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The pH Requirement for in Vivo Activity of the Iron-Deficiency-Induced "Turbo" Ferric Chelate Reductase[1]

A Comparison of the Iron-Deficiency-Induced Iron Reductase Activities of Intact Plants and Isolated Plasma Membrane Fractions in Sugar Beet

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The characteristics of the Fe reduction mechanisms induced by Fe deficiency have been studied in intact plants of *Beta vulgaris* and in purified plasma membrane vesicles from the same plants. In Fe-deficient plants the in vivo Fe(III)-ethylenediaminetetraacetic complex [Fe(III)-EDTA] reductase activity increased over the control values 10 to 20 times when assayed at a pH of 6.0 or below ("turbo" reductase) but increased only 2 to 4 times when assayed at a pH of 6.5 or above. The Fe(III)-EDTA reductase activity of root plasma membrane preparations increased 2 and 3.5 times over the controls, irrespective of the assay pH. The K_m for Fe(III)-EDTA of the in vivo ferric chelate reductase in Fe-deficient plants was approximately 510 and 240 μM in the pH ranges 4.5 to 6.0 and 6.5 to 8.0, respectively. The K_m for Fe(III)-EDTA of the ferric chelate reductase in intact control plants and in plasma membrane preparations isolated from Fe-deficient and control plants was approximately 200 to 240 μM. Therefore, the turbo ferric chelate reductase activity of Fe-deficient plants at low pH appears to be different from the constitutive ferric chelate reductase.

The existence in the roots of dicotyledonous plants of an obligatory Fe reduction step from Fe(III) to Fe(II) prior to uptake was first demonstrated by Brown and co-workers in 1972 (Chaney et al., 1972). Since then, an increase in the capacity to reduce Fe has been considered as an integral part of the so-called strategy I, which involves a number of mechanisms resulting in an improvement in Fe acquisition and is found in Fe-deficient dicots and non-Poaceae monocots (Marschner et al., 1986; Welkie and Miller, 1993; Marschner and Römheld, 1994). This strategy involves, besides the increase in the capacity to reduce Fe, morphological changes such as the development of root hairs and transfer cells and increases in the rates of excretion of diverse chemical species such as protons, phenolic compounds, and flavins (Marschner et al., 1986; Welkie and Miller, 1993; Marschner and Römheld, 1994). The relationships among all of these different responses within strategy I, however, are not clear. The increase in the capacity to reduce Fe is thought to be due to the existence of a Fe reductase enzyme in the PM of the deficient plants (Bienfait, 1985, 1988a; Cakmak et al., 1987).

The existence of not only one, but two, different Fe reductases in the plant root PM was postulated by Bienfait and co-workers in a series of papers (Bienfait et al., 1983, 1984, 1987; Bienfait, 1985, 1988a). A constitutive ("standard") reductase, present in the PM of all growing cells, would be able to reduce high potential acceptors such as ferricyanide. A second different ("turbo") PM chelate reductase would be induced by Fe deficiency and would be capable of reducing low potential Fe chelates (Bienfait, 1985, 1988a, 1988b). Sijmons et al. (1984) presented evidence indicating that cytoplasmic reduced pyridine nucleotides, possibly NADPH, may constitute the source of reducing power for the FC-R.

This view has been challenged in the last few years by using new methods to isolate pure PM. Plant PM preparations have been shown to possess one or more enzymes capable of transferring electrons to exogenous Fe compounds (Rubinstein and Luster, 1993; Moog and Brüggemann, 1994). NADH-dependent reduction of Fe chelates by isolated PM was first reported in strategy II plants (barley;

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Abbreviations: BPDS, bathophenantroline disulfonate; FC, ferric chelate; FCN, ferricyanide; FCN-R, ferricyanide reductase; FC-R, ferric chelate reductase; PM, plasma membrane; PTA, phosphotungstic acid.

Brüggemann and Moog, 1989) and subsequently in strategy I plants such as tomato (Buckhout et al., 1989; Brüggemann et al., 1990; Holden et al., 1991, 1992; Valenti et al., 1991), bean (Schmidt and Janiesch, 1991b), *Plantago* (Schmidt et al., 1990), *Geum urbanum* (Schmidt and Janiesch, 1991a), *Vigna unguiculata* (Brüggemann et al., 1993), and *Arabidopsis thaliana* (Moog et al., 1995). However, the PM from Fe-deficient plants usually exhibits FC-R activities biochemically similar to those of PM from control plants. All attempts to isolate an Fe-deficiency-induced PM FC-R biochemically distinct from that constitutively present in the PM of Fe-sufficient plants have failed so far (Rubinstein and Luster, 1993; Moog and Brüggemann, 1994).

Since the first paper by Chaney et al. (1972) more than 20 years ago, partial aspects of the enzymatic reduction of Fe by Fe-deficient plants and by PM have been described in many plant species (Marschner et al., 1986; Welkie and Miller, 1993). However, this information is still very fragmentary. The difficulty in achieving an unequivocal identification of the FC-R enzyme in the PM of Fe-deficient plants may arise from the fact that on only a few occasions have the activities of intact plants and of the PM been compared (see Moog and Brüggemann, 1994, and refs. therein).

On the other hand, most studies of the Fe-reduction mechanisms induced by Fe deficiency have been carried out with plants grown in zero- or low-Fe nutrient solutions with pH values ranging from 5.5 to 6.0 (Moog and Brüggemann, 1994), conditions that may bear little relationship to the Fe deficiency commonly found in field conditions, which most often occurs in plants grown in soils with high lime content and high pH. It is possible that mechanisms resulting from Fe-stress conditions under low pH may differ from those resulting from high pH.

In this paper we have studied the characteristics of the Fe reduction mechanisms in a plant species, *Beta vulgaris*, that is among the most efficient strategy I plants. We have characterized the Fe-reduction mechanisms in intact plants and in purified PM isolated by the two-phase partitioning method from the same plants and compared the biochemical characteristics of the FC-R activities in both materials. We have also compared Fe-deficient plants grown in the nutrient solution with or without a solid phase of $CaCO_3$ to ascertain whether the Fe-deficiency-induced FC-R activities are different in the presence and absence of $CaCO_3$.

MATERIALS AND METHODS

Plant Culture

Sugar beets (*Beta vulgaris* L. hybrid Monohil, Hilleshög, Landskröna, Sweden) were grown in a growth chamber. Seeds were germinated and grown in vermiculite for 2 weeks. Seedlings were grown for 2 more weeks in nutrient solution (in three-eighths-strength Hoagland nutrient solution with 22.4 μM Fe) and then transplanted (four plants per bucket) to 20-L buckets containing half-strength Hoagland solution with 0 or 44.8 μM Fe. Fe was added in the chelated commercial form Sequestrene 138 (Fe-EDDHA) from Ciba-Geigy (Barcelona, Spain) or as NaFe(III)-EDTA (Sigma). In some buckets with no Fe added, the pH of the nutrient solution was raised with 1 mM NaOH and 1 g/L of solid $CaCO_3$ to simulate conditions usually found in the field that lead to Fe deficiency; this treatment led to a constant pH of 7.7 throughout the 10- to 12-d growth period (Susín et al., 1994). Other Fe-deficient plants were grown in unbuffered solutions, resulting in a decrease of the solution pH, which reached pH 5.0 by d 8 (Susín et al., 1994). Plants were grown with a PPFD of 350 to 400 μmol photons m^{-2} s^{-1} at 25°C, 80% RH, with a photoperiod of 16 h light/8 h dark.

