphite furnace, because flame atomic absorption was too insensitive for the small amount of material available. Dried samples were digested with a mixture of concentrated nitric acid (200 μL) and perchloric acid (10 μL) at 70°C for 2 h and then heated to about 200°C to drive off the nitric acid. Digested samples were made up to 3 mL with water and analyzed by a standard additions method using an atomic absorption spectrophotometer equipped with a graphite furnace. Samples in the graphite furnace were ashed at 700°C and atomized at 2400°C, and $A_{403.1}$ was measured (Rothery, 1988). All glass and plasticware used for elemental determinations was washed in 20% (v/v) nitric acid and thoroughly rinsed with distilled water.

Ferric-Chelate Reductase Assay

Ferric-chelate reductase activity of Arabidopsis roots was assayed using methods based on those described by Welch et al. (1993). Excised roots were washed in 0.2 mM $CaCl_2$ solution for 5 min before being placed in 2 to 20 mL of assay solution (approximately 50 mg root tissue mL^{-1} assay solution). The assay solution comprised 0.2 mM $CaCl_2$, 5 mM Mes, 0.25 mM Na_2-bathophenanthrolinedisulfonic acid, and 0.1 mM Fe(III):EDTA at a pH of 5.5. The roots were placed on an orbital shaker (150 rpm) and incubated in darkness at 23°C for 0.5 to 2 h, depending on the ferric-chelate reductase activity of the roots. An assay solution without roots but treated the same as those with roots was used as a blank. At the end of the incubation, the A_{535} was determined. Preliminary experiments showed that the rate of reaction was constant for at least 2 h. At the end of the assay, roots were blotted dry and weighed. To test for the presence of reductants secreted by roots, excised roots were washed in 0.2 mM $CaCl_2$ solution for 5 min before being placed in 0.2 mM $CaCl_2$, 5 mM Mes, pH 5.5, and incubated for 1 h. At the end of the incubation, a subsample of the solution was reacted with the ferric-chelate reductase assay solution described above for 30 min, while the roots were incubated for a further 30 min in ferric-chelate reductase assay solution. At the end of the assays, the A_{535} was determined.

Genetic Crosses

To determine the genetics of *man1* mutants, seedlings from an F_2 population (derived from the third backcross of a *man1* mutant to wild type) were scored for the *man1* phenotype. Seedlings were grown on soil under fluorescent lighting (100–150 μmol photons m^{-2} s^{-1}; 16 h of light/8 h of dark) at 21°C for 14 d before being scored. Under these conditions, *man1* mutants were small, chlorotic plants segregating among larger, dark-green plants. Because the phenotype of *man1* seedlings varied under different conditions, *man1* and wild-type seedlings were grown alongside the segregating population to ensure that the phenotypes of the seedlings were correctly identified. A subsample of the segregating population was analyzed for shoot Mn concentration to determine whether that could be the cause

of the chlorosis. To determine the chromosomal location of *man1*, the mutant was crossed to Arabidopsis lines, which carried phenotypic mutations at known sites. In the F_2 generation seedlings that showed the *man1* phenotype (based on the presence of chlorosis) were selected and scored for the presence of the phenotypic markers.

RESULTS

Phenotype and Genetics of the *man1* Mutant

An Arabidopsis seedling with greater than normal Mn content in its leaves was identified in an M_2 population using a screening procedure based on x-ray fluorescence spectrometry described previously (Delhaize et al., 1993). Although Mn concentrations in leaves varied under different growth conditions, the mutant had greater leaf Mn concentration than wild type when grown on soil in a greenhouse (7.5-fold more than wild type, Table I), by hydroponic culture under artificial lighting (5.4-fold more than wild type, Table I), or on agar under artificial lighting (4.1-fold more than wild type: for 30-d-old seedlings grown on agar, *man1* had 650 ± 29 μg Mn/g dry weight, and wild type had 158 ± 6 μg Mn/g dry weight; means ± SE, n = 17). The mutant (backcrossed three times to wild type) was generally chlorotic when grown under artificial light on agar, on soil, or by hydroponic culture, but varied from pale-yellow to dark-green when grown on soil in a greenhouse. The reason for this variation in color is not known but may have been due to differences in light intensity or temperature. When grown on soil the mutant was smaller (Fig. 1) and flowered later than wild-type seedlings (*man1* mutants bolted 44 ± 1 d after planting, whereas wild-type bolted 36 ± 1 d after planting; means ± SE). The mutation *man1* was recessive and segregated as a single locus based on the chlorotic phenotype (a BC3F2 population segregated 343 wild type:102 chlorotic phenotypes; $\chi^2 = 0.97$ and P = 0.3–0.5 for a 3:1 ratio). Analysis of a subsample of the BC3F2 population for Mn concentrations in shoots showed

