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The Role of Iron-Deficiency Stress Responses in Stimulating Heavy-Metal Transport in Plants[1]

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Plant accumulation of Fe and other metals can be enhanced under Fe deficiency. We investigated the influence of Fe status on heavy-metal and divalent-cation uptake in roots of pea (*Pisum sativum* L. cv Sparkle) seedlings using Cd^{2+} uptake as a model system. Radiotracer techniques were used to quantify unidirectional ^{109}Cd influx into roots of Fe-deficient and Fe-sufficient pea seedlings. The concentration-dependent kinetics for ^{109}Cd influx were graphically complex and nonsaturating but could be resolved into a linear component and a saturable component exhibiting Michaelis-Menten kinetics. We demonstrated that the linear component was apoplastically bound Cd^{2+} remaining in the root cell wall after desorption, whereas the saturable component was transporter-mediated Cd^{2+} influx across the root-cell plasma membrane. The Cd^{2+} transport system in roots of both Fe-deficient and Fe-sufficient seedlings exhibited similar Michaelis constant values, 1.5 and 0.6 μM, respectively, for saturable Cd^{2+} influx, whereas the maximum initial velocity for Cd^{2+} uptake in Fe-deficient seedlings was nearly 7-fold higher than that in Fe-grown seedlings. Investigations into the mechanistic basis for this response demonstrated that Fe-deficiency-induced stimulation of the plasma membrane H^+-ATPase did not play a role in the enhanced Cd^{2+} uptake. Expression studies with the Fe^{2+} transporter cloned from Arabidopsis, *IRT1,* indicated that Fe deficiency induced the expression of this transporter, which might facilitate the transport of heavy-metal divalent cations such as Cd^{2+} and Zn^{2+}, in addition to Fe^{2+}.

Although abundant in the earth's crust, Fe predominates as insoluble Fe(III) precipitates and is largely unavailable to plants, especially at neutral or alkaline pH. Plants use two distinct strategies to assimilate Fe from the environment. The grasses release low-molecular-weight, high-affinity Fe(III)-chelating compounds called phytosiderophores, which solubilize ferric Fe in the rhizosphere and are recognized for uptake by specific membrane receptors (Römheld and Marschner, 1986; Chaney, 1987; Bienfait, 1988). Fe uptake in the dicots and the nongrass monocots is mediated by a plasma membrane-bound ferric reductase that transfers electrons from intracellular NADH (Buckhout et al., 1989) to Fe(III) chelates in the rhizosphere (Chaney et al., 1972). The ferrous ions (Fe^{2+}) released from the chelates by this process are subsequently transported into the cytoplasm via a separate transport protein (Kochian, 1991; Fox et al., 1996).

When Fe deficient, dicot and nongrass monocots stimulate a number of processes to enhance Fe accumulation from the soil. Fe deficiency induces a 5- to 10-fold stimulation of ferric reductase activity (Ambler et al., 1971; Chaney et al., 1972; Römheld and Marschner, 1979; Bienfait et al., 1983). Root-mediated acidification of the rhizosphere is an additional strategy used by Fe-deficient plants to enhance solubilization of Fe^{3+} from Fe hydroxides (Venkat Raju and Marschner, 1972; Brown and Jones, 1974). Finally, root Fe^{2+} influx is regulated by the Fe status of the plant. Fox et al. (1996) found that Fe-deficient pea (*Pisum sativum* L.) seedlings exhibit significantly higher rates of root Fe^{2+} influx than Fe-sufficient seedlings. In addition to these responses, which are usually linked specifically to Fe accumulation, tissue concentrations of other mineral elements also appear to be influenced by plant Fe status. Welch et al. (1993) demonstrated that the shoot concentrations of many divalent cations, including Cu, Mn, and Mg, increased in Fe-deficient pea seedlings. Rodecap et al. (1994) also reported that Fe-deficient Arabidopsis plants accumulated higher concentrations of Cd and Mg in racemes and seeds compared with Fe-sufficient plants.

In this study we investigated the influence of Fe status in stimulating heavy-metal uptake in pea using Cd uptake as a model system. Cd is a common environmental contaminant introduced into soils through anthropogenic activity. Cd contamination poses a serious hazard to human health, and uptake into plants is the primary avenue through which it can enter the food chain. Additionally, there has been considerable interest in the use of terrestrial plants for the remediation of surface soils contaminated with toxic heavy metals, although little is understood about plant mechanisms of heavy-metal hyperaccumulation.

We demonstrate that Fe deficiency elicits a large stimulation of Cd influx into roots of pea seedlings. We investigated the physiological basis of this enhanced Cd uptake through evaluation of Fe-deficiency stress responses that might play a role in enhanced heavy-metal absorption.

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DTNB, dinitrobenzoic acid; EDDHA, *N,N′*-ethylenebis-[(2-hydroxyphenyl)-Gly]; E_m, plasma membrane electropotential; FC, fusicoccin.

These include induction of a divalent cation transporter, induction of the plasma membrane H^+-ATPase, and induction of the plasma membrane ferric reductase. We found that Fe deficiency induced the expression of a Fe-transporter gene, which might facilitate the transport of heavy-metal divalent cations such as Cd^{2+} and Zn^{2+}, in addition to Fe^{2+}.

MATERIALS AND METHODS

Plant Material and Culture

At d 0, pea (*Pisum sativum* L. cv Sparkle) seeds were allowed to imbibe 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 roots were inserted through holes in black polyethylene seedling cups. The seedling cups were inserted into the covers of black polyethylene pots containing 5 L (five to seven plants per pot) 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; and $MgSO_4$, 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; and $NiSO_4$, 0.1. The solutions were supplemented with 10 μM Fe-EDDHA and 1 mM Mes buffer, adjusted to pH 5.5 with KOH. Nutrient solutions were changed on d 9 and 12. Fe-deficient plants were prepared by growing the seedlings in solutions without Fe from d 9 until d 14. Plants were grown in a controlled-environment growth chamber with a 16-h, 20°C day and an 8-h, 15°C night regime and a photon flux density of 350 μmol m^{-2} s^{-1}.