PM Isolation

Roots were harvested from control and Fe-deficient plants. Vesicles enriched in PM were purified from a root microsomal fraction by differential centrifugation in an aqueous-polymer two-phase system (Larsson, 1985; Brüggemann and Moog, 1989). Roots were homogenized using a mortar and pestle in a medium containing 250 mM Suc, 2 mM DTT, 4 mM EDTA, 5 mM ascorbate, 1 mM PMSF, 5% PVP, 0.2% BSA, and 0.2% casein in 50 mM Tris-HCl, pH 8.0. A ratio of 1 g of root fresh weight to 5 mL of homogenization medium was used. The homogenate was filtered through two layers of Miracloth (Calbiochem), and the filtrate was centrifuged at 10,000*g* for 10 min. The pellet was discarded, and the supernatant was centrifuged at 50,000*g* for 60 min to pellet the microsomal fraction.

The microsomal fraction was resuspended in 4 mL of a medium containing 250 mM Suc and 5 mM potassium-phosphate buffer, pH 7.0, and 3 mL were layered over an aqueous-polymer two-phase system, to yield a 12-g system with a final concentration of 5.7% dextran T500, 5.7% PEG 3350, 2.5 mM KCl, 5 mM Na ascorbate, 250 mM Suc, and 5 mM K-phosphate, pH 7.0. The partition was carried out three times (Larsson, 1985). The final upper PEG phase was diluted 6-fold with a buffer containing 10 mM Mops-Bis Tris Propane, pH 7.0, 3 mM EDTA, 250 mM Suc, and 5 mM Na ascorbate, and centrifuged at 50,000*g* for 60 min. The resulting pellet was resuspended in the same medium and stored in aliquots at −80°C. The introduction of ascorbate in the purification process was essential to maintain the activity of the PM marker.

The purity of the PM preparation obtained by aqueous-polymer two-phase partitioning was estimated from the enzymatic activities characteristic of PM and other organelles. The activity of the PM-associated, vanadate-sensitive, and Mg^{2+}-dependent ATPase was assayed by measuring the release of phosphate from ATP at pH 6.5 as described by Widell and Larsson (1990). The activities of nitrate-sensitive ATPase (O'Neill et al., 1983), latent IDPase (Lundborg et al., 1981), and Cyt *c* oxidase (Hodges and Leonard, 1974) were used as markers of tonoplast, Golgi apparatus, and mitochondria, respectively. Pi was determined by the formation of a colored phosphomolybdic complex (Widell and Larsson, 1990). Protein was determined by the method of Markwell et al. (1981).

EM and Morphometry

On-section staining with PTA was used as an additional specific marker for PM. Samples of microsomal fractions and purified PM were fixed in 2.5% glutaraldehyde/2% paraformaldehyde solution, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon. Thin sections were obtained, placed on nickel grids, incubated for 30 min in 1% periodic acid, and then stained with 1% PTA in 10% chromic acid. From this material at least 30 prints were taken for each treatment, and the purity of the PM was estimated by planimetry as described elsewhere (Serrano et al., 1994).

Fe-Reducing Capacities

The FC-R activity of roots of intact, illuminated plants was followed by measuring the formation of the Fe(II)-BPDS complex from Fe(III)-EDTA (Bienfait et al., 1983). Individual plants were transferred to 1-L beakers containing fresh nutrient solution (without Fe) supplemented with 300 μM BPDS, 500 μM Fe(III)-EDTA, and 5 mM Mes-KOH, pH 6.0. The pH of the assay solution was adjusted before adding Fe(III)-EDTA (Sigma) and BPDS (Sigma) from stocks (50 and 100 mM, respectively). The addition of Fe(III)-EDTA and BPDS did not modify the pH of the solution, which was also checked after introducing the plants and at the end of the experiment. Similar results were obtained when using solid Fe(III)NaEDTA instead of a Fe(III)NaEDTA stock. The beaker was fully covered with aluminum foil to exclude light, and the solution was aerated continuously. Aliquots were removed every 10 min and A_{535} was measured. An extinction coefficient of 22.14 mM^{-1} cm^{-1} was used for the estimation of reduced Fe. Usually, the reading at 20 min was used for estimation of the FC-R rates. When using Fe-deficient plants, it was possible to make several serial measurements, because the rates proceeded linearly for several hours (data not shown). Similar data were obtained when a different Fe-deficient plant was used for each measurement. With control plants, one measurement per plant was made, because the rates were not maintained after the 1st h (data not shown). Measurements were also made in the absence of plants to correct for any nonspecific Fe reduction. All measurements were performed at room temperature with a Shimadzu (Kyoto, Japan) model 2101 personal computer-controlled spectrophotometer using a 1-nm slit. In other experiments, the pH was varied in the range 3.0 to 8.5, using 5 mM phosphate buffer (pH 3.0–5.0), 5 mM Mes-KOH (pH 5.5–6.5), and 5 mM Tes-KOH (pH 7.0–8.5).

The FC-R activity of PM purified by two-phase partitioning was measured as described by Brüggemann and Moog (1989) by using 3 to 5 μg protein mL^{-1} in 10 mM Mops-Bis Tris Propane, pH 7.0, 250 mM Suc, 3 mM EDTA, 5 mM $MgCl_2$, 0.02% Triton X-100, 600 μM BPDS, 500 μM NADH, and 500 μM Fe(III)-EDTA. The reaction was performed in the dark for 15 min. The degree of reductase latency was determined by comparison of the enzyme activities in the presence and absence of detergent. The degree of latency was calculated as [(rate with detergent) − (rate without detergent)] × 100/(rate with detergent).

Estimation of the Free Ion Activities in Solution

The global speciation, including the free activities of the different ionic species in the nutrient solutions and the solutions used for the assay, was estimated by using the MINTEQA2 software (Allison et al., 1991).

RESULTS

The characteristics of the Fe-reducing activities in Fe-sufficient sugar beet plants were compared to those of plants grown without Fe under two different conditions. Some plants were grown in unbuffered nutrient solutions without Fe. These plants exhibited mild Fe chlorosis symptoms in their leaves, decreased the pH of their nutrient solutions down to values of 5.0 after 8 d of growth without Fe, and excreted flavin sulfates and riboflavin to the nutrient solution (Susín et al., 1994). In contrast, plants grown in nutrient solutions buffered at a pH of 7.7 with solid $CaCO_3$ exhibited advanced Fe chlorosis symptoms and accumulated flavins in the subapical root zone (Susín et al., 1994).

Development of the in Vivo Root FC-R Activity in Intact Plants

The root Fe(III)-EDTA reducing (FC-R) capacity was measured at pH 6.0 following the classical method of Bienfait et al. (1983). In the Fe-deficient plants, the FC-R activity increased progressively until approximately 9 d of Fe depletion and remained fairly constant from d 9 to d 20 (Fig. 1A). The Fe-sufficient, control plants exhibited a practically constant FC-R activity throughout the experimental period.

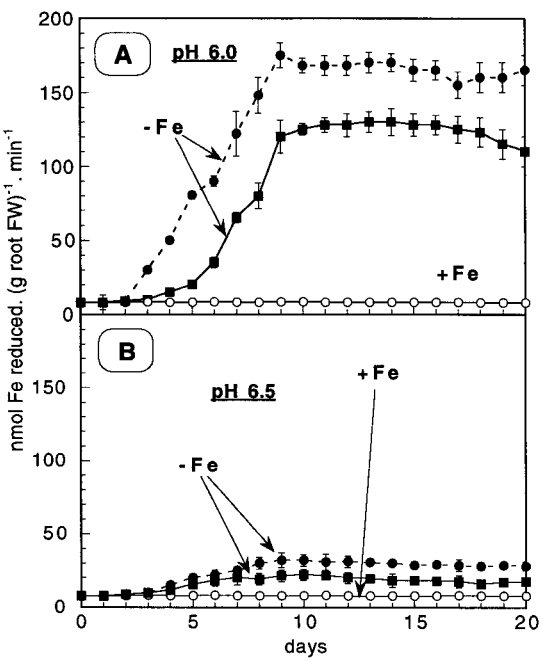

Figure 1. Time course of the development of the root FC-R in intact plants at 500 μM Fe-EDTA. A, FC-R at pH 6.0; B, FC-R at pH 6.5. Data are means ± SE of 12 replications. ■, Plants grown without Fe in the presence of $CaCO_3$; ●, plants grown without Fe in the absence of $CaCO_3$; ○, control plants grown with Fe. FW, Fresh weight.