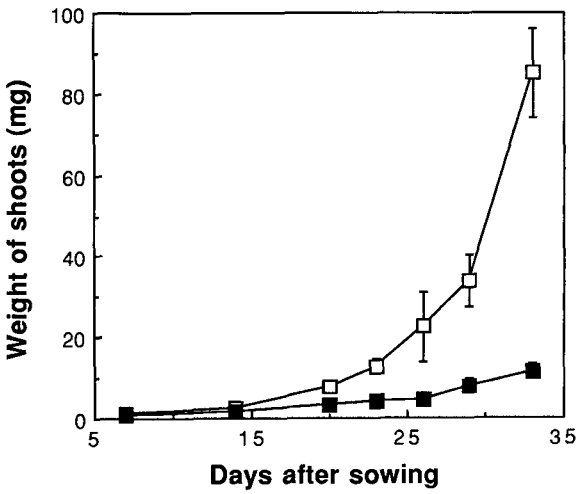

Figure 1. Shoot fresh weights of wild-type (□) and *man1* (■) seedlings grown on soil. The values are means of six seedlings, and error bars denote the SE.

that the chlorotic phenotype was associated with a high Mn concentration (seedlings with a chlorotic phenotype had 222 ± 16 [mean ± SE, n = 26; range 103–557] μg Mn/g dry weight, whereas seedlings with a wild-type phenotype had 20 ± 1 [mean ± SE, n = 46; range 12–39] μg Mn/g dry weight; control *man1* seedlings grown alongside had 149 ± 11 [mean ± SE, n = 10; range 117–213] μg Mn/g dry weight and control wild-type seedlings had 18 ± 4 [mean ± SE, n = 10; range 8–41] μg Mn/g dry weight). The *man1* locus was found to be located on the upper arm of chromosome 3 and was linked to the phenotypic marker *hy-2* (only one seedling showed the chlorotic *man1* phenotype among 85 homozygous *hy-2* seedlings in an F_2 population derived from a *hy-2* × *man1* cross for which the expected number for independent assortment of loci is approximately 21; P < 0.01).

Table I. *Concentrations of mineral nutrients in leaves, seed, and roots of wild-type and man1 mutants grown under various conditions*

Sample Type	Mg	P	S[a]	K	Ca[b]	Mn	Fe	Cu[c]	Zn
			% dry wt[d]				*μg/g dry wt*[d]		
Soil grown[e]									
Wild-type leaves	0.61 ± 0.05	0.67 ± 0.01	0.91 ± 0.02	3.56 ± 0.16	4.79 ± 0.13	47 ± 1	110 ± 8	14 ± 1	130 ± 5
man1 leaves	1.07 ± 0.01	0.65 ± 0.01	2.47 ± 0.19	3.49 ± 0.07	4.48 ± 0.18	351 ± 49	94 ± 6	65 ± 8	370 ± 59
Wild-type dry seed	0.27 ± 0.01	0.94 ± 0.01	0.90 ± 0.01	1.16 ± 0.01	0.60 ± 0.01	34 ± 2	103 ± 2	5 ± 1	60 ± 1
man1 dry seed	0.28 ± 0.01	0.92 ± 0.02	1.09 ± 0.01	1.11 ± 0.05	0.60 ± 0.01	54 ± 3	153 ± 9	13 ± 3	100 ± 6
Hydroponic culture[f]									
Wild-type leaves	1.15 ± 0.06	1.11 ± 0.06	n.d.[h]	1.05 ± 0.02	4.24 ± 0.25	113 ± 10	102 ± 13	n.d.	110 ± 7
man1 leaves	1.54 ± 0.01	0.97 ± 0.08	n.d.	0.99 ± 0.24	4.63 ± 0.09	613 ± 42	75 ± 5	n.d.	279 ± 36
Wild-type roots	0.37 ± 0.02	1.26 ± 0.02	n.d.	2.79 ± 0.27	n.d.	530 ± 90	370 ± 190	n.d.	200 ± 10
man1 roots	0.72 ± 0.21	1.49 ± 0.03	n.d.	2.39 ± 0.64	n.d.	5220 ± 330	4720 ± 1090	n.d.	1060 ± 230
Hydroponic culture[g]									
Wild-type leaves +Fe	0.83 ± 0.04	0.95 ± 0.02	n.d.	0.48 ± 0.13	3.04 ± 0.23	186 ± 7	71 ± 7	n.d.	54 ± 1
Wild-type leaves −Fe	1.05 ± 0.01	1.01 ± 0.02	n.d.	0.96 ± 0.03	2.95 ± 0.09	306 ± 34	30 ± 2	n.d.	83 ± 15