Arabidopsis thaliana (La-0) seeds were surface sterilized in 30% (v/v) bleach (5.25% sodium hypochlorite) and 0.01% Triton X-100 for 15 to 20 min and rinsed five times in sterile, 18-MΩ water. Seeds were vernalized overnight at 4°C, resuspended in a low-gelling-temperature agarose solution (1%), and placed on polypropylene screens submerged in nutrient solution in sterile Magenta Jars (Magenta Corp., Chicago, IL). Seedlings were grown in autoclaved nutrient solution consisting of the following nutrients in mM: KNO_3, 2; KH_2PO_4, 0.2; $MgSO_4$, 2; $(NH_4)_2SO_4$, 0.25; $Ca(NO_3)_2$, 1; $CaSO_4$, 1; and K_2SO_4, 1; and the following micronutrients in μM: H_3BO_3, 70; $MnCl_2$, 14; $CuSO_4$, 0.5; $ZnSO_4$, 1; Na_2MoO_4, 0.2; NaCl, 10; and $CoCl_2$, 0.01. The nutrient solution was supplemented with 10 g/L Suc, 1 mM Mes buffer, adjusted to pH 5.5 with KOH, and 50 μM filter-sterilized Fe-EDDHA. Fe-deficient seedlings were prepared by growing the seedlings in solutions without Fe from d 16 until d 21. Sterile plants were grown in an incubator at 20°C with a 16-h photoperiod at a light intensity of 60 μmol m^{-2} s^{-1}.

Root $^{109}Cd^{2+}$-Influx Experiments

Excised roots of 14-d-old pea seedlings were used for the Cd^{2+}-influx experiments. Root segments were excised from the older portion of the root system and consisted of a 5- to 20-mm section of primary root with 2 to 10 attached, intact lateral roots. Root segment fresh weights ranged from 0.1 to 0.4 g.

An uptake apparatus consisting of acrylic wells was used for the Cd^{2+}-influx experiments (Grusak et al., 1990). Hollow acrylic cylinders were fitted with mesh-covered rubber stoppers to allow vacuum withdrawal of solutions without disturbing the roots. The uptake solutions were aerated with acrylic tubing placed near the bottom of each well.

Root segments were gently inserted into each well using forceps and permitted to recover from excision for at least 30 min in an aerated pretreatment solution consisting of 5 mM Mes-Tris (pH 6.0), 1 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.2 mM $MgSO_4$, 0.1 mM K_2SO_4, and 0.2 mM NH_4NO_3. The pretreatment solution was then evacuated and replaced with fresh uptake solution of the same composition. Metabolic inhibitors, sulfhydryl reagents, or treatment cations were added to the uptake solutions 30 min before the introduction of radiolabeled ^{109}Cd. A 0.5-mL aliquot of the appropriate stock $Cd(NO_3)_2$ solution was added to each well immediately before addition of radiolabel to attain a desired Cd concentration between 1 and 100 μM. The 20-min uptake period was initiated by adding 50 μL of a radioactive stock solution ($^{109}CdCl_2$ in 0.1 N HCl) to each uptake well to attain a final radioisotope concentration of either 0.0025 or 0.01 μCi/mL. One-milliliter aliquots of the uptake solution were removed at 1 and 19 min into the uptake period to calculate an internal standard relating counts per minute to total Cd and also to determine the amount of substrate (Cd) depletion. After a 20-min uptake period, the uptake solution in the wells was replaced with ice-cold desorption solution consisting of 5 mM Mes-Tris (pH 6.0), 5 mM $CaCl_2$, and 100 μM $Cd(NO_3)_2$. After two 7.5-min desorption periods (15 min total desorption time), root segments were removed and blotted dry with paper towels. The section of primary root on each root unit was removed, and the lateral roots were weighed. Absorption of ^{109}Cd into the lateral roots was quantified via gamma detection using a counter (Auto-Gamma 5530, Packard, Downers Grove, IL).

Methanol:Chloroform Preparation of Root Cell Walls

To approximate the contribution of cell wall binding to Cd absorption, root cell wall preparations that maintained the morphologic and geometric characteristics of intact roots were obtained. Root systems were immersed in methanol:chloroform (2:1, v/v) solutions for 3 d and rinsed in several changes of distilled water for 1 d. This treatment has been shown to yield lipid-free cell wall preparations in maize while maintaining the structure and morphologic characteristics of an intact root (Hart et al., 1992).

Cd Accumulation in Roots and Shoots

Fe-deficient and Fe-sufficient plants were grown as described above. On d 12, 0.2 μM $Cd(NO_3)_2$ was added to the growth solution. Tissue was harvested on d 14. Aerial portions of the plant were rinsed in 18-MΩ water, blotted

dry, and placed in a bag to dry. Root systems were desorbed in a solution containing 1 mM $LaCl_3$ for 30 min, with the desorption solution replaced with fresh solution every 5 min (Reid et al., 1996). Dried tissue samples were wet digested in concentrated HNO_3 overnight at 100°C, then digested again with HNO_3 and $HClO_4$ at 200°C. Samples were resuspended in 5% HNO_3 and analyzed via simultaneous inductively coupled argon-plasma emission spectrometry (ICAP 61E trace analyzer, Themo-Jarrell Ashe, Franklin, MA).

Measurement of Exofacial Reduced Sulfhydryl Groups

Reduced sulfhydryl groups on the root surface (presumably on the plasma membrane surface and/or within the cell wall) of ± Fe-grown pea seedlings were measured with the membrane-impermeant, sulfhydryl-reactive reagent DTNB using a procedure modified from that of Welch and Norvell (1993). DTNB reacts with reduced sulfhydryl groups to form a yellow nitromercaptobenzoic acid anion product. Intact root systems were submerged in 100 mL of sulfhydryl reaction buffer (0.2 M Tris-HCl and 0.02 M Na-EDTA, adjusted to pH 8.2 with NaOH). The reaction buffer was purged with N_2 gas for 10 min before root submergence and during the reaction period. Each reaction period was initiated by adding 1 mL of a 10-mM DTNB solution to attain a final concentration of 100 μM. After a 15-min reaction period, absorbance values of aliquots of the assay solution were measured at 412 nm with a spectrophotometer (model DU 640, Beckman). The molar concentration of the nitromercaptobenzoic acid anion product was determined using a standard curve generated with fresh Cys stock solutions. Controls for each treatment in which DTNB was added to the solution after seedling removal were included to correct for soluble reducing agents excreted from the roots during the assay.

Measurement of Root-Cell E_m

Pea seedlings (14–15 d old) were secured in an acrylic chamber mounted on the stage of a microscope (model BH-2, Olympus). Roots were bathed in a solution identical to that used in the ^{109}Cd-uptake experiments (5 mM Mes-Tris [pH 6.0], 1 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.2 mM $MgSO_4$, 0.1 mM K_2SO_4, and 0.2 mM NH_4NO_3). Experiments with FC (10 μM) were performed in an unbuffered solution that also contained 0.06% (v/v) methanol. Cells of the root epidermis and cortex were impaled with microelectrodes at several locations at least 2 to 3 cm from the root apex using a hydraulically driven micromanipulator (model MO-204, Narashige USA, Greenvale, NY) mounted on the microscope stage. Membrane potentials were measured using a dual microprobe amplifier (model KS-700, World Precision Instruments, Inc., Sarasota, FL). Microelectrodes (tip diameter = 0.5 μm) were pulled from 1-mm-thick, single-barreled borosilicate glass capillary tubes with an internal filling fiber (World Precision Instruments), using a micropipette puller (model P-87, Sutter Instruments, Inc., Novato, CA). Electrodes were filled with 3 M KCl solution, adjusted to pH 2.0 to minimize tip potentials. A reference electrode (catalog no. 13-639-52, Fisher Scientific) was placed in the chamber housing the seedling to complete the electrical circuit.