At d 9 after the beginning of the experiment, the FC-R activity of roots from Fe-deficient plants had increased from 12- to 20-fold when compared with the Fe-sufficient controls (Fig. 1A). This Fe-deficiency-induced large increase in FC-R activity has been previously reported in other strategy I plant species and is usually referred to as "turbo" FC-R (see Moog and Brüggemann, 1994, for a thorough review). The FC-R activity was located in the subapical zone of the young roots, with the older root parts exhibiting very little FC-R activity (data not shown). The increase in FC-R activity was detectable on the 3rd d in the plants without $CaCO_3$, and 1 d later in the case of the plants with $CaCO_3$-buffered nutrient solutions (Fig. 1A). The rate of increase in FC-R activity was similar in both types of plants from approximately the 5th d of Fe deficiency.

The FC-R activities at d 8 were 80 to 150 and 8 nmol Fe reduced (g root fresh weight)$^{-1}$ min^{-1} in Fe-deficient and control plants, when measured in 500 μM Fe-EDTA and at pH 6.0. These rates are among the highest reported in the literature (Moog and Brüggemann, 1994). The final in vivo FC-R activity was 30% higher in plants grown without $CaCO_3$ in the medium than in those grown at high pH in the presence of a solid phase of $CaCO_3$ (Fig. 1). This indicates that growth in a high pH-buffered medium induced decreases in FC-R even after plants were transferred to a lower pH for the assay. It should be kept in mind, however, that these differences may be caused by differences in the general status of the plants in the two treatments, since plants were greener and bigger when no $CaCO_3$ was present.

pH Dependence of the in Vivo Root FC-R Activity in Intact Plants

The root FC-R activity of the intact Fe-deficient plants was much lower when assayed at pH 6.5 than when assayed at pH 6.0 (Fig. 1B). This occurred throughout the 20-d experimental period. The FC-R activity was increased in Fe-deficient plants only 2- to 4-fold compared to the controls when measured at pH 6.5 (Fig. 1B), which is in sharp contrast with the 12- to 18-fold increase found when the assay was done at pH 6.0 (Fig. 1A).

We assayed the FC-R activities in a broad pH range to look for possible differences in the behavior of Fe-deficient and control plants (Fig. 2). In Fe-deficient plants grown for 8 d in the absence of $CaCO_3$, the FC-R activity decreased little from pH 3.0 to pH 6.0 (Fig. 2A). However, the FC-R activity at pH 6.5 was much lower than at pH 6.0. Further decreases occurred at pH values higher than 6.5. In plants grown for 8 d at high pH in the presence of $CaCO_3$, there was no change in FC-R activity from pH 3.0 to pH 4.5. Approximately half of this FC-R activity was already lost at pH 6.0, and another 40% was lost at pH 6.5. Further decreases occurred at higher pH. In the Fe-sufficient, control plants, the FC-R activity did not change significantly from pH 3.0 to pH 6.5 but decreased at higher pH values.

That a significant part of the activity of the turbo FC-R depends on using a pH of 6.0 or below during the reduction measurements is clear when looking at the ratio between the FC-R activity in Fe-deficient and control plants.

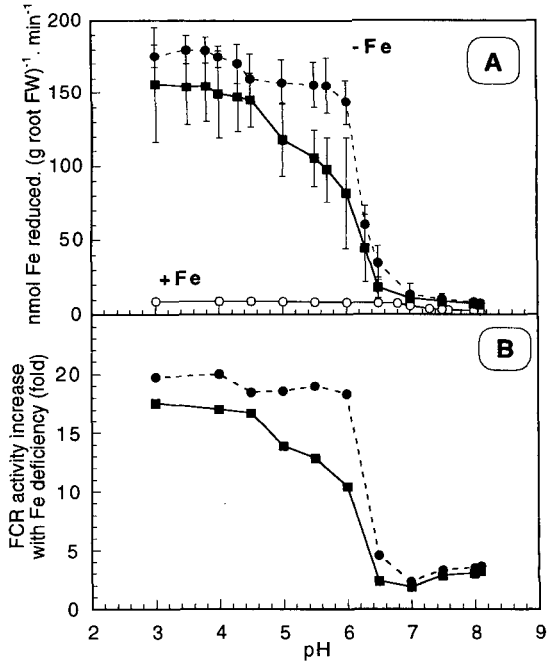

Figure 2. pH dependence of the root FC-R activities in intact plants submitted to Fe deficiency for 8 d using 500 μM Fe-EDTA. Data are means ± SE of 12 replications. A, Activities; B, ratios of activity in Fe-deficient plants to activity in control plants. Symbols as in Figure 1. FW, Fresh weight.

This ratio was between 2 and 4 in the pH range 6.5 to 8.0 and increased to values of 10 to 20 at a pH of 6.0 or below (Fig. 2B).

Excretion of reducing substances by plant roots occurred in Fe-deficient sugar beet but was minor when compared with the reducing power of intact plants. Excretion of reducing substances was measured in the reductase assay medium [without Fe(III)NaEDTA and BPDS] at different pH values for 1 h. After removing the plant, the medium was assayed for reducing activity by adding Fe(III)NaEDTA and BPDS. The excreted reducing substances reduced approximately 0.1 and 0.9 nmol Fe (g root fresh weight)$^{-1}$ min^{-1} in control and Fe-deficient plants, respectively. This is less than 2 and 4% of the minimal reducing power (i.e. when the assay was made at a pH of 6.5 or above) of intact control and Fe-deficient plants, respectively. At a pH of 6.0 or below, the excretion of reducing substances may be approximately 2 and 1% of the reducing activity of intact control and Fe-deficient plants, respectively.

In Vivo Fe Reduction by Intact Plants from Other Fe Sources

Two other Fe sources were used for measuring the Fe reduction activity as a function of pH. When using FCN, the in vivo FCN-R changed little from pH 5.0 to pH 7.5 in control and Fe-deficient plants, with Fe deficiency inducing approximately a 2-fold increase in activity (see below for the maximum rates). We also used $FeCl_3$ as an Fe source, and found activities (in nmol Fe reduced [g root fresh

weight]$^{-1}$ min^{-1}) of 18.5, 23.1, 24.7, and 19.2 (control plants) and 35.6, 41.2, 37.1, and 28.9 (Fe-deficient plants) at pH 5.0, 6.0, 6.5, and 7.5, respectively. Therefore, Fe deficiency increased the $FeCl_3$-reducing activities by 1.9-, 1.8-, 1.5-, and 1.5-fold at pH 5.0, 6.0, 6.5, and 7.5, respectively.

Enzymatic Characteristics of in Vivo Root FC-R in Intact Plants at Different pH Values

In further experiments, we used plants grown with $CaCO_3$, since this system is closer to the real conditions under which Fe deficiency occurs in the field. Both Fe-deficient and control, Fe-sufficient intact plants exhibited apparent Michaelis-Menten kinetics at pH 6.0 and 6.5. However, the FC-R activity was saturated at approximately 1000 μM Fe(III)-EDTA in Fe-sufficient plants assayed at pH 6.0 and 6.5 and in Fe-deficient plants at assayed at pH 6.5, whereas it did not saturate even at 2000 μM Fe(III)-EDTA in Fe-deficient plants assayed at pH 6.0 (Fig. 3). In control plants, the apparent K_m for Fe(III)-EDTA was in the range 229 to 236 μM, and the V_{max} was approximately 11 nmol Fe reduced (g root fresh weight)$^{-1}$ min^{-1} (Table I).