[a] S was not determined in ashed samples because of loss of volatile forms. [b] Roots were not analyzed for Ca because they were washed with $CaCl_2$ solution. [c] Cu was below the limits of detection for ashed samples. [d] Mean ± SE concentrations of three replicate samples. [e] Leaves were collected from 23-d-old plants grown in a greenhouse. [f] Fe was supplied as 20 μM Fe:EDDHA, and plants were harvested after 28 d of growth. [g] Wild-type *Arabidopsis* was grown with full nutrients, including 20 μM Fe:EDDHA for 28 d before treatments were imposed. Controls (+Fe) continued to receive 20 μM Fe, whereas Fe was omitted from the nutrient solution of the −Fe treatment for 6 d of additional growth. [h] n.d., Not determined.

Elemental Concentrations in Various Tissues of *man1* and Wild-Type Seedlings

In addition to having high Mn concentrations, leaves of *man1* seedlings grown on soil also had elevated concentrations of Cu (4.6-fold), Zn (2.8-fold), Mg (1.8-fold), and S (2.7-fold) (Table I) compared with wild-type seedlings. By contrast, Fe concentrations in leaves of *man1* mutants were similar to wild-type seedlings. The majority of the S was fully oxidized, probably as the sulfate ion, and the carbon-bonded S concentration was also elevated in the mutant (Table II). The elemental composition of dry seed of the *man1* mutant compared with wild type generally did not reflect the marked differences found in leaves (Table I). Seedlings of *man1* grown by hydroponic culture also showed elevated concentrations of Mn, Mg, and Zn (Table I; Cu and S could not be analyzed in these seedlings) compared with wild type, whereas roots accumulated very high concentrations of total Fe, Zn, and Mn. The high Fe concentration of *man1* roots, consisting of both apoplasmic and symplasmic Fe, was particularly interesting in view of the near-normal concentration of Fe in leaves.

The *man1* Mutant Shows High Constitutive Ferric-Chelate Reductase Activity in Roots

The ferric-chelate reductase activity in roots of *man1* seedlings was from 5- to 10-fold greater than wild-type seedlings when grown with an adequate supply of Fe as Fe:EDTA (Fig. 2). For both wild-type and *man1* seedlings, secreted reductants accounted for less than 7% (6.9 ± 1.0% for *man1* and 6.2 ± 0.8% for wild type; means ± SE) of the total ferric-chelate reductase activity of roots, with the majority of the activity being attributable to a membrane-bound ferric-chelate reductase. When seedlings grown with Fe for 20 d in hydroponic culture were transferred to Fe-free nutrient solution, they showed Fe-deficiency symptoms after 4 d of additional growth; after a total of 10 d without Fe, ferric-chelate reductase activity of wild-type roots increased about 4-fold, whereas the ferric-chelate reductase of the *man1* mutant remained unchanged (Fig. 2A). Manganese concentrations in leaves of seedlings grown with a high Fe supply were approximately 8-fold greater in *man1* mutants than in wild-type seedlings, reflecting the relative Mn concentrations of seedlings grown on soil. Fe-deficient wild-type seedlings had about 3-fold greater Mn concentrations in leaves than Fe-sufficient seedlings, but this was still considerably less than Mn concentrations in leaves of *man1* mutants. The increase in Mn concentrations in leaves of wild-type seedlings that were Fe deficient may have been due to the absence of EDTA in the minus-Fe treatment. EDTA is a nonspecific chelator of metal ions, and the concentration of available Mn in solution is likely to have been greater in the minus-Fe treatment, leading to greater uptake by the plants. To overcome this potential problem the more specific Fe chelator, EDDHA, was used in different experiments to assess the effect of Fe deficiency on Zn and Mn concentrations. Wild-type seedlings that were Fe deficient accumulated more K (2-fold), Zn (1.5-fold), and Mn (1.6-fold) in leaves than the Fe-sufficient controls grown with Fe:EDDHA (Table I). Ferric-chelate reductase activity of *man1* seedlings varied

