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Direct Measurement of ^{59}Fe-Labeled Fe^{2+} Influx in Roots of Pea Using a Chelator Buffer System to Control Free Fe^{2+} in Solution[1]

Tama C. Fox*, Jon E. Shaff, Michael A. Grusak, Wendell A. Norvell, Yona Chen, Rufus L. Chaney, and Leon V. Kochian

United States Plant Soil and Nutrition Laboratory, United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Cornell University, Ithaca, New York 14853 (T.C.F., J.E.S., W.A.N., L.V.K.); USDA-ARS, Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, 1100 Bates Street, Houston, Texas 77030–2600 (M.A.G.); Department of Soil and Water Sciences, Faculty of Agriculture, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel (Y.C.); and USDA-ARS, Environmental Chemistry Laboratory, Beltsville, Maryland 20705 (R.L.C.)

Fe^{2+} transport in plants has been difficult to quantify because of the inability to control Fe^{2+} activity in aerated solutions and nonspecific binding of Fe to cell walls. In this study, a Fe(II)-3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′4″-disulfonic acid buffer system was used to control free Fe^{2+} in uptake solutions. Additionally, desorption methodologies were developed to adequately remove nonspecifically bound Fe from the root apoplasm. This enabled us to quantify unidirectional Fe^{2+} influx via radiotracer (^{59}Fe) uptake in roots of pea (*Pisum sativum* cv Sparkle) and its single gene mutant *brz*, an Fe hyperaccumulator. Fe influx into roots was dramatically inhibited by low temperature, indicating that the measured Fe accumulation in these roots was due to true influx across the plasma membrane rather than nonspecific binding to the root apoplasm. Both Fe^{2+} influx and Fe translocation to the shoots were stimulated by Fe deficiency in Sparkle. Additionally, *brz*, a mutant that constitutively exhibits high ferric reductase activity, exhibited higher Fe^{2+} influx rates than +Fe-grown Sparkle. These results suggest that either Fe deficiency triggers the induction of the Fe^{2+} transporter or that the enhanced ferric reductase activity somehow stimulates the activity of the existing Fe^{2+} transport protein.

It was first suggested in 1937 that plants take up Fe preferentially as Fe^{2+} cations rather than Fe^{3+} complexes (Kliman, 1937). Since then, it has become clear that there are at least two Fe acquisition mechanisms in plants. Grasses (Poaceae) appear to absorb certain Fe(III)-chelates directly from the soil. Dicots and nongrass monocots, however, take up Fe via a two-step mechanism involving the reduction of Fe(III)-chelates followed by the influx of free Fe^{2+} ions (reviewed by Kochian, 1991). Support for the reductive uptake mechanism first came from Brown et al. (1971), who found that BPDS, a strong Fe(II)-chelator, inhibited Fe uptake into tomato roots. Chaney et al. (1972) demonstrated more conclusively that excess BPDS inhibited Fe uptake into soybeans by more than 99%. Allnutt and Bonner (1987b) and Varanini and Maggioni (1982) demonstrated a similar BPDS-mediated inhibition of Fe uptake into *Chorella* and *Vitis*, respectively. Römheld and Marschner (1983), using doubly labeled ^{59}Fe(III)-[^{14}C]EDDHA, presented strong evidence that Fe accumulated in roots, whereas the EDDHA was not rapidly absorbed. Similar patterns were also seen in *Chlorella* using ^{55}Fe-[^{14}C]citrate or ^{55}Fe-[^{14}C]rhodotorulic acid (Allnutt and Bonner, 1987a) and in *Vigna unguiculata* leaf mesophyll using ^{55}Fe-[^{14}C]citrate (Brüggemann et al., 1993). These and other results support a model for nonpoaceous plants in which Fe is first reduced at the cell surface followed by the influx of free Fe^{2+} into the cytosol. In this model, transplasma membrane Fe(III)-chelate reduction functions to remove Fe from its chelator and increase the activity of Fe^{2+} at the cell surface. The Fe^{2+} would then be transported into the cytosol via a separate transport protein.

Whereas Fe(III)-chelate reduction has been extensively characterized (reviewed by Moog and Bruggemann, 1995), Fe^{2+} influx has not. Two experimental limitations have previously prevented characterization of Fe^{2+} transport in multicellular roots: (a) Fe^{2+} activity is difficult to control because of the rapid reoxidation of Fe^{2+} to Fe^{3+} in aerobic environments (Stumm and Lee, 1961), and this makes it difficult to ascertain to what activity of Fe^{2+} plants are exposed; and (b) ^{59}Fe nonspecifically binds to cell walls, complicating measurements of Fe accumulation in roots. Thus, Fe influx into roots will be overestimated unless appropriate techniques are used to remove cell-wall Fe without compromising Fe transport into the symplasm.

The experimental decoupling of Fe^{2+} influx from Fe(III)-chelate reduction is critical for investigating the mecha-

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* Corresponding author; e-mail tcf2@cornell.edu; fax 1–607–255–2459.