Fe-deficient plants assayed at pH 6.5 exhibited a K_m value for Fe(III)-EDTA of 244 μM, similar to that of controls (Table I). This value is in the range reported for other plants (Moog and Brüggemann, 1994). The V_{max} values in these plants were 2.4-fold higher than the controls when assayed at pH 6.5 (26 nmol Fe reduced [g root fresh weight]$^{-1}$ min^{-1}; Table I). For the FC-R assay we used pH values of 7.0, 7.5, and 8.0 and found K_m (240, 223, and 256 μM, respectively) and V_{max} (25, 25, and 27 nmol Fe reduced [g root fresh weight]$^{-1}$ min^{-1}, respectively) values similar to those found at pH 6.5.

However, at pH 6.0 the Fe-deficient plants showed a K_m of 514 μM, indicating a lower affinity of the possible enzyme for the substrate than when assayed at higher pH values (Table I). This K_m value was found by using a linear fit ($r = 0.97$, $P < 0.01$). On the other hand, the data in Figure 3 may indicate that the existence of some sort of complex cooperativity cannot be completely ruled out. The V_{max} at pH 6.0 was 196 nmol Fe reduced (g root fresh weight)$^{-1}$ min^{-1}, a value much higher than that found at pH 6.5 (Table I). Therefore, when the measurements were carried out at pH 6.0, the increase in V_{max} in Fe-deficient plants was 19-fold higher than that of the controls. We also used for the assay pH values of 4.5, 5.0, and 5.5 and found K_m (525, 517, and 531 μM, respectively) and V_{max} (187, 199, and 201 nmol Fe reduced [g root fresh weight]$^{-1}$ min^{-1}, respectively) values similar to those found at pH 6.0.

Enzymatic Characteristics of in Vivo Root FCN-R in Intact Plants

The in vivo FCN reduction with NADH as electron donor was saturated at approximately 1000 μM FCN both in Fe-deficient and control plants (Fig. 4). The K_m values were in the range 218 to 226 μM FCN (Table I) in all cases, values quite similar to the 290 μM reported for Fe-deficient *G. urbanum* (Schmidt and Janiesch, 1991a). The V_{max} values were approximately 230 and 450 nmol Fe reduced (g root

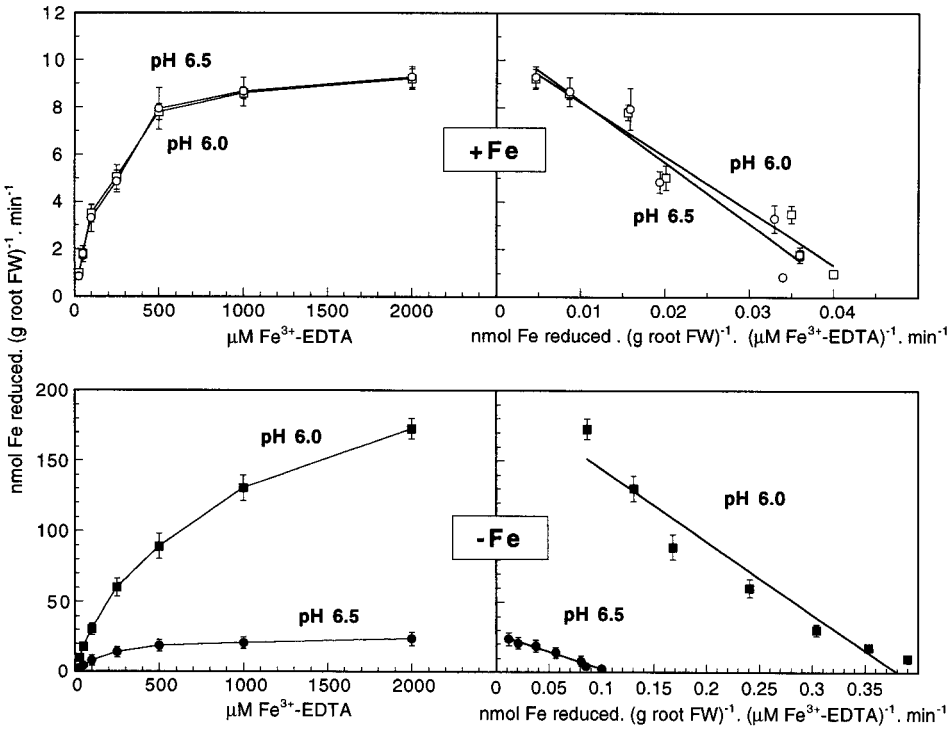

Figure 3. Dependence of the root FC-R activity in intact plants on the concentration of Fe(III)-EDTA and corresponding Eadie-Hofstee plots. Data are means ± SE of eight replications. Closed symbols, Fe-deficient plants; open symbols, controls; squares, pH 6.0; circles, pH 6.5. FW, Fresh weight.

Table I. *Enzymatic characteristics of the FC-R and FCN-R activities measured in vivo in intact plants*

K_m values are in μM, and V_{max} values are in nmol Fe reduced min^{-1} (g root fresh weight)$^{-1}$.

Treatment	Fe(III)-EDTA (n = 8)				FeCN (n = 8)			
	pH 6.5		pH 6.0		pH 6.5		pH 6.0	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
+Fe	236 ± 6	11 ± 1	229 ± 4	11 ± 1	218 ± 12	230 ± 17	221 ± 18	229 ± 15
−Fe	244 ± 23	26 ± 2	514 ± 18	196 ± 10	220 ± 23	460 ± 10	226 ± 16	448 ± 27
−Fe/+Fe	1.0	2.4	2.2	18.6	1.0	2.0	1.0	1.9

fresh weight)$^{-1}$ min^{-1} in control and Fe-deficient plants, respectively (Table I). Using pH values of 6.0 and 6.5 (Table I) or 5.0, 7.0, and 7.5 (data not shown) for the assay did not change the K_m or V_{max} values significantly.

Isolation and Characterization of PM-Enriched Preparations

PM vesicles were prepared using the two-phase partitioning method (Larsson and Møller, 1990). The purity of the PM fractions was tested by using marker enzymes. The PM fraction contained 75 to 85% of the specific vanadate-sensitive ATPase activity found in the two-phase system. The specific vanadate-sensitive ATPase activity was 4- and 3-fold higher than that of the dextran fraction in PM from control and Fe-deficient plants, respectively (Table II). The latency of the specific vanadate-sensitive ATPase activity was 86%. Contamination of the PM fraction by tonoplast, Golgi apparatus, and ER was not detectable or was very low (Table III). Contamination by mitochondrial membranes was estimated to be lower than 3 and 5% in PM from control and Fe-deficient plants, respectively (Table III). Furthermore, EM of the isolated fractions (Fig. 5) indicated that PM accounted for 94.3 ± 1.3 and 98.7 ± 0.8% of the total membranes in preparations from control and Fe-deficient plants, respectively. These data support the conclusion that the final fractions were highly enriched in PM vesicles.