Analysis of Fe-Transporter Expression

Polyadenylated mRNA from roots of ± Fe-grown Arabidopsis (21 d old, 5 d without Fe) and pea (13 d old, 4 d without Fe) was isolated as described by Sambrook et al. (1989). Aliquots of each mRNA preparation (1.9 μg for pea and 1.2 μg for Arabidopsis) were denatured, electrophoresed in a 1.2% agarose gel containing 1× Mops and 2.2 M formaldehyde (Sambrook et al., 1989), and transferred to a nylon membrane (HyBond N, Amersham). The mRNA was subsequently cross-linked to the membrane by baking for 2 h at 80°C. The membrane was prehybridized at 60°C in 5× SSC, 5× Denhardt's solution, and 0.5% (w/v) SDS plus 0.1 mg/mL denatured salmon-sperm DNA and probed with a genomic clone of the Arabidopsis *IRT1* gene (Eide et al., 1996).

The genomic clone was isolated from an Arabidopsis (La-0) genomic library using PCR. Primers were derived from the 20 nucleotides flanking the *IRT1* coding region (forward primer, 5′-CAAATTCAGCACTTCTCATG-3′; reverse primer, 5′-TTCCGCAATATCTGGAGTAT-3′). The resulting fragment was inserted into the TA cloning vector PCR2.1 (Invitrogen, Inc., San Diego, CA) and introduced into competent DH5α *Escherichia coli* cells. Transformed colonies were screened for β-galactosidase activity on Luria-Bertani medium plus 5-bromo-4-chloro-3-indolyl-β-D-galactoside and ampicillin. Plasmids were isolated, purified (Qiagen, Chatsworth, CA), and sequenced (ABI Prism, Perkin-Elmer). The *IRT1* genomic clone was radiolabeled with ^{32}P using the method of Feinberg and Vogelstein (1984), denatured at 100°C for 10 min, and hybridized to the membrane overnight at 60°C. After hybridization, the nylon membrane was washed twice for 20 min in 2× SSC, 0.1% SDS at 60°C, and exposed to x-ray film at −80°C.

RESULTS

Cd^{2+}-Uptake Experiments

The concentration-dependent kinetics of root Cd^{2+} influx in Fe-sufficient and Fe-deficient pea seedlings were graphically complex and nonsaturating. A least-squares procedure was used to test the fit of the Cd^{2+}-influx data to two different kinetic models, a three-parameter rectangular hyperbolic plus linear function (Eq. 1) or a linear function (Eq. 2; SigmaPlot, Chicago, IL):

$$J[Cd^{2+}] = k[Cd^{2+}] + \frac{V_{max}[Cd^{2+}]}{K_m + [Cd^{2+}]} \quad (1)$$

$$J[Cd^{2+}] = k[Cd^{2+}] \quad (2)$$

An analysis to find the best fit was performed for each of the treatments imposed. A kinetic model consisting of hyperbolic (saturable) and linear components was found to suitably describe Cd influx in the Fe-deficient roots (r^2 =

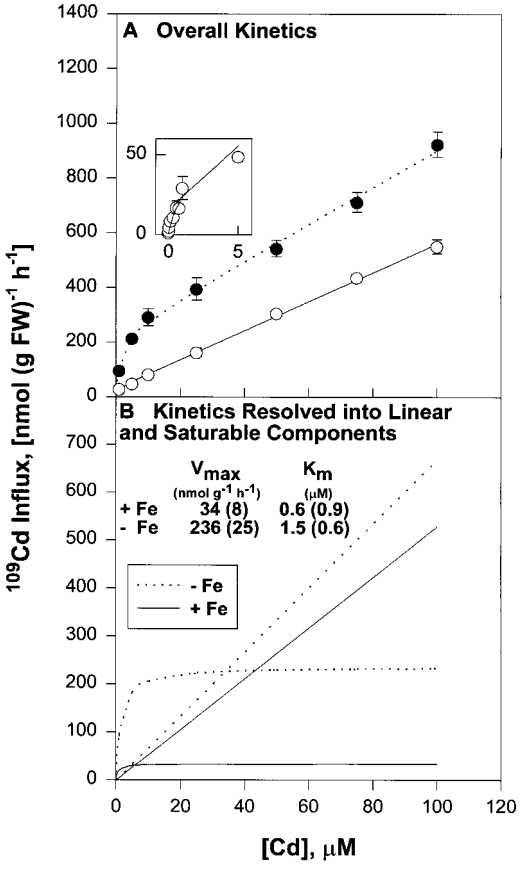

Figure 1. A, Concentration-dependent kinetics of root ^{109}Cd influx in ± Fe-grown pea seedlings. After a 30-min recovery from excision in pretreatment solution, root segments were exposed to radiolabeled uptake solutions for 20 min and then desorbed for 15 min. The inset depicts the concentration-dependent kinetics of Cd influx over the 0- to 5-μM concentration range in the +Fe-grown pea seedlings. Values are means expressed as nmol Cd^{2+} (g fresh weight [FW])$^{-1}$ h^{-1} (for +Fe [○], n = 3–6; for −Fe [●], n = 4–6). Error bars represent SE. B, The plots in A were mathematically dissected into their linear and hyperbolic components to estimate the kinetic parameters.

0.99). For Fe-sufficient roots, a linear function or a linear-plus-saturable function described the kinetics of Cd influx equally well (Fig. 1A; r^2 = 0.998); however, visual inspection of the data for Fe-sufficient roots (Fig. 1A, inset) shows the nonlinearity of the lower portion of the curve when the flux data for concentrations < 1 μM Cd are included. When these data are included, a linear-plus-hyperbolic equation describes the fitted function slightly better (r^2 = 0.999).