Abbreviations: BPDS, bathophenanthrolinedisulfonic acid; EDDHA, *N,N′*-ethylenebis[2-(2-hydroxyphenyl)-glycine]; ferrozine, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′4″-disulfonic acid.

nism(s) and regulation of Fe^{2+} acquisition. Previous studies of radiotracer Fe influx have generally involved the use of radiolabeled Fe(III)-chelates in which Fe accumulation depends on both Fe^{3+} reduction and Fe^{2+} influx (Chaney et al., 1972; Römheld et al., 1982; Römheld and Marschner, 1983; Allnutt and Bonner, 1987a; Chaney and Bell, 1987; Grusak et al., 1990a, 1990b). Recent developments of chelate buffer systems, which allow for the control of free metal ions in solution, now enable us to investigate Fe^{2+} influx while bypassing the Fe(III)-chelate reductase. Thus, we can design uptake solutions in which ^{59}Fe-labeled Fe^{2+} solutions are controlled at physiologically relevant activities. Just as pH buffers control the activity of free H^+ in solution via a combination of protonated and deprotonated forms of the buffer molecule, metal-chelates combined with excess free chelates can be used to buffer the activity of free metals in solution. Parker et al. (1995) used the computer speciation program GEOCHEM-PC to calculate free metal activities in the presence of chelators, buffers, and salts, while also taking into account pH and potential redox interactions. In this study, we used GEOCHEM-PC (Parker et al., 1995) and the Fe^{2+} chelator ferrozine to create solutions that buffer free Fe^{2+} in the nanomolar range, allowing $^{59}Fe^{2+}$ influx to be quantified while bypassing the root plasma membrane Fe(III)-chelate reductase. Nanomolar activities of free Fe^{2+} were chosen to reflect typical 0.1 to 1000 nM levels of Fe found in bulk soil solutions (Lindsay, 1991). This contrasts with most studies of Fe reduction and uptake, in which micromolar concentrations of Fe(III)-chelates were used (Chaney et al., 1972; Römheld et al., 1982; Römheld and Marschner, 1983; Allnutt and Bonner, 1987b; Chaney and Bell, 1987; Grusak et al., 1990a, 1990b).

In this study, we tested the validity of the ferrozine-buffered uptake solutions as a tool for quantifying Fe^{2+} influx and tested the efficacy of our desorption regime in removing nonspecifically bound Fe from roots. Upon verification of the technique, we quantified Fe^{2+} absorption and translocation in Fe-sufficient and Fe-deficient roots of *Pisum sativum* cv Sparkle and its Fe-hyperaccumulating mutant *brz*.

MATERIALS AND METHODS

Plant Material

Pea (*Pisum sativum* L. cv Sparkle [*brz,brz*]), an Fe hyperaccumulator also called E107, was originally obtained from cv Sparkle via ethyl methanesulfonic acid mutagenesis (Kneen et al., 1990). Both genotypes were soaked overnight in aerated distilled water, germinated in the dark for 2 d between filter paper, and transferred to nutrient solution as described previously (Grusak et al., 1990a). Mes-KOH (1 mM) was used to buffer the nutrient solution at pH 5.5. Fe levels varied with genotype; 10 μM Fe(III)-EDDHA was used for Sparkle to ensure Fe sufficiency, whereas the Fe hyperaccumulator *brz* was grown on 1 μM Fe(III)-EDDHA until d 10 and on 2 μM Fe(III)-EDDHA thereafter. This minimized Fe toxicity (bronzing on leaves) without creating visible signs of chlorosis. For Fe-deficient plants, seedlings were transferred to solutions without Fe (−Fe) for the final 3 to 4 d of growth. A maximum of five plants was grown in individual, black, 5-L polyethylene buckets as previously described (Grusak et al., 1990a). Solutions were changed on d 10, d 13, and, if necessary, on d 16. Fe(III)-EDDHA was prepared as described by Chaney and Bell (1987).

From d 3 until initiation of uptake experiments, plants were grown in a controlled environment growth chamber with a photon flux density of 350 $\mu E\ m^{-2}\ s^{-1}$ under a 20°C, 16-h day/15°C, 8-h night cycle. Whole plants, decapitated root systems, or excised root units of 15- to 17-d-old plants were used for Fe influx experiments. Root units were excised from the older portion of the root system and consisted of a 5- to 20-mm segment of primary root with 2 to 10 attached lateral roots. Root units were between 0.15 to 0.5 g fresh weight. Roots were allowed to recover from cutting in aerated pretreatment solution containing 5 mM Mes-Tris, pH 5.0, and 0.2 mM $CaSO_4$ for a minimum of 30 min. This also helped rinse the nutrient solution from the roots. Pretreatment solution was then removed by vacuum withdrawal and replaced with radiolabeled uptake solution.

Preparation of ^{59}Fe-Labeled Fe^{2+} Uptake Solution

GEOCHEM-PC was used to formulate ferrozine-buffered Fe^{2+} solutions with free Fe^{2+} activities of approximately 25, 100, and 250 nM free Fe^{2+}. The Fe(II)-ferrozine$_3$ binding constant was determined to be $10^{17.0}$. Briefly, ferrozine was recrystallized several times for purification. The proton formation constant was measured by titration in a constant ionic strength tetramethylammonium nitrate buffer, pH buffered using Mes. Competition between protons and Fe^{2+} ions was used to estimate the Fe^{2+}-binding constant with ferrozine. The binding constant was then confirmed by titrating solutions with different levels of Fe^{2+} and excess ferrozine and by comparing predicted pH and Fe(II)-ferrozine$_3$ color formation with measured values.

