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Induction of the Root Cell Plasma Membrane Ferric Reductase[1]

An Exclusive Role for Fe and Cu

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Induction of ferric reductase activity in dicots and nongrass monocots is a well-recognized response to Fe deficiency. Recent evidence has shown that Cu deficiency also induces plasma membrane Fe reduction. In this study we investigated whether other nutrient deficiencies could also induce ferric reductase activity in roots of pea (*Pisum sativum* L. cv Sparkle) seedlings. Of the nutrient deficiencies tested (K, Mg, Ca, Mn, Zn, Fe, and Cu), only Cu and Fe deficiencies elicited a response. Cu deficiency induced an activity intermediate between Fe-deficient and control plant activities. To ascertain whether the same reductase is induced by Fe and Cu deficiency, concentration- and pH-dependent kinetics of root ferric reduction were compared in plants grown under control, −Fe, and −Cu conditions. Additionally, rhizosphere acidification, another process induced by Fe deficiency, was quantified in pea seedlings grown under the three regimes. Control, Fe-deficient, and Cu-deficient plants exhibited no major differences in pH optima or K_m for the kinetics of ferric reduction. However, the V_{max} for ferric reduction was dramatically influenced by plant nutrient status, increasing 16- to 38-fold under Fe deficiency and 1.5- to 4-fold under Cu deficiency, compared with that of control plants. These results are consistent with a model in which varying amounts of the same enzyme are deployed on the plasma membrane in response to plant Fe or Cu status. Rhizosphere acidification rates in the Cu-deficient plants were similarly intermediate between those of the control and Fe-deficient plants. These results suggest that Cu deficiency induces the same responses induced by Fe deficiency in peas.

Plant Fe deficiency is a widespread agricultural problem, especially in alkaline soils where Fe, although abundant, is often insoluble. Ferric (III) Fe, the predominant oxidation state in well-aerated soils, becomes available through the pH-dependent dissolution of ferric hydroxides. Total soluble Fe, which consists of free Fe^{3+} and Fe^{2+} ions and their complexes, may be present in concentrations as low as 10^{-11} M in aerobic soils (Lindsay and Schwab, 1982). This level of soluble soil Fe is much lower than that required for optimal plant growth (Römheld and Marschner, 1986).

Plants exhibit distinct strategies to assimilate Fe from the environment. The grasses (Poaceae) release Fe(III)-chelating compounds, or phytosiderophores, that solubilize ferric Fe in the rhizosphere and are absorbed at the root surface as Fe(III)-chelate complexes (Römheld and Marschner, 1986; Chaney and Bell, 1987; Bienfait, 1988). The dicots and the nongrass monocots use a two-step mechanism of Fe uptake in which Fe is first reduced and subsequently absorbed as Fe^{2+} (Chaney et al., 1972). The requirement for Fe reduction likely results from the extremely low solubility of Fe(III) in the soil solution, particularly under alkaline conditions. In contrast, Fe(II) exhibits much greater solubility and is readily generated under mild reducing conditions. Fe-deficient plants induce a plasma membrane-bound ferric reductase that transfers electrons from intracellular NADH (Buckhout et al., 1989) or NADPH (Sijmons et al., 1984) to Fe(III)-chelates in the rhizosphere. Ferrous ion (Fe^{2+}) is subsequently released from the chelate and is believed to be transported into the cytoplasm via a separate transport protein (Kochian, 1991; Fox et al., 1996). Root-induced acidification of the rhizosphere, also stimulated by Fe deficiency, is a second strategy used by these plants to enhance solubilization of Fe^{3+} from Fe hydroxides (Venkat Raju and Marschner, 1972; Brown and Jones, 1974).

Induction of ferric reductase is a well-accepted response specific to Fe deficiency; however, recent evidence indicates that Cu deficiency also induces the plasma membrane ferric reductase in pea (Welch et al., 1993). Additionally, Jolley and Brown (1991) have reported that Zn deficiency may induce ferric reductase activity in some species of bean. In this study we investigated whether the status for mineral nutrients in addition to Fe and Cu also induces the ferric reductase in roots of *Pisum sativum* L. cv Sparkle. We tested a variety of macronutrient and micronutrient deficiencies for their ability to induce ferric reductase activity. We also conducted experiments to determine whether the same reductase is induced by both Fe and Cu deficiency. To accomplish this we compared the concentration-dependent and pH-dependent kinetics of ferric reduction in plants

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Abbreviations: BPDS, bathophenanthrolinedisulfonic acid; ICP-ES, inductively coupled argon plasma-emission spectrometry; ICP-MS, inductively coupled argon plasma-MS.

grown under control, −Fe, and −Cu conditions. Finally, we measured rhizosphere acidification in plants grown under the three regimes to determine whether this second response induced by Fe deficiency is also induced under Cu deficiency.