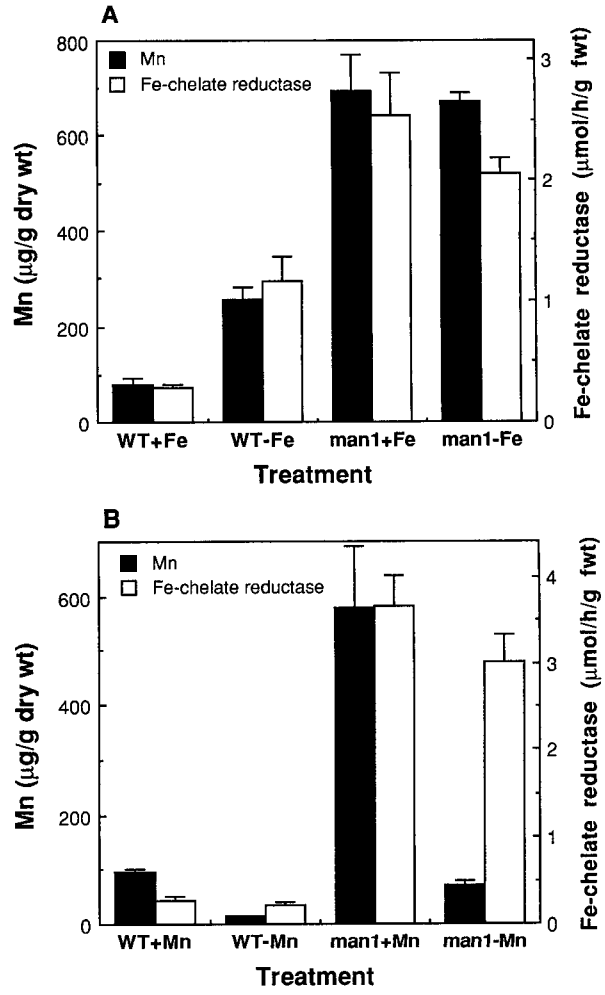

Figure 2. A, Effect of Fe supply on ferric-chelate reductase activities of roots and leaf Mn concentrations of wild-type and *man1* seedlings. All seedlings were grown with full nutrients, which included 20 μM Fe:EDTA, for 20 d and then were transferred to solutions that contained either no Fe (−Fe treatment) or 20 μM Fe (+Fe treatment) for a further 10 d of growth. Data are means ± SE of three replicate samples. B, Effect of Mn supply on root ferric-chelate reductase activity and leaf Mn concentration of wild-type seedlings and *man1* mutants. Seedlings were grown for 32 d in nutrient solution that either contained all nutrients (including 20 μM Fe:EDTA and 4 μM Mn) or all nutrients minus Mn. Data are means ± SE of three replicate samples. fwt, Fresh weight.

Table II. *Concentrations of fully oxidized (S^{6+}) and carbon-bonded S in leaves of wild-type and man1 seedlings grown on soil for 23 d*

Genotype	S^{6+} (Oxidized S)	Carbon-Bonded S
	% dry wt[a]	
Wild type	0.57 ± 0.01	0.35 ± 0.02
man1	1.83 ± 0.18	0.62 ± 0.03

[a] Mean ± SE concentrations of leaves taken from three replicate pots.

between experiments when plants were grown under different conditions, but was consistently greater (from 1.8- to 20-fold) than the activity of wild-type seedlings whether Fe was supplied in the growth culture as Fe:EDDHA or Fe:EDTA (Table III).