All uptake solutions contained 0.2 mM $CaSO_4$, 5 mM Mes-Tris (pH 5.0), 1.5 μM $Na_2S_2O_4$ (dithionite), and either 0.025 or 0.1 μCi/mL $^{59}FeCl_3$. Varying levels of $FeSO_4$ and ferrozine were added to yield specific Fe^{2+} activities as indicated below.

Radiolabeled uptake solution was prepared by adding freshly made 20 mM $Na_2S_2O_4$ solution (concentration in final uptake solution was 1.5 μM) to the appropriate volume of 100 μCi/mL $^{59}FeCl_3$ in 19 mM HCl; this was incubated for 10 min at room temperature. Dithionite reduces Fe(III) to Fe(II). The appropriate volume of freshly prepared 5 mM $FeSO_4$ stock was subsequently added. The solution was then vigorously vortexed to oxidize excess dithionite (Lambeth and Palmer, 1973), equilibrated for 10 min at room temperature, and transferred to foil-wrapped plastic bottles containing a solution consisting of 5 mM Mes-Tris (pH 5.0), 0.2 mM $CaSO_4$, and the appropriate concentration of ferrozine. Solutions were equilibrated for a minimum of 2 h in the dark and used the same day for Fe^{2+} influx experiments.

Radiolabeled Fe contributed no more than 2% of the total Fe pool in the uptake solution but was included in all calculations of added Fe. A solution at 24.4 nM free Fe^{2+} activity was created by combining 2.5 μM Fe(II) with 17.44 μM ferrozine. Eleven micromolar Fe(II) and 43.0 μM ferrozine were combined to create a 97.0 nM free Fe^{2+} activity solution. Finally, 35.0 μM Fe(II) and 115.0 μM ferrozine yielded a 257.2 nM free Fe^{2+} activity solution.

In experiments aimed at verifying that roots absorb free Fe^{2+} and not the Fe(II)-ferrozine$_3$ complex, we used two recipes to produce uptake solutions with similar Fe^{2+} activities and widely different concentrations of Fe(II)-ferrozine$_3$. Thus, for these experiments, when free Fe^{2+} was buffered at 24 nM, the Fe(II)-ferrozine$_3$ concentration was either 2.5 μM Fe(II)-ferrozine$_3$ (2.5 μM Fe and 17.44 μM ferrozine, 9.94 μM excess ferrozine, 24.4 nM free Fe^{2+}) or 4.85 μM Fe(II)-ferrozine$_3$ (4.85 μM Fe and 27.0 μM ferrozine, 12.45 μM excess ferrozine, 24.3 nM free Fe^{2+}). For the solutions containing 97 nM free Fe^{2+}, the Fe(II)-ferrozine$_3$ concentration was either 11.0 μM (11.0 μM Fe and 43.0 μM ferrozine, 10.0 μM excess ferrozine, 97.0 nM free Fe^{2+}) or 20.0 μM (20 μM Fe and 72.25 μM ferrozine, 12.25 μM excess ferrozine, 97.3 nM free Fe^{2+}).

Root $^{59}Fe^{2+}$ Influx Experiments

Two different systems were used for the Fe^{2+} absorption experiments. For the experimental results depicted in Figures 2 to 4, black polyethylene buckets (2.2 L) were divided into six vertical sectors via nylon mesh. A black polyethylene cover held the nylon mesh in place such that root units were separated from each other and from a central "column" in which tubing was added to allow solutions to be removed via vacuum withdrawal. When we compared uptake rates using 1, 2, or 3 root units per sector, we found that up to 2 root units could be added per sector without decreasing Fe^{2+} absorption via intraroot and interroot competition for low (nanomolar) activities of Fe^{2+}. A plastic aerator was guided into each sector via holes in the bucket lid. Root units were allowed to recover from excision in aerated pretreatment solution for a minimum of 30 min. Pretreatment solution was then removed via vacuum withdrawal, and radiolabeled uptake solution was added to start absorption experiments. At the end of each uptake period, root units were removed from random sectors (all root units were removed from that sector to minimize disturbance of remaining roots) and placed into 1-L desorption buckets containing 600 mL of aerated desorption solution for a 10-s rinse. Root units designated for further desorption were then transferred to fresh desorption buckets for 10 min in aerated desorption solution or for two successive 10-min desorptions. At the end of the desorption period, root units were blotted with paper towels, the primary root segment was removed, and lateral roots were weighed. Absorption of ^{59}Fe into lateral roots was quantified via γ detection using a Packard Auto-Gamma 5530 Counter. For these experiments, the desorption solution consisted of 5 mM Mes-Tris, pH 5.0, 5 mM $CaCl_2$, 5 mM sodium ascorbate, and 0.1 mM $FeSO_4$. Sodium ascorbate and $FeSO_4$ were added just before use. The desorption solution was then readjusted to pH 5.0 using HCl. All components of the uptake apparatus were acid washed and thoroughly rinsed before use.

For the data presented in Figure 5, an uptake system consisting of Plexiglas uptake wells was used (Grusak et al., 1990a). The bottoms of the uptake wells were fitted with mesh-covered rubber stoppers to allow vacuum withdrawal of solutions without disturbing the roots. The uptake solutions (70 mL) were aerated via a Plexiglas tube placed near the bottom of the well. One root unit was placed in each well and allowed to recover from excision for a minimum of 30 min in aerated pretreatment solution. Pretreatment solution was evacuated and replaced with radiolabeled uptake solution to start the absorption experiments. At the end of the uptake period, radioactive solutions were replaced with desorption solution for a 10-s rinse. Desorption solution was then evacuated for removal of root units or for replacement with fresh desorption solution. For Figures 4 and 5 and Tables I and II, two successive 10-min desorptions followed the 10-s rinse. For Figure 1, desorption occurred over a 60-min period with solution changes at 10 s, 10 min, and 20 min. Two-milliliter aliquots were removed from the desorption solution at 2.5, 5, 10, 20, 30, 40, and 60 min, and the radioactivity was counted via γ counting. At the end of the desorption period, solutions were drained and root units were removed and blotted for weighing and γ counting as described above.