Enzymatic Characteristics of the FC-R Activity in PM

The FC-R activity was investigated in PM preparations from Fe-sufficient and Fe-deficient roots. The optimal pH for FC-R activity was approximately 7.0, different from that found for intact plants (Fig. 6). For Fe(III)-EDTA as substrate, saturation occurred at approximately 1000 μM (Fig. 7). The K_m values for Fe(III)-EDTA were 220 and 200 μM for PM from control and Fe-deficient plants, respectively. These values are in the range of those reported for PM from other plant species (Moog and Brüggemann, 1994). The V_{max} values for FC-R activity with NADH as electron donor were 239 and 495 nmol Fe mg^{-1} protein min^{-1} in

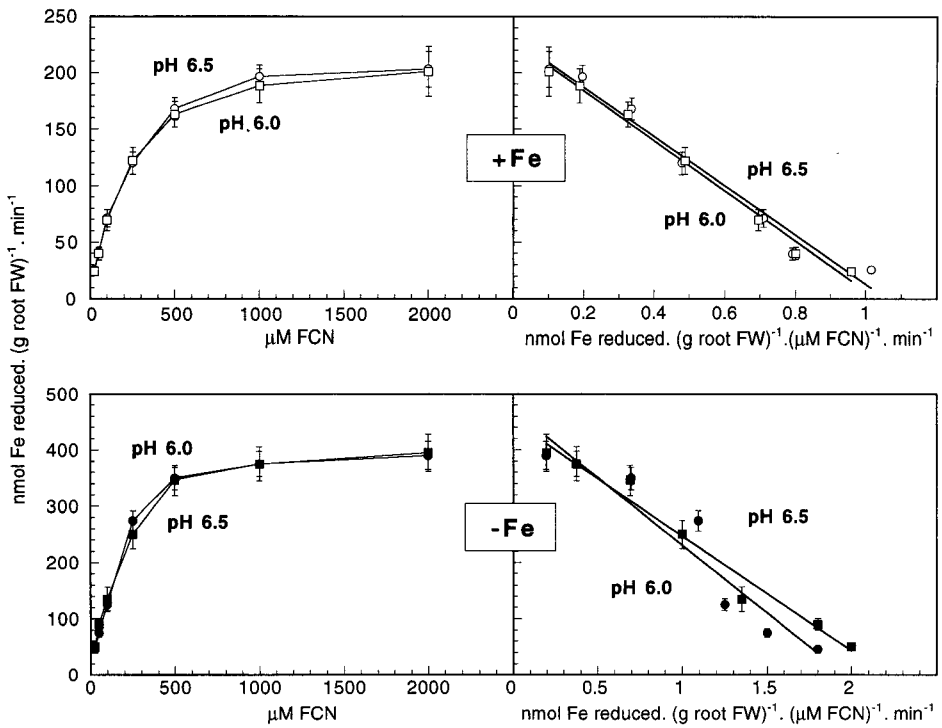

Figure 4. Dependence of the root FCN-R activity in intact plants on the concentration of FCN and corresponding Eadie-Hofstee plots. Data are means ± SE of eight replications. Symbols as in Figure 3. FW, Fresh weight.

Table II. *Protein recoveries and PM-associated activity during PM isolation*
Data are from a typical experiment. Other experiments gave similar results.

Fraction	+Fe			−Fe		
	Protein	Protein	ATPase activity	Protein	Protein	ATPase activity
	$\mu g\ \mu L^{-1}$	mg	nmol Pi min^{-1} mg^{-1} protein	$\mu g\ \mu L^{-1}$	mg	nmol Pi min^{-1} mg^{-1} protein
Homogenate	12.5	750	—	10.4	730	—
First centrifugation pellet	19.7	30.5	—	15.8	20.5	—
Supernatant	11.9	690	—	13.7	683	—
Microsomal fraction	8.8	24	92	7.3	27.4	149
PM vesicles	6.6	3.9	375 (4.1)	7.0	4.2	443 (3.0)
First lower phase	17.6	17.6	73.5 (0.8)	20.3	20.3	127 (0.8)
Second lower phase	1.6	0.7	24.5 (0.3)	3.0	1.2	33.5 (0.2)

control and Fe-deficient plants, respectively (Table IV). Therefore, the increase in the V_{max} of FC-R induced by Fe deficiency was approximately 2-fold. Similar K_m and V_{max} values were found for the pH range 5.0 to 7.5.

The kinetic analysis of the FC-R Eadie-Hofstee plots with varying concentrations of NADH (Fig. 8) gave K_m values for NADH of 108 and 125 μM for the PM preparations from control and Fe-deficient plants, respectively (Table IV). The V_{max} values were 219 and 446 nmol Fe mg^{-1} protein min^{-1} for PM from control and Fe-deficient plants, respectively. Detection of optimal FC-R activity required the presence of 0.02% (w/v) Triton X-100; FC-R latencies were 73 and 82% in control and Fe-deficient plants, respectively, when NADH was used as the electron donor (Table V). These data confirm that the binding site for NADH in PM vesicles is located in sites not exposed to the external medium.

When using NADPH as electron donor, the V_{max} was 20 and 9% of that found with NADH in control and Fe-deficient plants, respectively. The specific activity of the NADPH-dependent FC-R was not changed by Fe deficiency (Table IV). When NADPH was used, FC-R latencies were 20 and 21% for control and Fe-deficient plants, respectively (Table V).

Enzymatic Characteristics of the FCN-R Activity in PM

The FCN reduction by Fe-sufficient plasma membranes with NADH as electron donor was saturated at approximately 1000 μM FCN and showed similar kinetics for the two types of membranes (Fig. 9). The K_m values were 221 and 233 μM in PM from control and Fe-deficient plants, respectively (Table IV). The only K_m reported so far in the literature for the FCN-R in a strategy I plant was 208 μM for *G. urbanum* (Schmidt and Janiesch, 1991a). The V_{max} increased 3.5-fold with Fe deficiency, from 2649 to 9370 nmol Fe mg^{-1} protein min^{-1}.

The rate of NADPH-dependent FCN-R was 40 to 55% of the rate supported by NADH in both PM preparations. The specific activity of NADPH-dependent reduction was increased approximately 2.7-fold by Fe deficiency. Detection of optimal FCN-R activity also required the presence of Triton X-100, latencies obtained being in the ranges 38 to 51% (NADH) and 45 to 51% (NADPH) (Table V).

DISCUSSION

Most studies on the Fe-reducing activities induced by Fe deficiency have focused on very specific aspects of the reduction process. Moreover, these studies have been done with different plant species, resulting in very fragmentary information that makes comparisons between experiments very difficult (for a review, see Moog and Brüggemann, 1994). In the present work we have made a comprehensive study of the Fe-reducing mechanisms in *B. vulgaris*, which is among the most Fe-efficient species within strategy I (Nagarajah and Ulrich, 1966; Welkie and Miller, 1993). In this work we have characterized both the FC-R and FCN-R activities of Fe-deficient and Fe-sufficient intact sugar beet plants and compared their characteristics with those of the FC-R and FCN-R activities of PM prepared from the same plants.

Fe-deficient sugar beet plants exhibited an increase in in vivo FC-R activity of 10- to 20-fold over control values. This marked (turbo) increase is similar to those described for other plant species (Moog and Brüggemann, 1994). However, the data presented in this work indicate that the pH

Table III. *Activities of organelle markers during PM isolation*
Data are from a typical experiment. Other experiments gave similar results.