MATERIALS AND METHODS

Plant Material and Culture

Pea (*Pisum sativum* L. cv Sparkle) seeds were allowed to imbibe (d 0) overnight in aerated distilled water. Seeds were then placed between sheets of moistened filter paper in glass Petri dishes and germinated in the dark at 20°C. On d 3 seedling radicals were inserted through holes in black polyethylene seedling cups. The seedling cups were inserted into the covers of black plastic pots containing 2 L (one plant per pot) or 5 L (five to six plants per pot; ±Cu treatments only) of nutrient solution. The polyethylene seedling cups were filled with black polyethylene beads to prevent light exposure of the nutrient solution. In general, pea seedlings were grown in a modified Johnson's nutrient solution containing the following macronutrients in mM: KNO_3, 1.2; $Ca(NO_3)_2$, 0.8; $NH_4H_2PO_4$, 0.1; $MgSO_4$, 0.2, and the following micronutrients in μM: KCl, 50; H_3BO_3, 12.5, $MnSO_4$, 1; $ZnSO_4$, 1; $CuSO_4$, 0.4; Na_2MoO_4, 0.1; $NiSO_4$, 0.1. The solutions were supplemented with 1 mM Mes buffer, adjusted to pH 5.5 with KOH or $Ca(OH)_2$ (for ±K experiments) and either 10 μM Fe-EDDHA (Fe-*N,N'*-ethylenebis[2-(2-hydroxyphenyl)-glycine]; in ±K, ±Mg, ±Ca, ±Mn, or ±Cu experiments) or 15 μM Fe-EDTA (in ±Zn experiments). Seedlings were transferred to fresh nutrient solution on d 10, 13, and 16 and every 3 d thereafter until the end of the experiment. Plants were grown in a controlled-environment growth chamber with a 16-h/20°C day and 8-h/15°C night regime and photon flux density of 350 μmol m^{-2} s^{-1}.

Growth of Nutrient-Deficient Plants

Macronutrient- and micronutrient-deficient plants were grown by withholding the nutrient of interest from the growth solution. Macronutrients (K, Mg, or Ca) were withheld 10 d after seedling germination. Micronutrients (Cu, Zn, or Mn) were withheld 3 d after germination.

Zn-deficient plants were grown using a chelate-buffer technique in which an excess of free EDTA buffered several metal activities (Fe, Zn, Mn, Cu, and Ni) simultaneously. The growth solution consisted of the macronutrients described above and ±3 μM Zn-EDTA, ±15 μM Fe-EDTA, 0.5 μM Mn-EDTA, 3 μM Cu-EDTA, 0.1 μM Ni-EDTA, 20 μM free EDTA, 40 μM KCl, 12.5 μM H_3BO_3, and 0.1 μM Na_2MoO_4. Control and Fe-deficient plants were also grown using this chelate-buffer system.

Cu-deficient plants were grown by removing the cotyledons 9 d after germination, using ultrapure macronutrients in the growth solution (1 mM KNO_3, 0.8 mM $Ca[NO_3]_2$, 0.2 mM NH_4NO_3, 0.2 mM KH_2PO_4, and 0.2 mM $MgSO_4$), and adding 2 μM Na_2-EDTA to the growth solution 7 d after seedling germination to minimize Cu contamination via chelation by free EDTA.

Ferric Reductase Assays

Root-associated ferric reduction was measured using a spectrophotometer (DU 640, Beckman). Intact root systems were suspended in 200 mL of an aerated solution of 0.2 mM $CaSO_4$, 5 mM Mes (pH 5.5), 100 μM Fe-Na-EDTA, and 300 μM Na_2BPDS. For the ±Zn-grown plants, 5 μM free K_2-EDTA was also added to the assay solutions to scavenge contaminating Zn. The assay was conducted under low-light conditions and was terminated after 30 min by removing the roots from the assay solution. A_{535} values of aliquots of the assay solution were measured, and the molar concentration of the Fe(II)-$BPDS_3$ complex was calculated using the extinction coefficient 22.14 mM^{-1} cm^{-1}. The fresh weight of the intact root system was obtained nondestructively using an Archimedes' volume-displacement technique, which permitted the same plants to be monitored on consecutive days (Lang and Thorpe, 1989), with the exception of the ±Cu-grown plants, in which two replicates were assayed and harvested each day. Statistical calculations were performed using a Stat-Graphics analysis of variance (Manugistic, Rockville, MD).

Concentration-Dependent Kinetics Studies

Concentration-dependent ferric reductase activity in control, Fe-deficient, and Cu-deficient seedlings was determined using intact root systems of 18-d-old plants. The assay solutions consisted of 0.2 mM $CaSO_4$, 10 mM buffer(Homopipes[homopiperazine-*N,N'*-bis-2-(ethanesulfonic acid)], Mes, or Hepes), 400 μM Na_2BPDS, Fe-Na-EDTA (200 to 1400 μM), 5% excess K_2-EDTA to minimize nonspecific reduction, and varying concentrations of K_2SO_4 to adjust the total monovalent cation concentration to a constant value at all Fe-EDTA levels. To minimize variability, the same plants for each treatment were tested at all seven concentration levels. Control and Cu-deficient root systems were assayed in a 140-mL sample volume for 20 min. Fe-deficient root systems were assayed in a 195-mL sample volume for 10 min. Depletion of the substrate over the course of the assay was minimal and never exceeded 18%. Plants exhibited a small, linear decrease in ferric reductase activity over the course of an experiment. The change in activity ranged between 0.01 and 0.23% min^{-1} but was typically 0.1% min^{-1}. To correct reductase rates for this decrease, an assay using the 400 μM Fe level was performed at both the beginning and the end of the experiment. The proportional decrease in rate was used to adjust the rates at the other concentrations. Values for the kinetic parameters K_m and V_{max} were calculated using Hanes-Woolf transformations of the data. Statistical tests on the data were performed using analysis of variance (Minitab, State College, PA). Concentration-dependent kinetics experiments were performed at pH 4.5, 5.5, and 7.5.