Whole Plant Experiments

In translocation experiments, intact plants (four plants/3.5-L polyethylene bucket) were placed for 30 min in pretreatment solution and then transferred to radiolabeled uptake solutions for 20 min. Plants were then transferred to desorption solution for 20 min as described above. Entire roots and shoots were separated, blotted, weighed, and counted on a γ counter. All treatments were done in an environmental growth chamber at 20°C, 50% humidity, and 350 μE m^{-2} s^{-1} PAR.

^{59}Fe was quantified in lateral roots rather than entire roots in experiments contrasting Fe influx in detopped (but otherwise intact) roots and excised root units that had been reassembled into the geometry of intact roots. Roots were separated into 7 to 10 root units, and then roots were carefully reassembled by passing a commercial needle and thread through the longitudinal axis of the primary root of each root unit. This approximated the original geometry of the lateral roots in intact plants. After the radiotracer uptake and desorption, primary roots (and thread) were excised and discarded. The lateral roots were counted on a γ counter.

RESULTS

Adequacy of ^{59}Fe Desorption Regimes

Preliminary experiments suggested that the removal of nonspecifically bound ^{59}Fe from root units was facilitated by low levels of both a reductant and nonradioactive Fe^{2+} in the desorption solution. In Figure 1, the efficacy of two

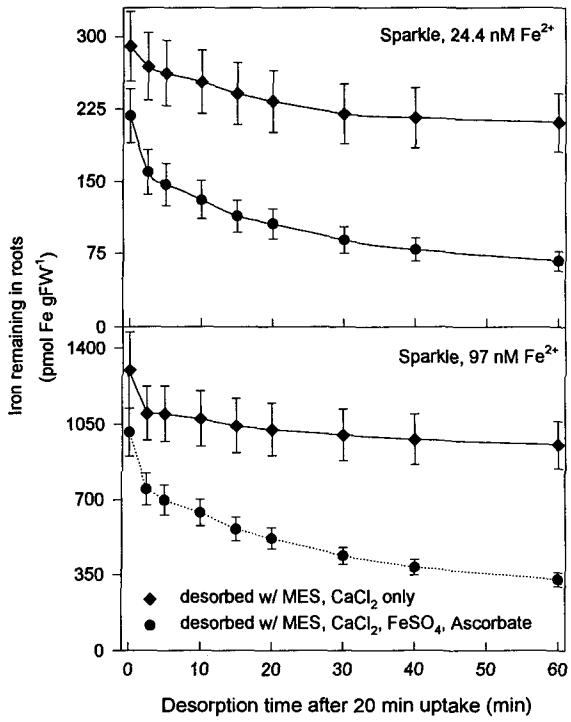

Figure 1. Desorption of Fe from the apoplasm of +Fe-grown Sparkle seedlings after 20 min of uptake in 24.4 or 97.0 nM free Fe^{2+}. Excised root units were briefly rinsed in desorption solution and then desorbed for up to 60 min. Solutions were changed at 10 and 20 min. Two-milliliter aliquots were taken from the desorption solution at each time for quantitation of ^{59}Fe. At 60 min, root units were blotted, and lateral roots were excised and weighed before γ counting. Values are expressed in pmol g^{-1} fresh weight (FW) and are means ± SE (n = 10).

different desorption solutions (± ascorbate and $FeSO_4$) to remove ^{59}Fe from Sparkle roots after a 20-min accumulation period in either 24.4 or 97.0 nM free Fe^{2+} is shown. Complete desorption solution containing 5 mM Mes-Tris, 5 mM $CaCl_2$, 5 mM sodium ascorbate, and 0.1 mM $FeSO_4$ removed much more Fe from root units than did desorption solution lacking ascorbate and $FeSO_4$. Most desorption occurred within the first 20 min, and we suggest that during this 20-min period primarily cell-wall ^{59}Fe was removed. Desorption from *brz* roots followed the same pattern (data not shown).

The amount of nonspecifically bound Fe removed from roots during the desorption period correlated with the activity of free Fe^{2+} ions in the uptake solution (Fig. 1). The amount of Fe desorbed from Sparkle and *brz* roots during the 20-min desorption period was 75 to 150 pmol Fe g^{-1} fresh weight following a 20-min absorption in 24.4 nM Fe^{2+}; this value was increased about 4-fold (300–500 pmol g^{-1} fresh weight per 20-min desorption) following a 20-min absorption in 97.0 nM Fe^{2+}.