Organelle	+Fe			−Fe		
	Microsomal fraction	PM fraction	Percent	Microsomal fraction	PM fraction	Percent
	nmol min^{-1} mg^{-1} protein			nmol min^{-1} mg^{-1} protein		
Mitochondria	1521	51	3	1729	86	5
Tonoplast	308	3.0	1	428	3.4	1
Golgi apparatus	31	—	0	23	—	0
ER	10.8	0.1	1	12.7	0.1	1

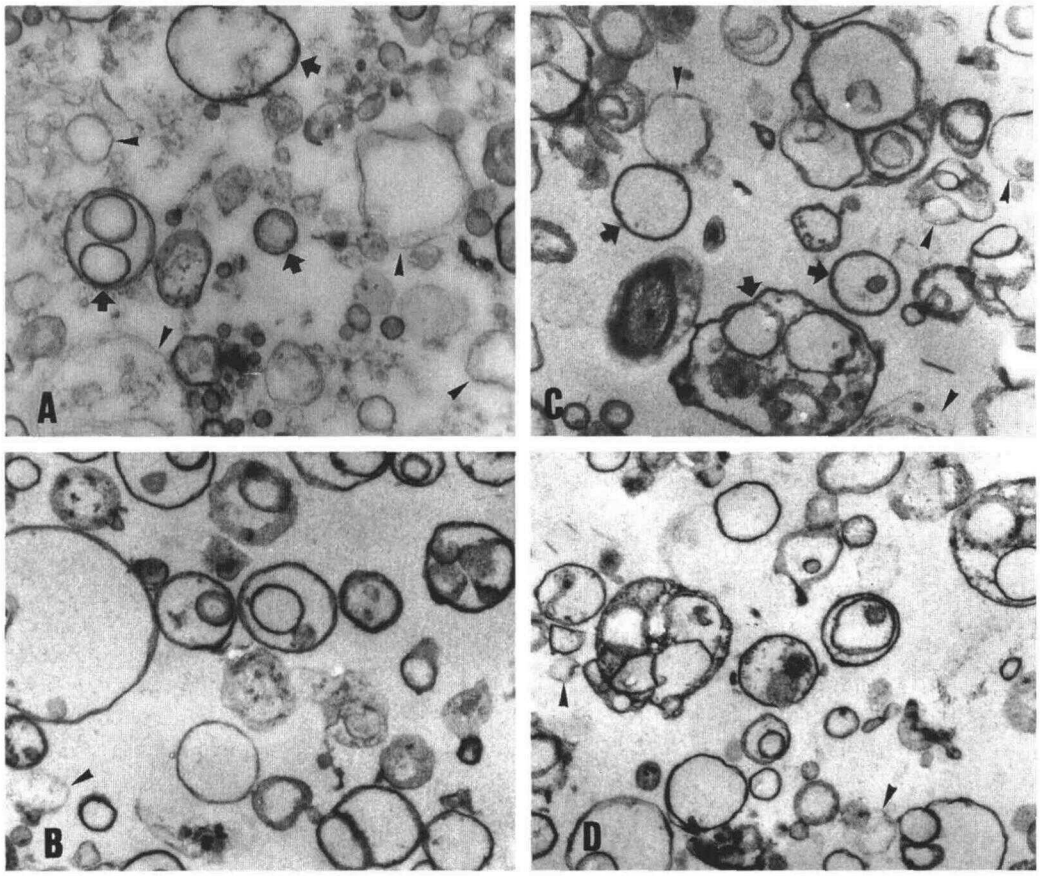

Figure 5. On-section staining with PTA of control (A and B) and Fe-deficient (C and D) plants. Microsomal fractions from control (A) and Fe-deficient plants (C) contain stained PM vesicles (arrows) and other, nonstained membranes (arrowheads). Most vesicles in the purified PM fractions from control (B) and Fe-deficient plants (D) were stained with PTA, indicating their PM origin; only a minor portion of the vesicles in these preparations were unstained (arrowheads). All sections, × 28,700.

dependence of the FC-R activity was very different in Fe-deficient and control plants. The turbo FC-R was readily observed in Fe-deficient plants when the pH used in the assay medium was 6.0 or below, but only a minor fraction of this activity remained at pH 6.5 or above. Conversely, the FC-R activity in intact control plants did not decrease significantly in the same pH range. Other in vivo Fe-reducing activities induced by Fe deficiency, such as the FCN-R or the reducing activity for $FeCl_3$, did not show such pH dependence.

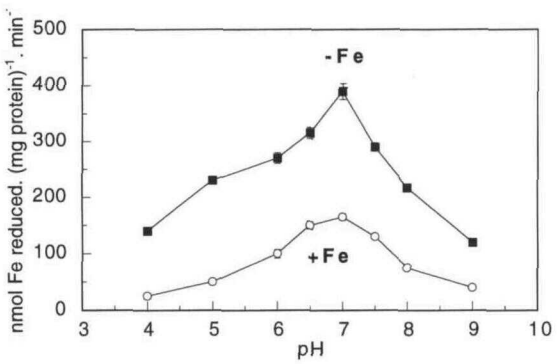

Figure 6. pH dependence of the root PM FC-R activity. Data are means ± SE of four replications. ■, Fe-deficient plants; ○, controls.

Preparations enriched in PM from sugar beet exhibit an FC-R activity similar to that described for PM preparations of other plant species (Moog and Brüggemann, 1994). PM preparations from Fe-deficient and control plants showed maximal activity at pH 7.0, a pH similar to those reported before for other plants. These maxima probably reflect the pH dependence of the cytoplasmic side of the enzyme (Moog and Brüggemann, 1994). On the other hand, the FC-R present in sugar beet PM appeared to be NADH dependent, and its high latencies indicate that transmembrane electron transport is needed for activity. Similar characteristics have been reported before for FC-R activities in plant root PM (Moog and Brüggemann, 1994).

So far there is little evidence in the literature that there could be differences in the in vivo pH dependence of the FC-R activities of Fe-deficient and Fe-sufficient intact plants. Decreases in the activity of the FC-R in Fe-deficient plants at high pH have been reported before for intact plants of peanut (Römheld and Marschner, 1983), apple (Ao et al., 1985), *Ficus benjamina* (Rosenfield et al., 1991), *G. urbanum* (Schmidt and Janiesch, 1991a), and *A. thaliana* (Moog et al., 1995). However, in most of these cases the pH

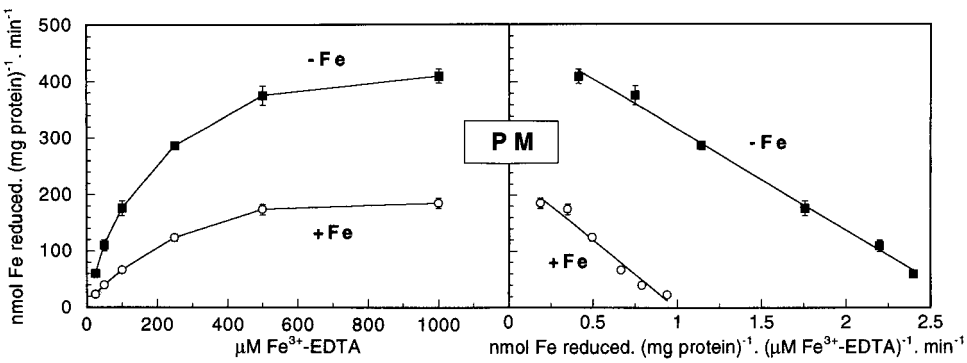

Figure 7. Dependence of FC-R activity by root PM vesicles on the concentration of Fe(III)-EDTA at pH 7.0 and corresponding Eadie-Hofstee plots. Data are means ± SE of eight replications. ■, Fe-deficient plants; ○, controls.

dependence of FC-R activity in control, Fe-sufficient plants was not reported. The only data available comparing pH-induced changes in FC-R activity with those of controls were reported for apple and indicate that the ratios of FC-R activity in Fe-deficient plants to FC-R activity in controls were 11.5, 6.6, 3.1, and 1.9 at pH 5.0, 6.5, 7.0, and 8.0, respectively (values for Fe[III]diethylenetriaminepentaacetate) (Ao et al., 1985). In our case, when using plants grown with $CaCO_3$, this ratio decreased from values of 10 at pH 6.0 to less than 3 at pH 6.5. The FC-R activity has been shown to be pH dependent in excised roots of bean (Cakmak et al., 1987) and rape (Toulon et al., 1992). In bean, the ratios of FC-R activity in Fe-deficient and control plants were approximately 20, 17, 12, and 5 at pH 6.0, 6.5, 7.0, and 7.5 (data estimated from figure 2 in Cakmak et al. [1987]). In rape, the decrease in FC-R activity was marked at pH values above 6.0, although no data were reported for control, Fe-sufficient plants.