The kinetic parameters (K_m and V_{max}) for the saturable system (Fig. 1B) were determined using the computer-generated fits of the data to linear-plus-saturable equations. Saturable Cd^{2+} influx for Fe-deficient and Fe-sufficient plants exhibited similar K_m values, 1.5 ± 0.6 and 0.6 ± 0.9 μM, respectively. The slopes of the linear components were also similar, 6.7 ± 0.35 nmol (g fresh weight)$^{-1}$ h^{-1} μM^{-1} for Fe-deficient roots and 5.3 ± 0.13 nmol (g fresh weight)$^{-1}$ h^{-1} μM^{-1} for Fe-sufficient roots. However, the Fe-deficient plants exhibited a nearly 7-fold higher V_{max} for uptake (236 ± 25 versus 34 ± 8 nmol [g fresh weight]$^{-1}$ h^{-1}). Thus, Fe deficiency stimulated a large increase in a high-affinity, saturable Cd-uptake system but had relatively little effect on the slope of the linear component. Cd^{2+} influx was also stimulated in roots of Fe-deficient seedlings at low Cd^{2+} concentrations (0–750 nM; Fig. 2). This Fe-deficiency-induced high-affinity system also appears to transport Zn. In a preliminary study performed at 1 μM Zn, ^{65}Zn influx was nearly 3 times higher in Fe-deficient roots (70 ± 7 versus 25 ± 1 nmol ^{65}Zn [g fresh weight]$^{-1}$ h^{-1}).

We tested the hypothesis that the saturable uptake represented carrier-mediated transport across the root-cell plasma membrane, whereas the linear component reflected apoplastic binding of Cd in the cell wall that was not removed during the desorption period. This model has been demonstrated for the uptake of several other divalent cations, including Zn^{2+} and putrescine, in roots of *Thlaspi caerulescens* and maize, respectively (DiTomaso et al., 1992; Lasat et al., 1996). This hypothesis was tested using several different experimental approaches. First, we examined the kinetics of Cd^{2+} uptake (or binding) with morphologically intact root cell wall preparations (methanol:chloroform-treated roots). A linear model was found to adequately describe Cd binding to methanol:chloroform-treated root cell wall preparations of both Fe-sufficient and Fe-deficient pea seedlings (Fig. 3, A and B; r^2 = 0.98 for −Fe and r^2 = 0.99 for +Fe roots). The slope of the concentration-dependent Cd binding in the cell wall preparations was significantly greater than that of the linear component for intact root segments (−Fe, 14 ± 0.7 versus 6.7 ± 0.3 nmol [g fresh weight]$^{-1}$ h^{-1} μM^{-1} for intact roots; +Fe, 23 ± 0.9 versus 5.3 ± 0.13 nmol [g fresh weight]$^{-1}$ h^{-1} μM^{-1} for

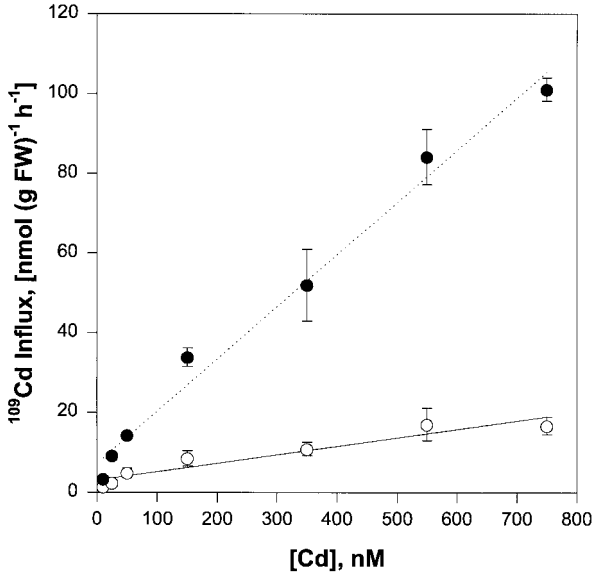

Figure 2. Concentration-dependent kinetics of root ^{109}Cd influx in ± Fe-grown pea seedlings (0–0.75 μM range). After a 30-min recovery from excision in pretreatment solution, root segments were placed in radiolabeled uptake solutions for 20 min and then desorbed for 15 min. Values are means expressed as nmol Cd^{2+} (g fresh weight [FW])$^{-1}$ h^{-1} (for +Fe [○], n = 6–9; for −Fe [●], n = 2–3). Error bars represent SE.

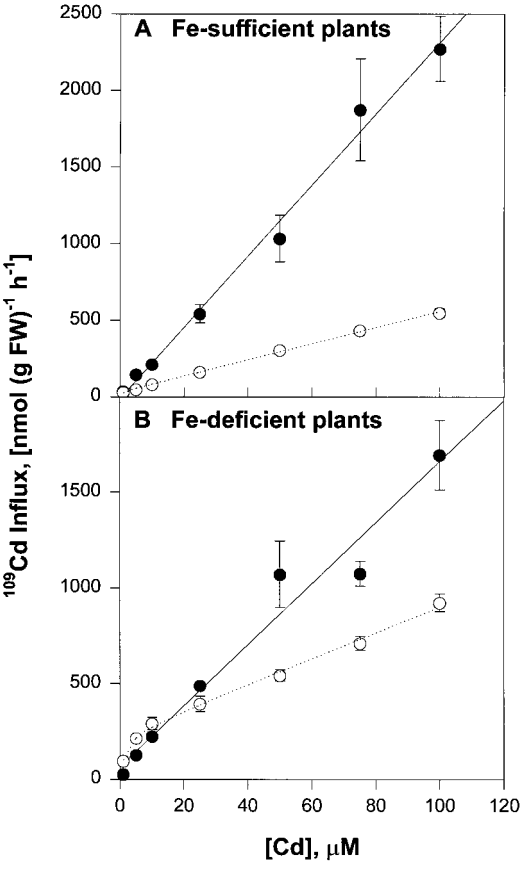

Figure 3. Concentration-dependent kinetics of ^{109}Cd influx in intact and methanol:chloroform-treated roots from +Fe-grown pea seedlings (A) and −Fe-grown pea seedlings (B). Excised roots were treated for 3 d in 2:1 (v/v) methanol:chloroform to remove protoplast material and rinsed for 1 d in H_2O. Root segments were then placed in radiolabeled uptake solutions for 20 min and subsequently desorbed for 15 min. Values are means expressed as nmol Cd^{2+} (g fresh weight [FW])$^{-1}$ h^{-1}. For +Fe: intact roots (○), n = 3 to 6 and cell wall only (●), n = 2 to 3; for −Fe: intact roots (○) and cell wall only (●), n = 4 to 6. Error bars represent SE.

intact roots). This difference in slope could be attributed to a greater exposure of potential binding sites in the root cell wall preparations resulting from removal of the root symplast.