Time courses of ^{59}Fe accumulation with no desorption or following one or two 10-min desorption periods are presented in Figure 2 (for accumulation from 24.4 nM Fe^{2+}) and Figure 3 (for accumulation from 97.0 nM Fe^{2+}). Theoretically, a time course of root accumulation for an ionic solute should consist of an initial rapid phase, representing entry into the apoplasm, followed by a slower linear phase, representing influx across the plasma membrane. This is what was seen for the time courses of accumulation for which the roots were only rinsed for 10 s after uptake (Figs. 2 and 3). Clearly, 10 s of desorption is inadequate for removing cell-wall radioiron, whereas a 10- or 20-min desorption period appears to remove most of the nonspecifically bound Fe. This holds true for both Sparkle and *brz* roots bathed in 24.4, 97.0, and 257.2 nM free Fe^{2+} (257.2 nM data not shown). When either a 10- or 20-min desorption period was used, the accumulation profile was linear over the first 30 to 60 min and extrapolated back to near the origin. The amount of Fe that was nonspecifically bound to the cell walls can be estimated by comparing the data in Figures 2 and 3 on Fe accumulation into root units after 10 s versus 20 min of desorption. The proportion of accumulated Fe that was adsorbed to the cell wall (as compared to Fe transport into the cytosol) was greatest for uptake from solutions with the lower Fe^{2+} activities, constituting about one-half of the total accumulated Fe for *brz* and Sparkle at 24.4 nM (Fig. 2) and Sparkle at 97.0 nM (Fig. 3) and less than one-third of the total accumulated Fe for *brz* at 97.0 nM and for *brz* and Sparkle at 257.2 nM Fe^{2+} (257.2 nM data not shown). This difference is likely due to a saturation of potential cell-wall-binding sites for Fe at higher free Fe^{2+} activities during the 20-min accumulation period.

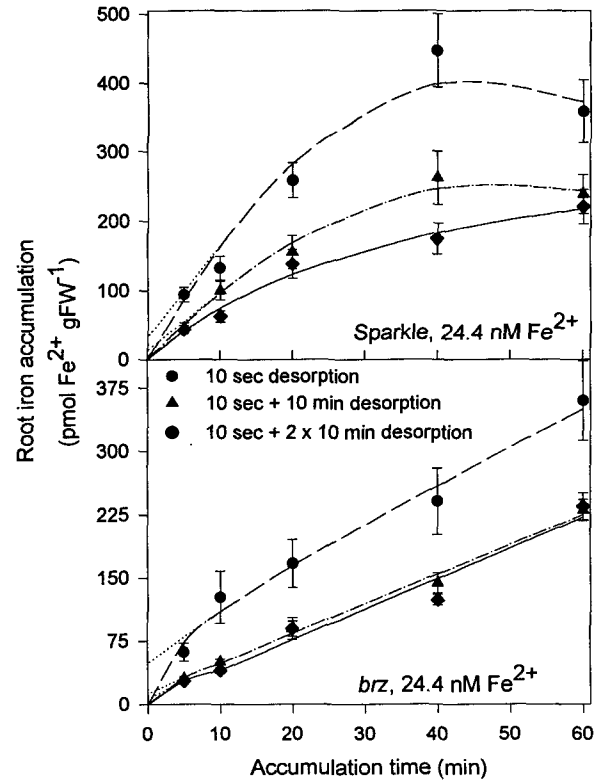

Figure 2. Time course of Fe^{2+} accumulation in +Fe-grown Sparkle and *brz* seedlings. Excised root units were placed in 24.4 nM Fe^{2+} uptake solution for up to 60 min and then desorbed for 10 s, 10 min, or 20 min. Values are expressed as pmol g^{-1} fresh weight (FW) and are means ± SE (Sparkle, n = 12; *brz*, n = 8).

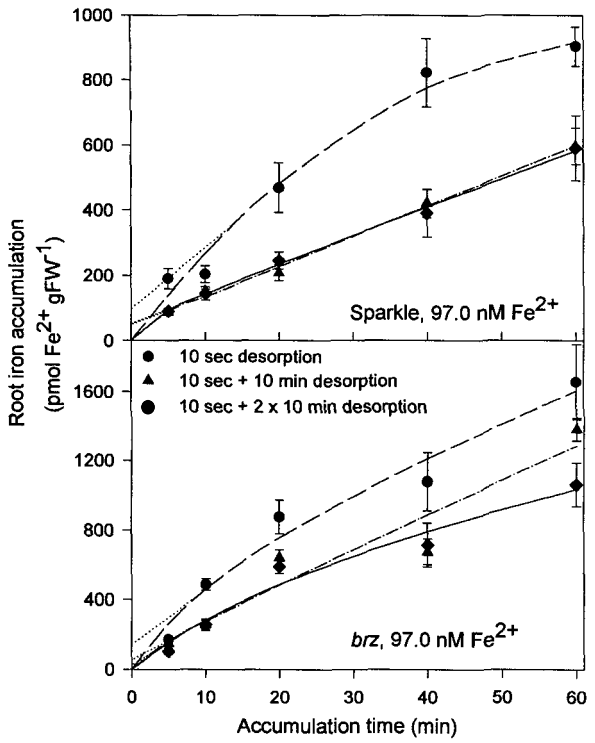

Figure 3. Time course of Fe^{2+} accumulation in +Fe-grown Sparkle and *brz* seedlings. Excised root units were placed in 97.0 nM Fe^{2+} uptake solution for up to 60 min and then desorbed for 10 s, 10 min, or 20 min. See Figure 2 legend for details. Values are expressed as pmol g^{-1} fresh weight (FW) and are means ± SE (Sparkle, n = 8–12; *brz*, n = 15–17).