The enzymatic characteristics of the in vivo FC-R induced by Fe deficiency depended markedly on the pH of the assay. We used two different assay pH values (6.5 and 6.0) to characterize these changes. When ferric reduction was assayed at a pH of 6.5 or above, the intact, Fe-deficient plants exhibited only a 2- to 3-fold increase in the V_{max} for FC-R activity when compared to Fe-sufficient plants. This increase was similar to the 2- to 3-fold increase found in PM preparations from Fe-deficient plants when compared to the controls. At these pH values the K_m for Fe(III)-EDTA of the FC-R in Fe-deficient, intact plants (244 µM) was similar to those of the "standard-type" FC-R found in intact, Fe-sufficient plants (236 µM) and in PM preparations isolated from Fe-deficient and Fe-sufficient plants (200–220 µM).

In contrast to the modest increase in FC-R activity at higher pH values, when assayed at pH 6.0 or below, Fe-deficient intact plants exhibited 19-fold (turbo) increases in their V_{max} for FC-R activity. In contrast, the PM preparations from Fe-deficient plants still showed a 2- to 3-fold increase in V_{max} at this pH when compared to the controls. Since the FC-R activity is located in a relatively small area in the distal part of the root, 19-fold increases in FC-R activity on a fresh weight basis mean that the increase in FC-R activity in the cells expressing the turbo FC-R were possibly 1 to 2 orders of magnitude larger. When a pH of 6.0 or below was used, the turbo FC-R also had a larger K_m for Fe(III)-EDTA (514 µM) than intact control plants (229 µM) and isolated PM from Fe-deficient and control plants (200–230 µM).

The kinetic characteristics of FCN-R activity did not change from pH 5.0 to 7.5. The K_m values of the FCN-R of intact plants and PM preparations were approximately 220 µM in both Fe-deficient and Fe-sufficient plants in the pH range studied. In PM vesicles, FCN-R activity appeared to be NADH dependent. In contrast to what has been reported before for other plant species (Moog and Brüggemann, 1994), the FCN-R activity in PM exhibited increases with Fe deficiency when assayed with either NADH or NADPH (3.5- and 2.7-fold, respectively).

The stability of the complex Fe(III)-EDTA in solution depends on the composition of the medium. The complex Fe(III)-EDTA can be very stable at high pH values (even at pH 13) if no competing cations other than H^+ are present (Lucena et al., 1987, 1988). However, in the presence of other cations with a good affinity for EDTA, such as Zn (or Mn under certain redox conditions), Fe is displaced and can precipitate at pH values higher than 6.2 (Lindsay and Norvell, 1969). At a pH of approximately 7.2, Ca-EDTA is the most important EDTA species in a calcareous soil. However, the complex Fe(III)-EDTA seems to be quite

Table IV. *Enzymatic characteristics of the FC-R and FCN-R activities measured in PM vesicles*

K_m values are in µM, and V_{max} values are in nmol Fe reduced min^{-1} mg^{-1} protein.

Treatment	Fe(III)-EDTA (n = 12)		NADH (n = 12)		NADPH (n = 8)		FeCN (n = 5)	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
+Fe	220 ± 25	239 ± 30	108 ± 6	219 ± 10	111 ± 7	43 ± 7	221 ± 37	2649 ± 138
−Fe	200 ± 30	495 ± 58	125 ± 17	446 ± 17	115 ± 3	40 ± 5	233 ± 50	9370 ± 190
−Fe/+Fe	0.9	2.1	1.2	2.0	1.0	1.0	1.0	3.5

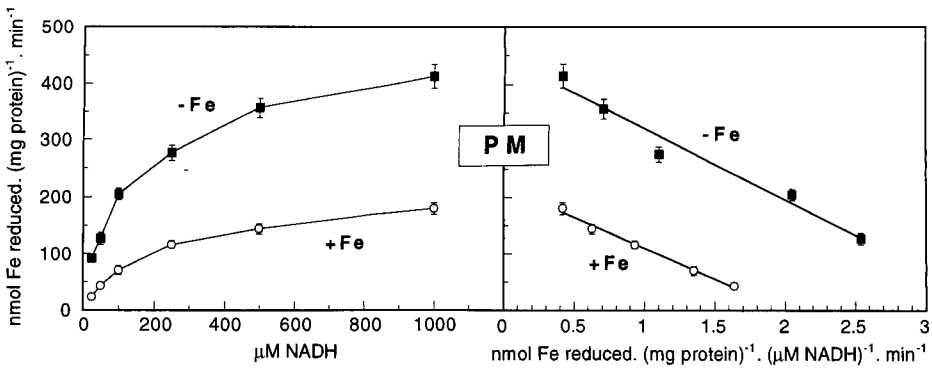

Figure 8. Dependence of the FC-R activity in root PM on the concentration of NADH at pH 7.0 and corresponding Eadie-Hofstee plots. Data are means ± SE of eight replications. ■, Fe-deficient plants; ○, controls.

stable under the conditions used for the reductase assay, where total Fe was high (500 μM) and the amount of other competing cations was very low (Zn was only 0.4 μM). By using the MINTEQA2 software, we estimated that the major form of Fe(III)-EDTA in the assay solution at pH values between 5.0 and 7.0 was Fe(III)-EDTA$^-$, with only a minor amount of Fe(III)OH-EDTA^{2-} being present (Table VI). The estimated free activity of Fe^{3+} was less than 1 pM at these pH values. The low amount of Zn present was unable to displace a significant amount of Fe. Ca can still compete with Fe, but this effect is significant only at pH values higher than 8.2 (Table VI). Therefore, it is unlikely that any major change occurred in the chemistry of the Fe(III)-EDTA in the assay solution from pH 6.0 to pH 6.5, when the FC-R activity switched from the turbo to the constitutive type. This view is supported by the pH dependence of the FC-R in isolated PM vesicles, which show a distinct optimum at pH 7.0, with assay conditions similar to those used for the in vivo experiments.

The pH dependence of the FC-R activity in excised rape roots has been hypothesized to be a consequence of the acquisition by Fe(III)-EDTA of one negative charge as the pH varied from 6.2 to 8.2, since the pK_a (the pH needed in order to have 50% of the molecule in each of the two chemical species) for Fe(III)-EDTA was estimated to be approximately 7.2 for an ionic strength of approximately 10 mM (Toulon et al., 1992). However, the pK_a of the pair Fe(III)-EDTA$^-$/Fe(III)OH-EDTA^{2-} has been estimated by several authors to be between 7.4 and 7.65 for an ionic strength of 100 mM (Martell and Smith, 1974), and approximately 8.0 for an ionic strength of 10 mM, similar to that used in the assay medium in the present work (16.5 mM). By using the MINTEQA2 software, we estimated that the pK_a for Fe(III)-EDTA was higher than 8.0 (Table VI).

Data previously presented by Schmidt and Janiesch (1991a) indicated that the maximal rate of reduction by *G. urbanum* plants was attained at pH 5.0, and that the K_m of the FC-R increased with an increase in pH, with K_m values of 90 to 110 μM Fe at pH 4 to 5 and between 550 and 800 μM Fe at pH 7 to 8. Such K_m increases have been interpreted by Moog and Bruggemann (1994) as due to poor stability of Fe(III)-EDTA at high pH (Chaney, 1988). However, our data for Fe-deficient sugar beet indicate that the K_m decreased with an increase in pH.