We next investigated the influence of different ion-transport inhibitors on the concentration-dependent kinetics of Cd uptake. $LaCl_3$, a Ca^{2+}-channel blocker, was found to inhibit both kinetic transport components (Fig. 4). The best fit was a linear function (r^2 = 0.9) with a reduced slope (1.8 ± 0.01 versus 6.09 ± 0.45 nmol [g fresh weight]$^{-1}$ h^{-1} μM^{-1} for the control). This result is consistent with a model in which La^{3+} may both displace Cd^{2+} from the cell wall, decreasing the slope of the linear component, and compete with Cd^{2+} for a plasma membrane transporter, abolishing the saturable component. In Cd^{2+}-binding experiments with root cell wall preparations, inclusion of 0.2 mM La^{3+} in the uptake solution effectively inhibited Cd^{2+} association with the cell wall (data not shown).

Treatment with the respiratory inhibitor KCN (0.5 mM; data not shown) or the protonophore CCCP (20 μM; Fig. 5) specifically abolished the saturable Cd^{2+}-influx component but had no effect on the linear component. A linear function was found to be the best-fit model (r^2 = 0.95 for CCCP-treated roots), providing additional evidence that saturable Cd^{2+} uptake represents transporter-mediated Cd influx across the root-cell plasma membrane. Treatment with the same concentration of KCl (0.5 mM) was noninhibitory (data not shown), which indicates that inhibition of the saturable component for Cd^{2+} influx by KCN was not a consequence of K^+-induced depolarization of the transmembrane electrical potential.

The Mechanistic Basis of Enhanced Cd Uptake

As mentioned in the introduction, there are several possible explanations for the Fe-deficiency-induced stimulation of heavy-metal transport observed here. These include (a) induction of the plasma-membrane H^+-ATPase, which could stimulate cation uptake; (b) induction of a transporter (such as *IRT1*), which could mediate Cd^{2+} influx; and (c) induction of the ferric reductase, which could play a direct or indirect role in influencing ion transporters in the root-cell plasma membrane. Experiments were conducted to investigate all three of these scenarios.

Role of the Plasma Membrane H^+-ATPase in Stimulating Cd^{2+} Influx

We explored the possibility that Fe-deficiency-induced H^+-ATPase activity, resulting in increased H^+ extrusion

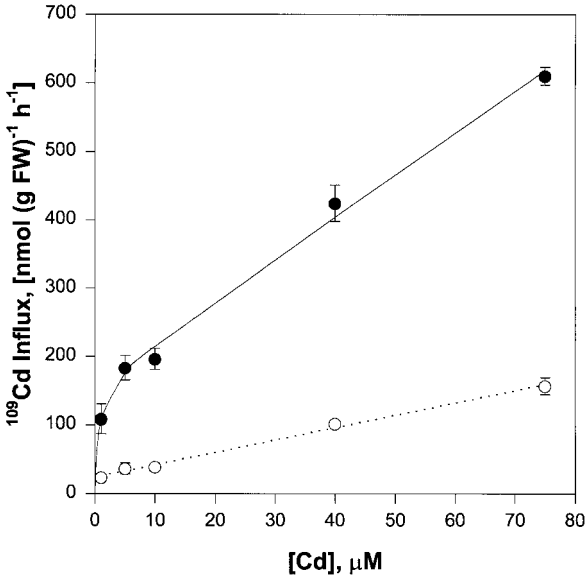

Figure 4. Concentration-dependent kinetics of ^{109}Cd influx into roots of −Fe-grown pea seedlings treated with $LaCl_3$, a Ca^{2+}-channel blocker. After excision, root segments were pretreated for 30 min in 0.2 mM $LaCl_3$, and then placed in radiolabeled uptake solution containing 0.2 mM $LaCl_3$ for 20 min, and subsequently desorbed for 15 min. Values are means expressed as nmol Cd^{2+} (g fresh weight [FW])$^{-1}$ h^{-1} (n = 4). ●, Control roots; ○, +La^{3+}. Error bars represent SE.

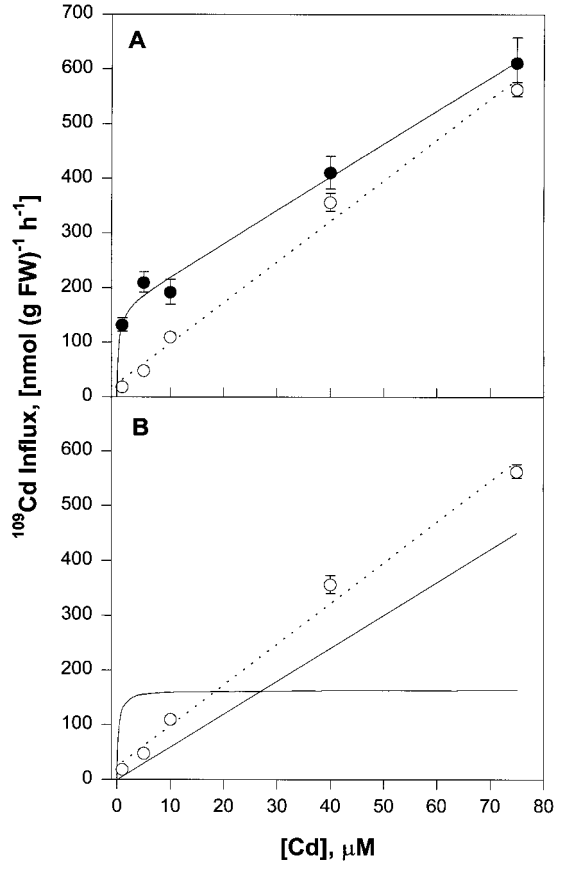

Figure 5. A, Concentration-dependent kinetics of ^{109}Cd influx in −Fe-grown pea seedling roots treated with the protonophore and metabolic inhibitor CCCP. After excision, root segments were pretreated for 30 min in 20 μM CCCP, then placed in radiolabeled uptake solution containing CCCP for 20 min, and subsequently desorbed for 15 min. ●, −CCCP; ○, +CCCP. B, The concentration-dependent kinetics of root ^{109}Cd influx for CCCP-treated pea seedlings. The Cd^{2+}-uptake kinetics for −CCCP-treated seedlings (solid lines) were dissected into linear and hyperbolic components. ○, +CCCP. Values are means expressed as nmol Cd^{2+} (g fresh weight [FW])$^{-1}$ h^{-1} (n = 4). Error bars represent SE.