Mn Deficiency Does Not Induce Ferric-Chelate Reductase Activity in Wild-Type Seedlings

In several plant species, Cu deficiency and, to a lesser extent, Zn deficiency induce ferric-chelate reductase activity in roots (Jolley and Brown, 1991; Norvell et al., 1993; Welch et al., 1993). It was of interest to determine whether Mn deficiency in wild-type Arabidopsis seedlings also induced ferric-chelate reductase activity in roots. It was hypothesized that Arabidopsis seedlings normally regulate Mn uptake through ferric-chelate reductase activity and that in the mutant this regulation is lost, resulting in constitutive expression of ferric-chelate reductase activity and continual Mn uptake beyond requirements. Wild-type seedlings grown in solution culture for 32 d without Mn showed symptoms of Mn deficiency and had leaf Mn concentrations (Fig. 2B, 13.0 ± 1.0 μg/g dry weight) that are considered to be deficient for many plant species (Reuter and Robinson, 1986). Ferric-chelate reductase activity did not increase in roots of these Mn-deficient seedlings (Fig. 2B), suggesting that the Mn status of Arabidopsis seedlings does not influence ferric-chelate reductase activity in roots. Although the *man1* mutant grown in the absence of Mn had lower Mn concentrations in leaves than *man1* mutants grown with Mn (Fig. 2B), these seedlings would not be considered to be Mn deficient. Regardless of the Mn supply, *man1* mutants had approximately 10-fold greater ferric-chelate reductase activity in roots than wild-type seedlings.

Table III. *Relative activity of ferric-chelate reductase in roots of man1 mutant compared with wild-type roots in seedlings grown under a range of conditions*

Growth Conditions and Age of Plants	Relative Ferric-Chelate Reductase Activity
	× *greater activity in man1 than wild-type roots*
Hydroponic culture[a]	
19 d	22.7
24 d	2.4
31 d	9.2
38 d	1.8
Agar grown[a]	
17 d	2.7
22 d	1.9
25 d	8.8
30 d	4.7
Immersed culture[b]	
11 d	13.3
15 d	11.8

[a] Fe supplied as 20 μM Fe:EDDHA. [b] Fe supplied as 20 μM Fe:EDTA. Seedlings were grown immersed in nutrient solution as described previously (Delhaize and Randall, 1995), and solutions were changed after 4 d of initial growth and every 2 d thereafter.

DISCUSSION

In this paper I describe a mutation of Arabidopsis that results in the accumulation of a range of metals and S in roots and leaves. Arabidopsis seedlings that are homozygous for the *man1* mutation share some characteristics with the pea E107 mutant. However, although both mutations cause high ferric-chelate reductase activity and accumulation of total Fe in roots, the E107 mutant accumulates excessive Fe concentrations in shoots (Grusak et al., 1990a; Welch and LaRue, 1990), whereas *man1* mutants have near-normal Fe concentrations in shoots (Table I). Both mutants accumulate a range of cations in shoots, but these cations differ between the two mutants. The pea mutant accumulates primarily Fe, Mn, Mg, and K (Welch and LaRue, 1990), whereas the Arabidopsis mutant accumulates Mn, Zn, Cu, and Mg. Despite the differences observed between the two systems, the *man1* mutant provides supportive evidence that ferric-chelate reductase activity correlates with the uptake of Fe and other cations. Further evidence of this relationship is provided by the increased accumulation of K, Zn, and Mn in the leaves of Fe-deficient wild-type seedlings (Table I). The site of Fe accumulation in *man1* roots is not known and could either be apoplasmic (occurring within cell walls or on the root surface) or symplasmic. In either case, the observation that *man1* roots have high total concentrations of Mg, Zn, Mn, and Fe, but Fe is the only metal found in near-normal concentrations in leaves, suggests that either the uptake of external Fe or the translocation of internal Fe differs from that of the other metals.

Although we found that *man1* seedlings have elevated ferric-chelate reductase activity in roots compared with wild-type seedlings, this activity varied between experiments (Table III) and may be subject to the developmental stage of the plant and growth conditions. For example, Grusak (1995) showed that ferric-chelate reductase of pea roots varies over a 10-fold range throughout the life cycle of peas continuously supplied with Fe. It remains to be shown that the consistent accumulation of divalent cations can be attributed solely to the ferric-chelate reductase activity. It is possible that the differences in ferric-chelate reductase activity are associated with differences in growth rates between wild-type and mutant seedlings (Fig. 1) and not due to the mutation per se. Moog et al. (1995) suggested that, since the elongation and maturation zones of roots are the major sites of Fe reduction, large root systems consisting of a greater proportion of older root than small root systems may effectively reduce the activity of ferric-chelate reductase when expressed on a fresh weight basis. Despite this possible confounding factor, we found that *man1* seedlings do not increase their ferric-chelate reductase activity when Fe deficient, suggesting that the enzyme is either constitutively expressed or unable to be induced by Fe deficiency.