These data suggest that a 20-min desorption period in a solution containing ascorbate and nonradioactive Fe^{2+} is adequate for removing most Fe nonspecifically adsorbed to the cell walls. Thus, a 20-min desorption period was used for all subsequent uptake and translocation experiments.

Fe^{2+} Influx Depends on Temperature

Fe influx was measured concurrently at either 22 or 2°C. Cold-treated root units were incubated in precooled (2°C) pretreatment, uptake, and desorption solutions. As shown in Figure 4, Fe^{2+} uptake at 2°C was dramatically inhibited in Sparkle and *brz* root units (approximately 70% inhibition). A similar degree of inhibition occurred for uptake from solutions containing either 24.4 or 97.0 nM free Fe^{2+}. These data suggest that Fe^{2+} influx depends on metabolism and, along with the data from the previous desorption experiments (Figs. 1–3), suggest that the Fe^{2+} accumulation measurements as described in this study represent Fe^{2+} influx across the plasma membrane rather than nonspecific binding of Fe to the roots.

Fe^{2+} Influx Correlates with Free Fe^{2+} Activity

Because the uptake solutions contained low activities of Fe^{2+} ion and much higher levels of Fe(II)-ferrozine$_3$, an

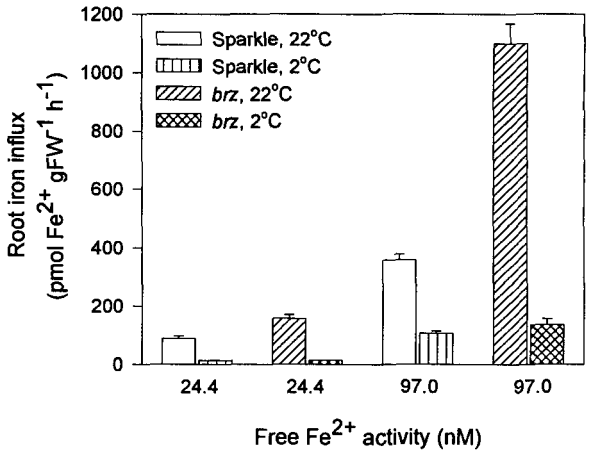

Figure 4. Comparison of Fe^{2+} influx at 22 or 2°C in excised root units from +Fe-grown Sparkle and *brz* seedlings. Pretreatment, uptake, and desorption solutions were precooled to 2°C or kept at 22°C. After a 30-min recovery from excision in 22°C pretreatment solution, 2°C root units were precooled for 30 min, incubated in uptake solution for 20 min, and then desorbed twice for 10 min each time (all procedures at 2°C). Values are expressed as pmol g^{-1} fresh weight (FW) h^{-1} and are means ± SE (n = 5).

experiment was conducted to verify that Fe^{2+}, and not Fe(II)-ferrozine$_3$, was the Fe species transported into roots. Figure 5 indicates that Fe influx was identical in roots bathed in the two uptake solutions in which free Fe^{2+} was approximately 24 nM, but total Fe(II)-ferrozine$_3$ was either 2.5 or 4.85 μM (varying the total Fe and excess ferrozine to create similar free Fe^{2+} activities). Similarly, no significant differences were observed for Fe^{2+} influx when measurements were made using solutions containing a free Fe^{2+} activity of approximately 97 nM free Fe^{2+}, with total Fe(II)-ferrozine$_3$ at either 11 or 20 μM. Thus, Fe^{2+} influx clearly

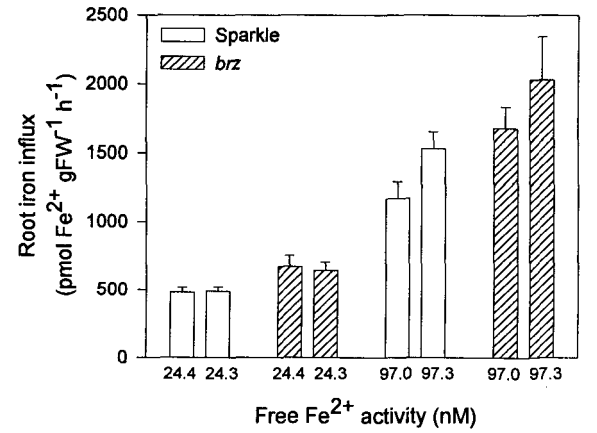

Figure 5. Comparison of Fe^{2+} influx using two chelate buffers that yield similar Fe^{2+} activities with very different (2-fold) concentrations of Fe(II)-ferrozine$_3$. The similar influx of Fe^{2+} from both chelate-buffered solutions for a particular Fe^{2+} activity suggests that Fe^{2+} and not Fe(II)-ferrozine$_3$ was transported across the root cell plasma membrane. Values are expressed as pmol g^{-1} fresh weight (FW) h^{-1} and are means ± SE (n = 10).

correlates with the amount of free Fe^{2+} ions in solution rather than the total chelated Fe concentration.

Fe Is Translocated during Short-Term Fe Influx Experiments

Fe influx and translocation was quantified in intact pea plants grown in the presence or absence of Fe (Table I). The amount of Fe absorbed and the percentage of Fe translocated to the shoots depended on the physiological state of the seedlings. Fe-deficient Sparkle plants were more active in taking up Fe than were Fe-sufficient Sparkle plants. A slight decrease in influx occurred in Fe-deficient *brz* plants, as compared to Fe-sufficient *brz* plants, yet both Fe-sufficient and Fe-deficient Fe-hyperaccumlating *brz* seedlings exhibited constitutively higher Fe influx than Fe-sufficient Sparkle plants.