and possible hyperpolarization of the E_m, played a role in stimulating Cd influx. If enhanced proton efflux during Fe deficiency were involved in stimulating Cd influx, we would expect FC, a fungal toxin that stimulates P-type H^+-ATPases, to stimulate Cd uptake in +Fe-grown roots. In preliminary electrophysiological experiments, we found that FC stimulated the pea root plasma membrane H^+-ATPase very rapidly, hyperpolarizing the root-cell E_m by approximately 40 mV within 30 min. However, we found no effect of FC on Cd influx in +Fe-grown pea seedlings under either unbuffered or strongly buffered conditions, which would presumably eliminate the effect of proton gradients and allow us to look exclusively at the effect of E_m on Cd^{2+} influx (Table I). We also investigated the role of H^+ gradients on enhanced Cd^{2+} influx by using highly pH-buffered uptake solutions to abolish H^+ gradients in the unstirred layer adjacent to the root. We had previously demonstrated using pH microelectrodes that inclusion of

Table I. *Effect of FC on Cd^{2+} accumulation in +Fe-grown pea seedling roots*

Root segments were pretreated for 30 min after excision in buffered (10 mM Mes-Tris, pH 6.0) or unbuffered solution with or without 10 μM FC, incubated in radiolabeled uptake solution containing 0 or 10 mM buffer, 0 or 10 μM FC, and 1 or 10 μM $Cd(NO_3)_2$ for 20 min, and desorbed for 15 min. Values are means (n = 4–5). Values in parentheses represent SE.

Treatment	Cd^{2+} Influx			
	Unbuffered		10 mM Buffer	
	1 μM Cd	10 μM Cd	1 μM Cd	10 μM Cd
	nmol (g fresh wt)$^{-1}$ h^{-1}			
Control	17 (7)	73 (11)	19 (3)	67 (9)
+FC	12 (1)	75 (3)	17 (4)	78 (11)

10 mM Mes-Tris buffer abolished H^+ gradients generated in this unstirred layer (L.V. Kochian, unpublished results). Therefore, these highly buffered solutions were used to abolish enhanced H^+ gradients established along roots of −Fe-grown plants. This treatment was found to have no effect on Cd influx in Fe-deficient plants (Fig. 6). We also compared measurements of root-cell membrane potentials in ± Fe-grown seedlings to determine if H^+-ATPase-mediated hyperpolarization of the root-cell membrane potential was correlated with enhanced Cd^{2+} influx, but we found no difference in membrane potential values between Fe-deficient and Fe-sufficient pea seedlings (−102 ± 4 mV for +Fe-grown seedlings versus −95.5 ± 4 mV for −Fe-grown seedlings; n = 21).

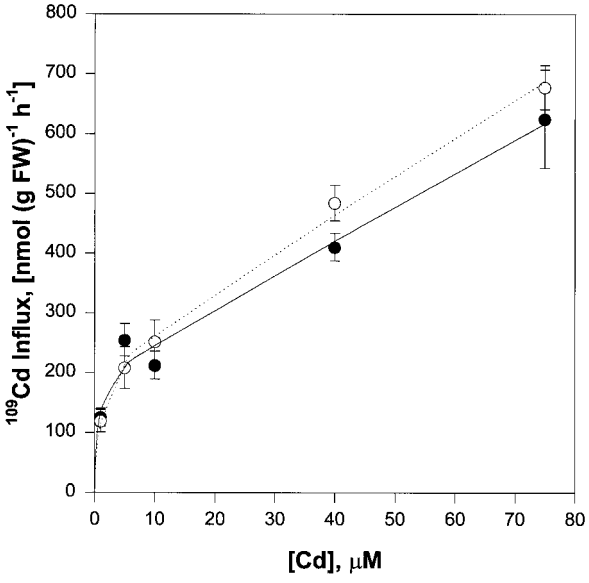

Figure 6. Concentration-dependent kinetics of root ^{109}Cd influx in −Fe-grown pea seedlings in buffered (10 mM Mes-Tris, pH 6.0) and unbuffered uptake solution. After excision, root segments were pretreated for 30 min in buffered (10 mM; ○) or unbuffered (0 mM; ●) solutions, placed in fresh buffered or unbuffered radiolabeled uptake solution for 20 min, and subsequently desorbed for 15 min. Values are means expressed as nmol Cd^{2+} (g fresh weight [FW])$^{-1}$ h^{-1} (n = 4). Error bars represent SE.

Role of Fe-Transporter Induction in Stimulating Cd^{2+} Influx

IRT1, a gene that encodes an Fe^{2+} transporter presumably involved in Fe acquisition in nongrass plants, was recently cloned in Arabidopsis (Eide et al., 1996). We used PCR to obtain an IRT1 genomic clone from Arabidopsis. This clone was sequenced and found to be identical to the *IRT1* cDNA (Eide et al., 1996), with the addition of two introns. Northern-blot analysis with the *IRT1* genomic clone revealed hybridization to polyadenylated RNA from Fe-deficient but not Fe-sufficient roots of both Arabidopsis and pea seedlings (Fig. 7). Our results also corroborate the findings of Eide et al. (1996) showing specificity of *IRT1* expression to roots but not to shoots of −Fe Arabidopsis (data not shown). This result suggests that expression of the *IRT1* homolog in pea is induced in an Fe-deficiency-dependent fashion as observed in Arabidopsis, and this could account for the enhanced ability of Fe-deficient pea seedling roots to absorb Cd^{2+}.

Role of the Plasma Membrane Ferric Reductase in Stimulating Cd^{2+} Influx

We explored the possibility that redox modification of a divalent cation carrier may be involved in stimulated uptake in −Fe-grown plants via enhanced activity of the plasma membrane ferric reductase (Welch et al., 1993). One might expect that if a stimulated ferric reductase either directly or indirectly altered the redox status of sulfhydryl groups that play a role in the functioning of a divalent cation transporter, then Cd^{2+} influx via this system would be sensitive to inhibition by sulfhydryl-binding compounds. However, we found that the enhanced Cd^{2+} influx in roots of Fe-deficient seedlings was unaffected by the inclusion of either permeant (0.3 mM *N*-ethylmaleimide) or impermeant (2 mM *p*-chloromercuribenzene sulfonic acid) sulfhydryl-modifying reagents in the uptake solution (data not shown). Sulfhydryl-containing reducing agents such as DTT and β-mercaptoethanol were not used for these experiments because they could potentially interfere with uptake by binding to Cd^{2+}.