Although the *man1* mutant "hyperaccumulated" several metals compared with wild-type Arabidopsis plants, it does not fit into the category of plants defined as hyperaccumulators. Baker and Brooks (1989) defined Mn hyperaccumulators as plants able to accumulate Mn in shoots to

concentrations in excess of 10,000 $\mu g/g$ dry weight. This Mn concentration is more than 10-fold greater than that found in the leaves of the *man1* mutant (Table I). Furthermore, the accumulation of Mn on a per plant basis is similar for both wild-type and *man1* seedlings. Table I shows that leaves of *man1* accumulate approximately 10-fold greater Mn concentrations than wild type, but this was offset by the smaller size of *man1* seedlings (Fig. 1): 35-d-old *man1* shoots were about 11% of the fresh weight of wild-type shoots.

In addition to metals, *man1* mutants accumulated high concentrations of S in leaves compared with wild-type seedlings (Table I). This contrasts with the E107 mutant of pea, which does not overaccumulate S under a variety of growth conditions despite showing other similarities to the *man1* mutant (Welch and LaRue, 1990). The bulk of the S in leaves was fully oxidized and was probably in the form of sulfate (Table II). A proportion of the S accumulated by the mutant could be due to the plant balancing its excess cation uptake with sulfate ions. However, the amounts of sulfate accumulated were in excess of that required to balance the excess cations accumulated by *man1* mutants. Alternatively, it is possible that the mutation affects a gene involved in sulfate uptake or metabolism and that uptake of cations is increased as a response by the plant to balance its excessive anion uptake with cations. However, the cations that are accumulated, other than Mg, are micronutrients and do not contribute greatly to balancing the excess anions. Carbon-bonded S concentrations are also greater (approximately 2-fold) in *man1* mutants compared with wild-type seedlings (Table II). A proportion of this S fraction may be due to increased synthesis of metallothioneins and phytochelatins. Both of these classes of compounds contain a large proportion of Cys, and phytochelatin synthesis is induced in plants by exposure to excess metal ions (Robinson et al., 1993), whereas metallothionein mRNAs are induced either by excess Cu (Zhou and Goldsbrough, 1994) or by Fe deficiency (Robinson et al., 1993). The induction of metallothionein mRNA by Fe deficiency is thought to be due to increases in intracellular Cu as a result of increased ferric-chelate reductase activity of roots. The accumulation of metal ions in leaves of *man1* mutants is likely to have induced the synthesis of these compounds.

The accumulation of metals and S in leaves and roots of *man1* seedlings was not reflected in the elemental composition of seed (Table I). This indicates that the translocation of mineral nutrients to developing seed is under different regulation than that to the remainder of the shoot and that the concentration of elements in the seed does not simply reflect concentrations in the leaves. This is consistent with the observation of Grusak (1994) that the pea mutant E107, which can accumulate up to 50-fold greater Fe in its leaves compared with wild-type plants, does not accumulate Fe in the developing ovule.

The chlorotic phenotype and excessive accumulation of Cu by *man1* mutants are also characteristics of a recently described mutant of Arabidopsis, *cup1*. van Vliet et al. (1995) speculated that the excessive accumulation of Cu in *cup1* may be associated with a primary defect in Fe metabolism, resulting in a functional Fe deficiency and constitutively high Fe-chelate reductase activity in roots. Despite these similarities, the *cup1* locus is located on chromosome 1, whereas the *man1* locus is on chromosome 3, indicating that these are mutations of different genes. Further analysis of these mutants and the construction of *cup1/man1* double mutants should shed further light on the functions of the wild-type genes and their interactions. The pea mutant E107 is thought to be defective in a gene involved in Fe homeostasis, resulting in uncontrolled uptake of Fe and other cations through constitutively high ferric-chelate reductase activity (Grusak et al., 1990a). Similarly, the *man1* mutation may also be interpreted as affecting a regulatory gene for Fe homeostasis, although in this case the mutation would somehow result in accumulation of total Fe into roots but not into leaves. Further work on the *man1* mutant will be needed to clarify whether the mutation affects a regulatory gene for the uptake of a range of divalent cations possibly acting through ferric-chelate reductase activity, or whether the excessive accumulation of these cations is due to pleiotropic effects of a mutation affecting a different function.

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