pH Dependence of Ferric Reductase Activity

The pH dependence of ferric reductase activity in Cu-deficient, Fe-deficient, and control seedlings was also compared. Ferric reductase activity was measured at five different pH levels using Homopipes-KOH (homopiperazine-*N,N'*-bis-

2-[ethanesulfonic acid]-KOH) (pH 4.5), Mes-KOH (pH 5.5, 6.0, and 6.5), and Hepes-KOH (pH 7.5). Assays were conducted as described in "Concentration-Dependent Kinetics Studies" using 400 μM Fe-Na-EDTA, a concentration chosen because it was close to the K_m at pH 5.5. The pH 5.5 level was repeated at the end of the experiment to determine the small attenuation of reductase rate with time.

Tissue Mineral Analysis

Young and old leaf tissue samples from Mg- and K-deficient plants and their corresponding controls were dry-ashed at 550°C for 24 h. The samples were subsequently digested with concentrated HNO_3 at 120°C to dryness, dry-ashed again for 24 h, and resuspended in 3 N HNO_3. Digestates were analyzed for K, Mg, Ca, Zn, Mn, Cu, and Fe via simultaneous ICP-ES (ARL model no. 34,000, Thermo-Jarrell Ashe, Franklin, MA). Tissue from the Ca-, Zn-, Mn-, and Cu-deficient plants and corresponding controls was wet-digested in concentrated HNO_3 overnight at 100°C and then further digested with HNO_3/$HClO_4$ at 200°C. Digestates were resuspended in 5% HNO_3 and analyzed for K, Mg, Ca, Zn, Mn, Cu, and Fe via ICP-ES (Mn- and Ca-deficient plants were analyzed using an ICAP-61, radial plasma, Thermo Jarrell-Ashe; Zn- and Cu-deficient plants were analyzed via ICAP 61E Trace Analyzer, axial plasma, Thermo-Jarrell Ashe). Tissue samples from control and Cu-deficient plants were analyzed for Cu by ICP-MS (ELAN 500 ICP-mass spectrometer, Perkin Elmer).

Rhizosphere Acidification Experiments

Intact root systems were incubated with stirring in a beaker containing 250 mL of unbuffered nutrient solution (without Mes or KH_2PO_4). The roots were separated from the stir bar with a polypropylene screen. A pH electrode with a chart recorder output was placed in the stirring solution. The change in solution pH was determined over the initial 2 min following plant insertion into the assay solution. The root systems were removed from the solution, blotted dry, and weighed. The buffering capacity of the solution was determined by titrating with a known amount of KOH, and the corresponding change in pH was recorded. Using these values, we calculated the proton efflux in μmol H^+ (g fresh weight)$^{-1}$ h^{-1}.

RESULTS

Tissue analysis from control and nutrient-deficient pea seedlings performed using ICP-ES and ICP-MS indicated that each mineral deficiency of interest was successfully achieved (Table I). The plant status for mineral nutrients other than the target nutrient (K, Ca, Mg, Mn, Zn, Cu, and Fe) was unaffected by the treatments in mature shoot tissues. Macronutrient- and micronutrient-deficient (K, Mg, Ca, Mn, Zn, Cu, or Fe) pea seedlings exhibited elevated ferric reductase activity only in response to Fe or Cu deficiency (Figs. 1 and 2).

We used excess chelate (EDTA) to buffer the Zn activity of control and −Zn growth solutions to investigate the effect of Zn status on reductase activity (Fig. 2B). Both control and Zn-deficient seedlings exhibited slightly elevated reductase activity relative to control levels of seedlings grown in nonchelate-buffer systems (+Mg, +K, +Ca, +Mn, and +Cu). However, both of these rates were considerably lower than those exhibited by Fe-deficient plants grown in an analogous chelate-buffer system (0.1–0.4 μmol (g fresh weight)$^{-1}$ h^{-1} for ±Zn-grown plants versus 1.2 ± 0.06 μmol (g fresh weight)$^{-1}$ h^{-1} for −Fe-grown plants). We attribute the higher basal level of reductase activity to the lower overall metal activities maintained by the chelate-buffer system. Also noteworthy is a transient increase in ferric reductase induction in the +Zn-grown seedlings on d 23. Older plants from some of the other growth regimes (−Mn and controls) also exhibited transient increases in reductase activity (data not shown). These peaks in activity coincided with the onset of flowering and corroborate recent evidence of a developmental induction of reductase activity during flowering and subsequent seed-pod fill (Grusak, 1995).

The pH dependence of root ferric reductase activity in control, Cu-deficient, and Fe-deficient pea seedlings is depicted in Figure 3. In all three treatments, maximal reductase activity occurred at pH 5.5 to 6.0. The decrease in rate observed at pH 7.5 likely reflects a combination of enzyme

Table I. *Effect of specific mineral nutrient deficiencies on leaf tissue mineral concentrations*

ICP-ES and ICP-MS (for Cu) analysis of leaf tissue shows that deficiencies were achieved for the specific mineral nutrient withheld in each experiment. Tissue was harvested on d 19 (±Cu), d 21 (−Ca), d 23 (−Mg and −K), d 25 (+Mg, +K, and ±Zn), d 29 (−Mn), and d 31 (+Mn and +Ca). The values are means with SE values in parentheses. For +Mg and +K, n = 4; for −K and −Mg, n = 5; for −Ca, n = 6; for +Ca and ±Mn, n = 7; for +Zn, n = 9; for −Zn, n = 10; and for ±Cu, n = 2.