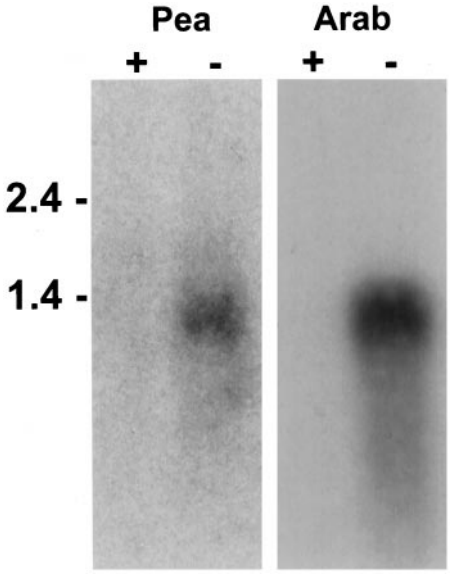

Figure 7. Regulation of *IRT1* mRNA levels by Fe availability in Arabidopsis and pea seedlings. Polyadenylated RNA from ± Fe-grown roots of Arabidopsis (1.2 μg) and pea (1.9 μg) was probed with the Arabidopsis *IRT1* genomic clone. RNA was prepared from 21-d-old Arabidopsis plants grown for 5 d without Fe or 13-d-old pea plants grown for 4 d without Fe.

In yeast it has been suggested that Fe deficiency increases the number of reduced sulfhydryl groups on the outer face of the plasma membrane, which might play a role in the uptake of Fe and other mineral ions (Lesuisse and Labbe, 1992). We found that Fe deficiency elicited a 50% increase in reduced sulfhydryls on the root surface (0.15 ± 0.004 μmol reduced sulfhydryl groups [g fresh weight]$^{-1}$ for −Fe-grown roots versus 0.10 ± 0.007 μmol g^{-1} fresh weight for +Fe-grown roots).

Cd Accumulation in Roots and Shoots

The concentrations of various metals in roots and shoots of seedlings exposed to 0.2 μM $Cd(NO_3)_2$ for 2 d are presented in Table II. Enhanced Cd accumulation was apparent in roots but not in shoots of Fe-deficient seedlings. An approximately 2-fold greater Cd concentration was observed in the roots of Fe-deficient plants relative to Fe-sufficient plants. In shoots, however, Cd levels were lower in the Fe-deficient plants relative to control plants. Zn followed the same pattern as Cd, exhibiting enhanced accumulation only in roots of Fe-deficient plants and slightly lower concentrations relative to the control plants in the shoots. The concentrations of certain other metals, such as Cu and Mn, increased in both roots and shoots of Fe-deficient plants relative to control plants.

DISCUSSION

This study provides evidence for the induction of transporter-mediated root Cd^{2+} influx in Fe-deficient pea seedlings. The effect of Fe deficiency on the concentration-dependent kinetics of root Cd^{2+} influx was a specific and significant stimulation of a saturable Cd^{2+} influx (Figs. 1 and 2). This result is consistent with a model in which transporters are deployed at a greater density on the plasma membrane under Fe-deficient conditions, or existing transporters are modified to exhibit higher activity. Because the kinetics for Cd^{2+} influx were complex and appeared to be the sum of saturable and linear functions, a number of different approaches were used to determine the mechanistic basis for these two kinetic components of Cd^{2+} influx.

Several pieces of evidence indicated that the saturable system represented true trans-plasma membrane Cd^{2+} influx. First, the selective abolition of the saturable component by the metabolic inhibitors CCCP and KCN provides strong evidence that saturable, transporter-mediated uptake is distinct from apoplastic Cd^{2+} binding and accounts for the differences in Cd^{2+} influx between Fe-sufficient and Fe-deficient plants. Also, La, a Ca^{2+}-channel blocker that also exhibits very high affinity for negatively charged sites

Table II. *Tissue metal accumulation in ± Fe-grown pea seedlings exposed to 0.2 μM Cd*

Seedlings were grown hydroponically in modified Johnson's nutrient solution. Fe was withheld from the growth solutions of −Fe seedlings on d 9. On d 12, 0.2 μM $Cd(NO_3)_2$ was added to the growth solution. Tissue was harvested on d 14. Values represent means (n = 3). Values in parentheses represent SE.

Treatment	Tissue	Content			
		Cd	Zn	Cu	Mn
		μg (g dry wt)$^{-1}$			
Control	Shoots	4.2 (0.1)	171 (12)	10 (0.3)	75 (7.5)
−Fe	Shoots	1.5 (0.2)	125 (13)	21 (2.4)	102 (11)
Control	Roots	147 (50)	250 (62)	29 (6.4)	150 (29)
−Fe	Roots	279 (6.5)	478 (21)	213 (9.8)	244 (44)

on the cell wall, partially inhibited both the saturable and the linear components of Cd^{2+} influx. This result further reinforces the existence of two discrete components of influx. We would expect La to both interfere with transporter-mediated uptake and, as a trivalent cation, to bind more effectively than Cd^{2+} to cell wall surfaces. Finally, only a linear kinetic phase of $^{109}Cd^{2+}$ binding was observed with morphologically intact root cell wall preparations. This linear component was similar in root cell walls of Fe-sufficient and Fe-deficient plants. Together, these results provide strong evidence for Fe-deficiency-mediated induction of a saturable Cd^{2+}-transport system, with linear Cd^{2+} "uptake" reflecting apoplastic Cd^{2+} binding that remains after desorption. The linear component of Cd^{2+} binding most likely consists of ionic interaction of Cd^{2+} with carboxyl and/or sulfhydryl groups contained in cell wall constituents such as hemicelluloses and cell wall proteins. Longer desorption periods might eventually remove this bound Cd^{2+} from the cell wall, but uptake and desorption periods must be kept short to ensure that unidirectional influx, and not efflux, of radiolabeled Cd is being measured. Although much of the Cd^{2+} is only weakly bound to the cell wall by electrostatic forces, a portion of the apoplastically bound Cd^{2+} may be irreversibly bound, because Cd^{2+} could form covalent bonds with sulfhydryl and other functional groups within the cell wall.

Although roots of Fe-deficient plants exhibit enhanced Cd uptake during both short-term radiotracer studies and long-term tissue-accumulation experiments, shoots from Fe-deficient seedlings exhibited lower Cd accumulation than control treatments in long-term experiments. When a number of different micronutrient metals were measured in Fe-deficient plants, Cd and Zn accumulation was found to be stimulated in roots but not in shoots; however, Cu and Mn accumulation was stimulated in both roots and shoots. We do not yet fully understand what governs the differential distribution of metals in plant tissues. Nicotianamine, a nonprotein amino acid thought to regulate root and shoot metal levels, could be involved in mediating Cd partitioning to the different tissues. In addition, variable phytochelatin affinity for specific metals such as Cu or Cd could also determine how much of each metal is translocated to the shoot. For example, much of the Cd entering roots of Fe-deficient plants could be sequestered in the vacuole as the Cd-phytochelatin complex and would not be available for translocation to the shoot.

Possible Mechanisms of Enhanced Cd^{2+} Uptake

We explored three potential scenarios that could explain the observed induction of heavy-metal transport in Fe-deficient pea seedlings: induction of the proton pump, induction of a divalent cation transporter, and induction of the ferric reductase. Our investigations into the mechanistic basis for the Fe-deficiency-induced stimulation of heavy-metal influx in pea point to the induction of a divalent cation transporter as well as the possible involvement of sulfhydryl modification.