Both Sparkle and *brz* seedlings, grown in the presence or absence of Fe, translocated a significant proportion of Fe (5–16%) during the relatively brief 20-min uptake/20-min desorption periods (Table I). Fe-deficient Sparkle seedlings translocated a higher percentage of absorbed Fe to the shoots (12–14%), compared to Fe-sufficient Sparkle seedlings (6–9%). Fe-sufficient *brz* plants again were similar to Fe-deficient Sparkle plants in that they translocated a higher percentage of absorbed Fe to the shoots (14–16.5%).

Fe^{2+} Accumulation Differs in Cut Root Units versus Intact Plants

The data in Figures 1 to 5 were obtained using excised root units, consisting of a portion of the older primary root with 3 to 10 lateral roots attached. Intact plants, however, were used for the translocation experiments shown in Table I. We found that influx rates were generally higher in excised root units compared to intact plants. This could potentially be due to either excision injury or a decrease in intraroot competition for Fe^{2+} ions. To test this, we measured Fe^{2+} influx in whole, decapitated root systems as compared to decapitated root systems that were excised into 7 to 10 root units and then reassembled back into the original geometry by sewing the primary root segments together (Table II). Intact plants have roots that are geometrically close to each other as compared to the 3 to 10 lateral roots contained in the root unit. On the other hand, excision injury could influence Fe^{2+} influx in excised root units. It should be noted that decapitation of the entire root system had no effect on root Fe^{2+} influx (data not shown). These results indicate that excision injury of the primary root did not stimulate Fe^{2+} influx into the lateral roots attached to that primary. Instead, the stimulated Fe^{2+} influx in excised root units versus whole root systems is probably due to reduced intraroot competition for the diffusion-limited substrate (Fe^{2+} ions).

Table I. *Measurement of root Fe^{2+} influx and transport to the shoot of intact pea seedlings*

Fe influx was quantified in whole roots and shoots. Fe influx units are expressed as pmol g^{-1} fresh wt h^{-1} and are means ±SE.

Type	n[a]	Free Fe^{2+}	Fe Uptake	Percentage in Shoots
		nM		
+Fe Sparkle	7	24.4	44.1 ± 3.9	6.0 ± 0.9
−Fe Sparkle	4	24.4	142.9 ± 5.3	14.1 ± 2.7
+Fe *brz*	5	24.4	115.2 ± 14.6	14.0 ± 0.7
−Fe *brz*	4	24.4	77.8 ± 6.4	5.2 ± 0.3
+Fe Sparkle	4	97.0	152.9 ± 11.1	9.0 ± 1.7
−Fe Sparkle	8	97.0	402.2 ± 14.5	12.0 ± 1.0
+Fe *brz*	4	97.0	443.8 ± 26.2	16.5 ± 0.4
−Fe *brz*	4	97.0	273.5 ± 15.0	7.2 ± 0.3

[a] *n*, Number of replicate measurements.

Table II. *Measurement of root Fe^{2+} influx in detopped intact or excised pea roots from 97.0 nM free Fe^{2+} uptake solutions*

Fe^{2+} influx into detopped but otherwise uninjured roots was compared with roots that were cut into 7 to 10 root units and then reassembled back to the original geometry of the intact root system by sewing the primary root material together with needle and thread. Fe influx was quantified in entire lateral roots only. Fe influx units are expressed in pmol g^{-1} fresh wt h^{-1} and are means ±SE (n = 5).

Type	Detopped Roots	Reassembled Roots
Sparkle	456 ± 131	711 ± 95
brz	1249 ± 142	1422 ± 159

DISCUSSION

Uncoupling Fe^{2+} influx from Fe(III)-chelate reduction is critical for elucidating and characterizing the mechanism(s) of Fe^{2+} influx in organisms that use the reduction-based form of Fe uptake. We used the computer speciation program GEOCHEM-PC to design uptake solutions in which the free Fe^{2+} ion activity was buffered at physiologically relevant levels; this allowed us to use radiotracer flux techniques to study root Fe^{2+} influx.

Removal of apoplasmic ^{59}Fe is critical to accurately quantify Fe^{2+} influx into roots. Previous studies have indicated that ^{59}Fe can bind tightly to root cell walls. For example, Römheld and Marschner (1983) incubated isolated cell-wall material from both monocot and dicot roots, including Fe-sufficient and Fe-deficient peanut, and +Fe-sufficient sunflower, corn, barley, and sorghum in a solution containing doubly labeled ^{59}Fe(III)-[^{14}C]EDDHA. ^{59}Fe strongly labeled the cell-wall material after a 10-h incubation, whereas ^{14}C-labeling was 10% or less of Fe binding, suggesting that the Fe was separated from its strong chelator before and/or during cell-wall binding. In addition, the binding of Fe to the cell walls correlated with the cation exchange capacity of the cell-wall material. We developed a desorption solution methodology that removes most nonspecifically bound Fe from the apoplasm, allowing a more accurate quantitation of Fe^{2+} transport across the root-cell plasma membrane. The method presented here bypasses the need to subtract 2°C data to correct for nonspecific binding of ^{59}Fe to cell walls (Allnutt and Bonner, 1987a) while accurately determining Fe^{2+} uptake into complex higher plant roots.