Nutrient Withheld	Control Treatment		Deficient Treatment	
	Young leaves	Old leaves	Young leaves	Old leaves
	μg (g dry wt)$^{-1}$			
K	21,300 (6200)	14,700 (2740)	5940 (1360)	3580 (570)
Mg	3100 (220)	3010 (780)	361 (50)	1780 (570)
Ca	5500 (694)	63,000 (5120)	84 (3)	9030 (1930)
Mn	33 (2.5)	187 (16)	9 (1.0)	20 (1.5)
Zn	34 (0.6)	47 (1.7)	7 (0.7)	9 (0.8)
Cu	3.5 (0.24)	1.9 (0.15)	0.24 (0.01)	0.39 (0.03)

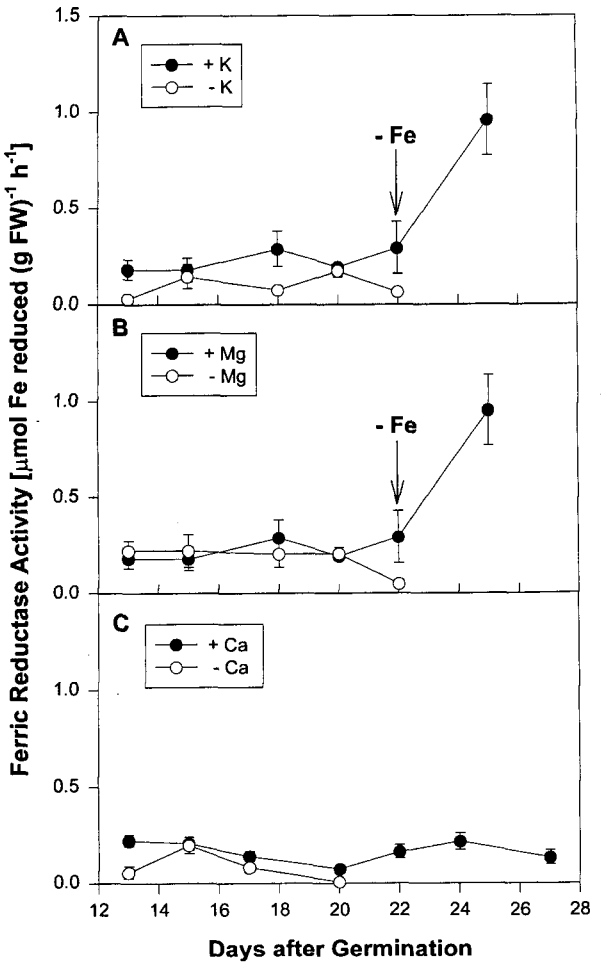

Figure 1. The influence of various macronutrient deficiencies (−K, −Mg, and −Ca) on ferric reductase activity. Nutrient-sufficient and K-, Mg-, and Ca-deficient seedlings exhibited no differences in ferric reductase activity. Control seedlings grown previously on full-nutrient media were subjected to −Fe conditions on d 22 and exhibited induced ferric reductase activity by d 25. Pea seedlings were grown in nutrient solutions from which either K, Mg, or Ca was withheld starting 10 d after germination. Ferric reductase activity was measured spectrophotometrically by suspending intact roots in an aerated solution of 0.2 mM $CaSO_4$, 5 mM Mes (pH 5.5), 100 μM Fe-EDTA, and 300 μM BPDS. The same intact plants were used sequentially throughout the course of the experiment. Root weights were measured in vivo via volume displacement. Visible deficiency symptoms were first observed on d 17 (−Ca: loss of turgor, dimpled leaves, stunting, and chlorotic spots in the younger leaves) and d 22 (−Mg: necrosis in the interveinal areas of old and mature leaves; −K: necrotic spots and tip scorch in the old and mature leaves). Values are means expressed as μmol (g fresh weight)$^{-1}$ h^{-1}. For +Mg and +K, $n = 4$; for −K and −Mg, $n = 5$; for −Ca, $n = 6$; and for +Ca, $n = 7$. Error bars represent SE. FW, Fresh weight.

properties and partial instability of the Fe-EDTA substrate at high pH. Although some precipitation of ferric hydroxides may have occurred, the reaction times used were short, and there was no evidence of colloid formation. Thus, we believe the decline in reductase activity was real and not caused by substrate limitation.