We investigated the possibility that enhanced heavy-metal accumulation in response to Fe deficiency involves the increased activity of the plasma membrane H^+ pump, either through generation of a H^+ gradient or through hyperpolarization of the E_m. To test whether the H^+ pump or consequences of the pump play a key role in energizing heavy-metal absorption, we used buffers to abolish the enhanced H^+ gradient in Fe-deficient plant roots, and FC to stimulate the H^+-ATPase in Fe-sufficient plant roots. We also compared measurements of root-cell membrane potentials in ± Fe-grown pea seedlings to determine if H^+-ATPase-mediated hyperpolarization of the root-cell membrane potential could stimulate Cd^{2+} influx. Neither treatment had any effect on heavy-metal absorption, and membrane potentials were found to be the same in ± Fe-grown seedlings. We concluded that the proton pump does not appear to be involved in enhancing heavy-metal accumulation under Fe deficiency.

We also investigated whether enhanced activity of the Fe-deficiency-induced plasma membrane ferric reductase may stimulate heavy-metal accumulation, perhaps by modulating the redox state of a divalent cation transporter. Modification of the redox status of critical sulfhydryl groups involved in the gating of ion-channel proteins has been reported in the literature and is a potential mechanism for reductase-mediated regulation in absorption. Bertl and Slayman (1990) described a cation channel in the yeast vacuolar membrane that required very high (and non-physiological) Ca^{2+} levels for activation (1 mM), but was activated by much lower levels of Ca^{2+} when exposed to sulfhydryl-reducing agents such as DTT or β-mercaptoethanol. In addition, Lesuisse and Labbe (1992) reported in yeast that Fe deficiency resulted in higher levels of reduced sulfhydryl groups at the exofacial plasma membrane surface, as well as higher concentrations of GSH

within the cell. They speculated that the yeast ferric reductase may play a general role in regulating the redox status of cells, in addition to facilitating Fe uptake. Welch et al. (1993) further hypothesized that the plasma membrane ferric reductase may serve a regulatory function in gating channels involved in divalent-cation influx, either directly, by affecting the redox status of critical sulfhydryl groups in the membrane, or indirectly, by increasing levels of GSH in the cell, which could in turn influence ion channels. However, recent work with a recessive mutant in Arabidopsis, *frd1*, in which ferric reductase activity is impaired, suggests that on the contrary, the ferric reductase may not be involved in regulating divalent cation influx. Under Fe deficiency, *frd1* exhibits accumulation of Mn, Cu, and Zn to levels even higher than that of the Fe-deficient wild type (Yi and Guerinot, 1996). It is clear that this area awaits further research.

In this study we used roots treated with sulfhydryl-modifying agents to test whether redox modification is involved in stimulating metal influx, and we also compared exofacial reduced sulfhydryl groups in roots of ± Fe-grown pea seedlings. The significant increase in reduced sulfhydryl groups on the root surface of −Fe-grown pea seedlings suggests that some type of sulfhydryl modification of plasma membrane components may occur under Fe deficiency and may partially account for the enhanced Cd influx.

Molecular studies of the recently cloned Fe^{2+}-transporter gene *IRT1* from Arabidopsis provide compelling support for the induction of a divalent-cation transporter by plant Fe status (Eide et al., 1996). *IRT1* encodes a high-affinity Fe^{2+} transporter that functionally complements yeast mutants defective in both high- and low-affinity Fe transport. *IRT1* is highly expressed under Fe deficiency and possibly transports other divalent cations, including Cd, Co, Mn, and Zn. To determine whether the *IRT1* ortholog in pea is also induced by Fe deficiency, we used an *IRT1* genomic clone from Arabidopsis to probe root poly(A^+) mRNA from ± Fe-grown pea and Arabidopsis. This clone hybridized to an approximately 1.3-kb transcript from both pea and Arabidopsis roots that is induced under Fe deficiency. This result suggests that induction of a Fe transporter could account for enhanced uptake of divalent cations, including Cd, in Fe-deficient plants.

IRT1 is a member of a family of closely related transporter genes from a diverse group of eukaryotic organisms, including fungi, yeast, nematodes, plants, and mammals. This family also includes the yeast Zn-regulated transporters *ZRT1* and *ZRT2* (Zhao and Eide, 1996a, 1996b). It is interesting that, although *IRT1* is a functional Fe transporter in yeast mutants defective in Fe transport, the predicted amino acid sequence of *IRT1* has no similarity to that of endogenous yeast Fe transporters (Askwith et al., 1994; Dix et al., 1994; Eide et al., 1996; Stearman et al., 1996). Yeast has two separate Fe-uptake pathways, a high-affinity (K_m = 0.15 μM) and a low-affinity (K_m = 40 μM) Fe transporter. The *FTR1* gene product, a putative high-affinity transporter that is highly selective for ferric Fe, interacts with the *FET3* gene product, a multicopper oxidase localized to the plasma membrane, to facilitate high-affinity Fe^{3+} transport (Askwith et al., 1994; Stearman et al., 1996). The *FET4* gene in yeast is required for low-affinity Fe uptake and encodes a ferrous transporter that can also transport other metals such as Co^{2+} and Cd^{2+} (Dix et al., 1994). Yeast *fet3fet4* mutants defective in both high- and low-affinity Fe-transport systems are extremely sensitive to Fe limitation but can be functionally complemented with the Arabidopsis *IRT1* gene.

The transporter activity we have characterized in the roots of Fe-deficient pea seedlings exhibits high substrate affinity (0.9–1.5 μM) for Cd. As mentioned in "Results," this Fe-induced system also effectively transports Zn. Based on our result that Fe deficiency induces *IRT1* expression and on our preliminary results indicating that a pea *IRT1* ortholog complements yeast Fe- and Zn-transport mutants, we speculate that Cd may be transported by a high-affinity Fe transporter. A future research priority will be to test whether yeast Fe-transport mutants bearing the pea *IRT1* ortholog have the ability to transport a range of other heavy metals. This future work should more comprehensively address the question of the transport specificity of this system; that is, in plants using a reductase-based mechanism of Fe uptake, is the Fe^{2+} transporter specific for Fe or can it transport other micronutrients and/or heavy metals? Our findings of a correlation between enhanced Cd influx and induction of a high-affinity Fe transporter under Fe deficiency suggest that the Fe transporter might be a relatively nonspecific divalent-cation transporter. A more thorough characterization of this transporter will have important implications for both plant-micronutrient nutrition and heavy-metal remediation of contaminated soils using plants.

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