Ascorbate facilitates removal of Fe from the root, presumably by chelation and/or reduction of oxidized Fe in the cell wall. The mechanism of Fe oxidation/precipitation onto the root surface during the uptake period is not

known at this time. Even though the Fe(II)-chelate buffer system should tend to keep Fe in the reduced Fe(II) state, there must be physiological and/or chemical processes occurring within the cell wall that facilitate Fe oxidation and precipitation. At this time, to our knowledge these processes have not been identified.

The dramatic inhibition of Fe^{2+} uptake at 2°C compared to 22°C suggests that Fe influx is metabolically dependent. The residual Fe influx remaining at 2°C probably reflects plasma membrane influx that continues to occur at 2°C. The decreased trans-plasma membrane electrical potential at 2°C may be sufficient to maintain a significant electrochemical potential driving force for influx of divalent cations. Alternatively, the Fe accumulation at 2°C may represent a pool of apoplasmic Fe that is difficult to desorb from the cell wall, although the linear accumulation of Fe versus time in Figures 2 and 3 does extrapolate close to the origin. The data presented here emphasize the importance of removing nonspecifically bound ^{59}Fe from the apoplasm before accurate quantitation of Fe^{2+} influx can be made.

Intact seedlings also translocate a significant proportion of absorbed Fe to the shoots. If one makes the assumption that Fe translocated to the shoot must cross the endodermis and, thus, must at least cross the endodermal cell plasma membrane to reach the xylem, these results also provide circumstantial evidence that the $^{59}Fe^{2+}$ uptake studied here was true transport into the root symplasm rather than nonspecific binding to the root apoplasm.

We observed that excised root units had higher and more variable rates of uptake than intact plants. Part of the heterogeneity could be due to the variable expression of Fe deficiency stress responses along the root. This has been shown for the induction of Fe(III)-chelate reductase activity in dicot roots (Grusak et al., 1990a). In intact plants, this variability would become averaged by measuring influx into the entire root system. Also, ethylene appears to be involved in the pathway linking the sensing of Fe deficiency with the induction of Fe deficiency responses in dicots (Romera and Alcántara, 1994). It is unknown, however, whether ethylene could have such a rapid effect on Fe^{2+} transport, considering that root units were used for transport assays within 2 h of excision. If isolation of roots from phloem sources was to have an effect, it would be expected to decrease rather than increase Fe^{2+} transport. Instead, excision appeared to promote Fe^{2+} influx. It has been shown that excision injury decreases cation influx for other cations such as K^+ (Kochian and Lucas, 1985); thus it appears unlikely that the stimulation is due to excision injury. Also, it has been shown that after excision cation influx can recover to pre-excision values (Kochian and Lucas, 1985), and our root material was allowed to recover from cutting for a minimum of 30 min in an aerated buffered $CaSO_4$ solution prior to Fe uptake. More likely, excised root units show higher Fe^{2+} influx rates than intact plants because there is less intraroot competition for Fe in these root units. Absorption of Fe^{2+} at low nanomolar levels may cause a depletion zone to be created in the unstirred layer of solution surrounding the roots of intact plants, particularly when lateral roots are physically close together in the nutrient solution. Slower mixing combined with slow dissociation of Fe(II)-ferrozine$_3$ could effectively decrease the amount of available Fe^{2+} in the solution close to the root surface where influx occurs. Excised root units, however, consist of only 3 to 10 widely spaced lateral roots, so that depletion zones would be less likely to overlap. Artificially clumping root units into a small space of the uptake well decreased Fe^{2+} influx rates by as much as 50% (data not shown), suggesting that this phenomenon may occur. At this point, we can only speculate on the nature of the variability in Fe^{2+} influx rates in excised root units and intact plants.

We found that Fe^{2+} influx rates are influenced by the nutritional state of the roots. Specifically, Fe-deficient Sparkle seedlings exhibited higher influx rates than did Fe-sufficient seedlings. Fe-hyperaccumulating *brz* seedlings, which constitutively express high rates of Fe(III)-chelate reduction and Fe accumulation, also consistently exhibited elevated rates of Fe^{2+} influx. Thus, the pleiotropic, single gene mutation in *brz* causes high levels of expression of all Fe deficiency responses measured to date, leading to Fe hyperaccumulation. There are two possible reasons for the linkage of increased Fe^{2+} influx with stimulated Fe(III)-chelate reductase activity. First, it is possible that Fe deficiency induces the Fe^{2+} transport protein, as well as the Fe(III)-chelate reductase. Second, there is evidence that when the Fe(III)-chelate reductase or acidification is induced the uptake of a number of divalent cations (Zn^{2+}, Cu^{2+}, Mg^{2+}) is increased, possibly through biochemical stimulation of a divalent cation channel (Welch et al., 1993). Increased Fe^{2+} influx may be another example of this response.

In conclusion, Fe^{2+} transport into roots appears to involve a plasma membrane transporter similar to other known transporters, in that ion influx is metabolically dependent and depends on the activity of free Fe^{2+} ions in the external solution. The methods presented here are now being used to characterize concentration-dependent kinetics of Fe^{2+} influx as a tool for determining the characteristics of the Fe^{2+} influx system, such as whether Fe^{2+} influx occurs via a channel or another type of transporter and the specificity of this transport system.

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