Figure 4 depicts the concentration-dependent kinetics of ferric reduction at pH 5.5 in Cu-deficient, Fe-deficient, and control seedlings. The concentration-dependent kinetics of ferric reduction were also performed at pH 4.5 and 7.5

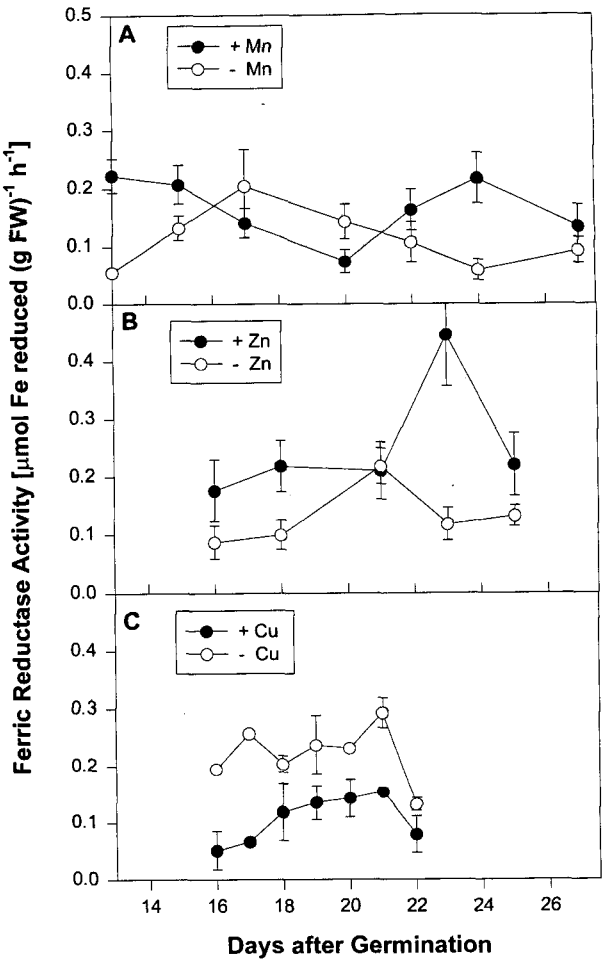

Figure 2. The influence of various micronutrient deficiencies (−Cu, −Mn, and −Zn) on ferric reductase activity. Only Cu deficiency stimulated reductase activity to levels significantly higher than control levels (2- to 4-fold stimulation, significant at $P < 0.00001$). Mn and Zn deficiencies did not induce elevated ferric reductase activity. Plants were grown as described in Figure 1, except micronutrients were withheld 3 d after seedling germination. Free EDTA was used to buffer Zn activity to 10^{-10} M (−Zn) or 3 μM (+Zn). Fe-deficient plants grown in chelate-buffer conditions induced reductase activity to 1.2 μmol Fe reduced (g fresh weight)$^{-1}$ h^{-1} by d 15 (not shown). Cu-deficient plants were grown by removing the cotyledons on d 9, using ultrapure macronutrients, and adding 2 μM free EDTA to minimize Cu contamination in the growth solution. Reductase activity was measured in the same plants over time as described in Figure 1, except in the ±Cu-grown plants, in which two replicates were assayed and harvested each day. Visible symptoms of Mn deficiency were observed on d 24 (chlorosis in the interveinal areas of young leaves), and symptoms of Zn deficiency were observed on d 18 (stunting, proliferation of bulbous secondary lateral roots, and chlorotic interveinal areas on younger leaves). Visible symptoms of Cu deficiency were never observed. Values are means expressed as μmol (g fresh weight)$^{-1}$ h^{-1}. For ±Mn, $n = 7$; for +Zn, $n = 9$; for −Zn, $n = 10$; and for ±Cu, $n = 2$. Error bars represent SE. FW, Fresh weight.

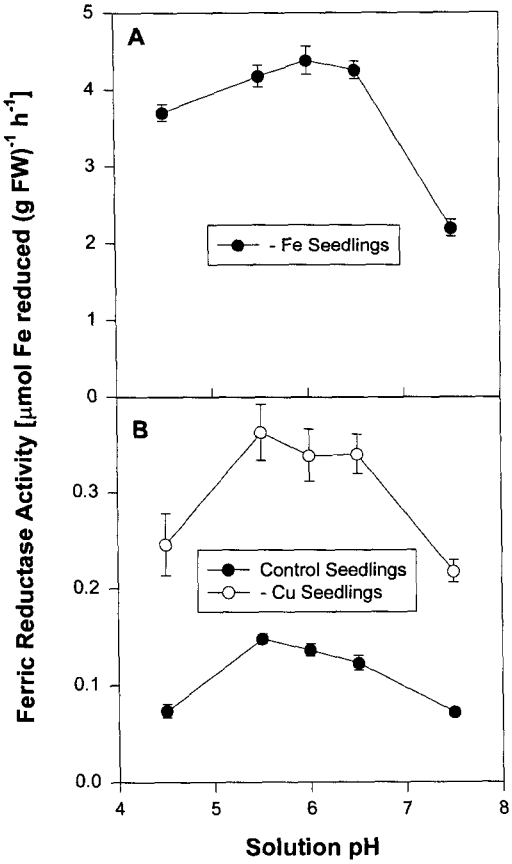

Figure 3. pH dependence of ferric reductase activity in control, Fe-deficient, and Cu-deficient pea seedlings. All three treatments share a pH optimum of 5.5 to 6.0. Values are means expressed as μmol (g fresh weight)$^{-1}$ h^{-1}. For −Cu and control, n = 5; and for −Fe, n = 10. Error bars represent SE. FW, Fresh weight.

(data not shown). In all cases the concentration dependence followed Michaelis-Menten kinetics. The kinetic parameters obtained from Hanes-Woolf transformations of the Michaelis-Menten kinetic curves are summarized in Table II. At all pH levels, the V_{max} of the Cu deficiency-induced reductase was between the maximal rates of the control reductase and the Fe deficiency-induced reductase. At pH 5.5, there were no major differences in the K_m value for ferric reductase activity among treatments; however, the K_m value of the Cu-deficient seedlings was slightly lower than those of the control and Fe-deficient seedlings. Although this difference was statistically significant, it is unclear whether the slightly higher affinity of the Cu-deficient plants for the ferric chelate substrate is physiologically relevant.

An additional trend of note is the significant effect of pH on K_m. As the pH was increased from 5.5 to 7.5, the K_m increased significantly for all three treatments. As the pH was decreased from 5.5 to 4.5, the K_m also decreased in both the Cu-deficient and control plants. The magnitude of this effect of pH on K_m value was quite different for each treatment. The effect of pH on K_m was most pronounced for control roots (an approximately 10-fold increase in K_m from pH 4.5 to 7.5) and had a smaller effect in Cu-deficient and Fe-deficient roots.

The rates of rhizosphere acidification observed in Fe-deficient, Cu-deficient, and control seedlings are summarized in Table III. As expected, Fe deficiency induced a dramatic stimulation in root H^+ efflux (a 3.5-fold increase). As was found for induction of ferric reductase activity, Cu deficiency induced an intermediate increase in rhizosphere acidification (a 2-fold increase).

DISCUSSION

What Mineral Nutrient Stresses Induce Ferric Reductase in Plants?

We sought to establish whether other mineral nutrient deficiencies could induce ferric reductase activity and to determine whether Cu and Fe deficiency induced activity of the same enzyme. We found that Fe and Cu deficiencies play an exclusive role in eliciting elevated activity of ferric reductase in intact root systems of pea seedlings. We believe that Cu deficiency may cause localized Fe deficiency in the plant that consequently induces ferric reductase

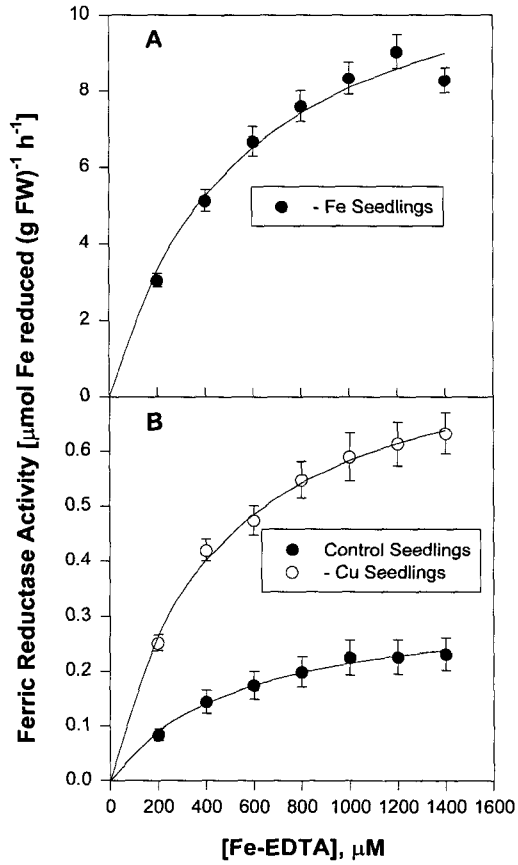

Figure 4. Concentration-dependent kinetics of ferric reductase activity at pH 5.5 in control, Fe-deficient, and Cu-deficient pea seedlings. Values are means expressed as μmol (g fresh weight)$^{-1}$ h^{-1}. For −Fe and −Cu, n = 9; and for control, n = 8. Error bars represent SE. FW, Fresh weight.

Table II. *Summary of kinetic parameters describing concentration-dependent ferric reductase activity at pH 4.5, 5.5, and 7.5 in control, Cu-deficient, and Fe-deficient pea seedlings*

Values for V_{max} and K_m were derived from Hanes-Woolf transformations of each Michaelis-Menten kinetic curve. Values are means with SE values in parentheses. For pH 4.5 experiments, $n = 4$ for control and −Cu, and $n = 5$ for −Fe; for pH 5.5 experiments, $n = 8$ for control, and $n = 9$ for −Cu and −Fe; for pH 7.5 experiments, $n = 5$ for control, −Cu, and −Fe.

Treatment	pH 4.5		pH 5.5		pH 7.5	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
	μmol (g fresh wt)$^{-1}$ h^{-1}	μM	μmol (g fresh wt)$^{-1}$ h^{-1}	μM	μmol (g fresh wt)$^{-1}$ h^{-1}	μM
Control	0.19 (0.03)	211 (86)	0.33 (0.04)	545 (27)	0.64 (0.11)	2405 (702)
−Cu	0.82 (0.05)	335 (30)	0.84 (0.06)	435 (19)	1.00 (0.11)	1435 (85)
−Fe	5.83 (0.32)	788 (53)	12.5 (0.39)	565 (32)	10.7 (0.97)	2125 (94)

activity. Cu deficiency also stimulated rhizosphere acidification, another Fe-stress response commonly exhibited by dicots. Although both responses of Cu-deficient plants are clearly more pronounced than those of control plants, they appear to be somewhat attenuated relative to those of Fe-deficient plants. It is unclear what accounts for these differences in magnitude of the responses.

Our results correspond well with what is known about Fe transport mechanisms in other organisms. Oxidoreductive mechanisms of Fe transport, based on a membrane-bound ferric reductase and a transmembrane ferrous transporter, appear to be conserved among groups of eukaryotes as disparate as yeast, plants, and mammals. Like plants, yeasts also use a reductase-based system of Fe transport. In yeast, molecular studies suggest that there are ferric reductase proteins coupled to both high-affinity (K_m 0.15 μM) and low-affinity (K_m 40 μM) Fe transporters (De Silva et al., 1996). Mammals use a transferrin-based system of Fe transport, but recent evidence has demonstrated that the activity of oxidoreductive mechanisms of Fe transport is similar to those in plants and yeast (De Silva et al., 1996). In mice, reduction is required for Fe transport from the intestinal lumen into mucosal cells (Raja et al., 1992). NADH-dependent reduction also appears to be required for the release of Fe(III) from transferrin receptors in endocytic vesicles and subsequent Fe(II) transport into the cytoplasm (Nuñez et al., 1990; Watkins et al., 1991, 1992).

The animal literature provides compelling evidence for a physiological relationship between Cu and Fe metabolism. Cu-deficient swine exhibited severe Fe deficiency-like symptoms, which could be alleviated by administration of ceruloplasmin (Ragan, 1969). Ceruloplasmin, a Cu-containing ferroxidase, mediates the transfer of Fe from tissue to transferrin in the plasma via an obligatory oxidation step (Lee, 1968). These studies suggest an important functional role for Cu in ceruloplasmin, which in turn mediates Fe homeostasis in mammals. In addition, Cu enhances transferrin-mediated Fe release into the cytoplasm in mammalian cell cultures (Percival, 1992).

Further evidence indicates the involvement of Cu in yeast Fe metabolism. The yeast high-affinity Fe uptake system requires Cu. Mutations in the *FET3* gene, which encodes a ceruloplasmin-like multi-Cu oxidase, abrogate high-affinity Fe accumulation (Askwith et al., 1994). Lesions in genes that have products involved in the delivery of Cu to FET3, including *CTR1*, which encodes a plasma membrane Cu transporter, and *CCC2*, an intracellular Cu-transporting ATPase, also disrupt proper functioning of the high-affinity Fe transport system (Dancis et al., 1994; Yuan et al., 1995). The ferroxidase-containing domain of FET3 is localized to the external cell surface, where it putatively functions to reoxidize ferrous Fe made available by plasma membrane ferric reductases (De Silva et al., 1995). Ferric reduction and subsequent reoxidation by FET3 may be required to confer selectivity for Fe(III) transport through a high-affinity Fe(III) transporter, FTR1 (Stearman et al., 1996).

Cu also plays a role in ferric reductase activity in yeast. MAC1, a transcription factor, has a Cys-rich Cu-binding domain and has been shown to regulate transcription of the ferric reductase gene *FRE1* (Jungmann et al., 1993). The *FRE1* gene is negatively regulated by both Fe and Cu (Jungmann et al., 1993), and the *FRE1* gene product can function as a Cu(II) reductase as well as an Fe(III) reductase (Hassett and Kosman, 1995).

Table III. *Rhizosphere acidification rates in intact root systems of control, Cu-deficient, and Fe-deficient pea seedlings*

Rates of rhizosphere acidification are the means of 41 or 42 replicates; the values in parentheses represent SE.

Treatment	Rhizosphere Acidification
	μmol (g fresh wt)$^{-1}$ h^{-1}
Control	0.35 (0.06)
−Cu	0.75 (0.10)
−Fe	1.21 (0.10)

Do Cu Deficiency and Fe Deficiency Induce the Same Ferric Reductase?

Our results point to the stimulation of a single ferric reductase enzyme in response to either Cu or Fe deficiency because of the similarity in K_m values and pH optima among the treatments. We found the V_{max} of Cu deficiency-induced reductase activity to be intermediate between Fe deficiency-induced and control reductase maximal rates. This relationship was consistent over the broad pH range tested. This result is compatible with a model in which varying amounts of ferric reductase enzyme are deployed on the membrane in response to changes in nutritional status.

The apparent K_m values we obtained were about 1 order of magnitude higher than those obtained by previous

workers using other species. Chaney (1989) reported a K_m of 55 μM in intact root systems of Fe-deficient *Arachis hypogea*. Moog et al. (1995) found a K_m of 45 μM in intact root systems of *Arabidopsis thaliana*. Higher K_m values have been documented in intact root systems of *Phaseolus vulgaris* (120 μM; Bienfait et al. [1983]), *Lycopersicon esculentum* (230 μM; Brüggemann et al. [1990]), and *Geum urbanum* (87 μM; Schmidt and Janiesch [1991]). Our results were most similar to those of Susín et al. (1996), who reported a K_m of 514 μM in Fe-deficient *Beta vulgaris*. Although we observed significant differences among K_m values of the different treatments, we are unsure whether these reflect relevant physiological differences, since the values all lie within the same order of magnitude.

We found solid agreement of the pH optimum of 5.5 for ferric reduction in pea with results from other species. Optimal pH values ranging from 5 to 5.5 have been described in intact root systems of *A. thaliana* (Moog et al., 1995), *A. hypogeae* (Römheld and Marschner, 1983), *Malus domestica* (Ao et al., 1985), and *G. urbanum* (Schmidt and Janiesch, 1991). The pH optimum of *Ficus benjamina*, pH 5.5 to 6.7, was slightly higher (Rosenfield et al., 1991). Susín et al. (1996) found a strong pH dependence for Fe deficiency-induced ferric reductase activity. In that study, ferric reductase activity was highest between pH 5.0 and 6.0, but when the pH was increased to 6.5, both K_m and V_{max} in the Fe-deficient plants reverted to control values. The authors concluded that the constitutive and induced ferric reductase are different enzymes and that the induced ferric reductase is only active at low pH. However, Holden et al. (1991) have provided convincing evidence against the induction of a novel reductase under Fe deficiency. Plasma membrane fractions from both Fe-deficient and Fe-sufficient tomato roots analyzed by IEF exhibited the same number of ferric reductases with the same pI values but different apparent activities, suggesting a single enzyme induced to different levels under control and −Fe conditions.

In several treatment comparisons in Table II, we observed decreasing affinity of the enzyme for the substrate with increasing pH. Schmidt and Janiesch (1991) demonstrated a similar pattern in *G. urbanum*. Higher enzyme affinity at low pH could be attributed to increased shielding of negative charges on the membrane by protons and hence less electrostatic repulsion of the negatively charged substrate Fe-EDTA. The pH-dependent alterations in K_m value could also be ascribed to electrostatic changes in the Fe-Na-EDTA substrate. Chemical speciation calculations showed that at pH 4.5 and 5.5 the predominant form of the substrate would be Fe-EDTA^{-1}. However, at pH 7.5 approximately 50% of the EDTA associated with Fe would be as Fe(OH)-EDTA^{-2}, which would be more strongly repulsed by the negatively charged membrane surface and would result in a decrease in enzyme affinity. Furthermore, the Fe(OH)-EDTA^{-2} species may not be reduced as readily by the ferric reductase as Fe-EDTA^{-1}. A decrease in the concentration of the reactive substrate could result in an increase in the apparent K_m.

At high pH it is difficult to uncouple the true properties of the enzyme from partial instability of the Fe-EDTA. Although there was no evidence of Fe-oxide precipitation during the short reaction times, the decrease in V_{max} and the increase in K_m for ferric reduction at pH 7.5 could both be attributed to instability of the Fe-EDTA substrate. We used the GEOCHEM-PC chemical speciation program (Parker et al., 1995) to demonstrate the limited stability of EDTA at pH 7.5. The degree of instability depends on the solubility of the hydrous Fe oxides that may have formed, as well as on the kinetics of formation.

At pH 4.5 the control and Cu-deficient plants exhibited a higher affinity for the substrate, whereas the Fe-deficient plants exhibited a lower affinity for the substrate. One interpretation of this result is that the ferric reductase may exhibit slightly different properties depending on its cytoplasmic or apoplasmic environment. Alterations in redox potential, membrane potential, or rhizosphere acidification associated with Fe deficiency may confer unique conformational changes in the ferric reductase that in turn alter the apparent K_m.

Cu Deficiency Stimulates Rhizosphere Acidification

We found that Cu deficiency stimulated an increase in proton extrusion to a level intermediate between those of Fe-deficient and control plants. Although rhizosphere acidification varied significantly with treatment, the elevated proton extrusion was not always correlated with high ferric reductase activity (data not shown). However, this result is not altogether unexpected in light of circumstantial evidence that indicates a physiological uncoupling of these two Fe-stress responses. Grusak and Pezeshgi (1996) have demonstrated a temporal separation in expression of the two responses, in which elevated proton extrusion precedes the onset of enhanced ferric reductase activity by several days. Furthermore, localization of reductase activity and proton fluxes along the root surface showed a spatial separation of the two responses (Grusak et al., 1989). In Fe-deficient pea seedlings, reductase activity was localized along the surface of lateral roots except for the tip, whereas proton efflux mapped along the root surface using extracellular ion-selective microelectrodes was enhanced along the entire surface, including the tip. A similar pattern for Fe deficiency-induced H^+ efflux was recently observed by analyzing expression of plasma membrane H^+-ATPase (*AHA*) gene promoters fused to the *GUS* gene in transgenic *A. thaliana* (M.L. Guerinot, personal communication). Plants bearing the *AHA2-GUS* construct exhibited high expression along the root, including the tip, when subjected to −Fe conditions. In addition, a recessive mutant in Arabidopsis exhibiting greatly reduced ferric reductase activity, *frd1–1*, still exhibited normal induction of rhizosphere acidification under Fe deficiency (Yi and Guerinot, 1996). This suggests that the *FRD1* gene product is not involved in acidification and that the two responses may be regulated independently.

In summary, we have demonstrated that elevated ferric reductase activity is induced exclusively by Fe and Cu deficiency. Cu deficiency also stimulates enhanced rhizosphere acidification, another response associated with Fe

deficiency. Rates of Cu deficiency-induced ferric reductase and acidification were consistently intermediate between those of nutrient-sufficient and Fe-deficient plants. The similarity of pH optima and K_m values of ferric reductase induced by either treatment strongly suggests that a common enzyme is induced in response to both Fe and Cu deficiency. The large differences in V_{max} between the treatments suggest that varying amounts of the same enzyme may be deployed on the plasma membrane in response to plant Fe or Cu status.

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