Our data suggest that the mechanism responsible for the turbo FC-R activity in Fe-deficient, intact plants at pH 6.0 or below was different from that present at higher pH values. Furthermore, our data indicate that the FC-R activity in Fe-deficient, intact plants at pH 6.5 or above was biochemically similar to the constitutive FC-R present in the control plants at any pH. This would support the theory put forward by Bienfait (1985) that a turbo FC-R activity was indeed present in Fe-deficient plants, although from our data it appears that it would be active only at a pH of 6.0 or below. This contrasts with the current view that possibly there is no turbo FC-R (Brüggemann et al., 1990), a view supported by the fact that no biochemically distinct enzyme could be found in PM preparations iso-

Table V. *FC-R and FCN-R latencies in PM preparations*

Measurements were made with 500 μM NADH or NADPH and 500 μM KFeCN or Fe(III)-EDTA, pH 7.0. Measurements are in nmol Fe reduced min^{-1} mg^{-1} protein. T-X100, Triton X-100.

Electron Acceptor	Treatment	Electron Donor					
		NADH			NADPH		
		+T-X100	−T-X100	Percent latency	+T-X100	−T-X100	Percent latency
Fe(III)-EDTA	+Fe	160 ± 14	43 ± 5	73	35 ± 2	28 ± 2	20
	−Fe	380 ± 25	70 ± 9	82	38 ± 2	30 ± 2	21
	−Fe/+Fe	2.4	2.3		1.1	1.1	
FeCN	+Fe	1915 ± 105	1194 ± 75	38	1054 ± 80	514 ± 50	51
	−Fe	6556 ± 225	3183 ± 184	51	2816 ± 200	1558 ± 250	45
	−Fe/+Fe	3.4	2.7		2.7	3.0	

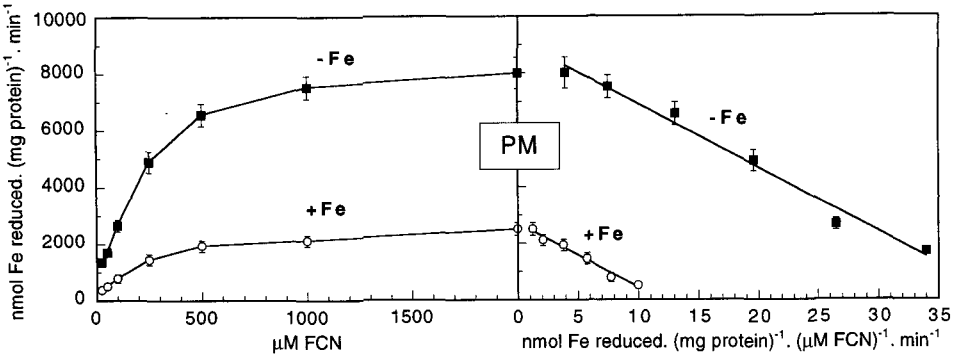

Figure 9. Dependence of the FCN-R activity in root PM on the concentration of FCN at pH 7.0 and corresponding Eadie-Hofstee plots. Data are means ± SE of six replications. ■, Fe-deficient plants; ○, controls.

lated from Fe-deficient plants when compared to the controls (Brüggemann and Moog, 1989; Holden et al., 1991; Rubinstein and Luster, 1993; Moog and Brüggemann, 1994).

In view of the data available, several possible mechanisms could be responsible for the turbo FC-R. The first possibility is in line with the classical Bienfait theory that a distinct turbo FC-R enzyme, different from the standard FC-R present in controls and capable of reducing Fe at high rates, is active in intact, Fe-deficient plants. From our data, this enzyme would have a significantly lower affinity for Fe-EDTA than the standard FC-R and its activity could be strongly inhibited with a small shift in pH, from 6.0 to 6.5. An alternative explanation would be that in Fe-deficient plants the standard FC-R may, in intact roots in a narrow pH range, undergo a modification that lowers its affinity for Fe-EDTA and markedly increases its capacity to reduce Fe.

Any of these mechanisms could be mediated by pH through charge changes in the surface and/or the enzyme at the apoplastic side of the PM. A low pH may reduce the electrostatic repulsion between charged group(s) at the cell wall, the PM, or the FC-R complex of Fe-deficient plants and the negatively charged major species, Fe(III)-EDTA$^-$. This may in turn influence the access of the reducing site of the FC-R complex to the Fe(III) ion. That substantial changes occur in the functioning of root plasma membrane of Fe-deficient sugar beet when pH changes from 6.5 to 6.0 is likely, since massive excretion of accumulated flavin sulfates from the roots of Fe-deficient plants grown at high pH occurs between pH 6.5 and pH 6.0 (Susín et al., 1993, 1994). However, the fact that the root FC-R activity of Fe-deficient plants growing in unbuffered solution (which do not accumulate flavins; Susín et al., 1994) was approximately 30% higher than that of plants growing at high pH (which exhibit a large accumulation of flavins; Susín et al., 1993) indicates that the accumulation of total flavins per se was not crucial in determining FC-R activity. One may speculate that a change in membrane permeability, perhaps mediated by the opening of membrane channels or pores, could lead both to the availability of internal reducing power and to the excretion of accumulated flavins.

A crucial question that has not been resolved yet is why a turbo-type FC-R activity cannot be observed in PM preparations. It has been suggested that this is a consequence of the localization of the turbo FC-R, which is restricted to a relatively small area in the root surface around the root tip. Isolation of PM from the whole of the root would, according to this view, result in a major dilution of the turbo FC-R, whose characteristics would be masked by those of the constitutive FC-R present in all PM (Moog and Brüggemann, 1994). However, this hypothesis overlooks the fact that in most cases the FC-R activity is measured on a root fresh weight basis and not on an area basis. For instance, in sugar beet we found that in response to Fe deficiency the FC-R activity had a 10- to 20-fold increase in intact plants (on a root fresh weight basis) and only a 2-fold increase in PM vesicles (on a protein basis). Therefore, for the dilution theory to hold, the amount of PM protein per unit of root fresh weight must be 5- to 10-fold higher in the controls than in the Fe-deficient plants. However, both the total protein per unit of root fresh weight and the relative

Table VI. *Equilibrium concentrations of different EDTA species in the pH range 5.0 to 8.0 in the conditions used for the FC-R assay, predicted using MINTEQA2 (pe + pH = 14), where pe = Eh/59.2. Eh is the redox potential in mV.*

Data are in micromolar.

pH	FeEDTA	Fe(OH)EDTA	$Fe(OH)_2EDTA$	ZnEDTA	CuEDTA	MnEDTA	CaEDTA
5.0	499	0	0.0	0.32	0.07	0.11	0.0
5.5	498	1	0.0	0.36	0.10	0.24	0.1
6.0	495	4	0.0	0.38	0.13	0.49	0.2
6.5	486	12	0.0	0.39	0.16	0.91	0.4
7.0	461	36	0.1	0.39	0.17	1.55	0.7
7.5	397	97	1.2	0.40	0.18	2.44	1.6
8.0	271	211	8.3	0.40	0.18	3.63	5.1

amount of PM per total protein (data not shown) were similar in control and Fe-deficient roots. Therefore, other possible explanations should be taken into consideration. A second obvious possible alternative is that an as-yet unidentified cofactor necessary for the functioning of the turbo FC-R may be lost during the preparation of PM fractions. A possible candidate for this may be some cytoplasmatic cofactor needed for the activity of the turbo FC-R.

An important conclusion that can be drawn from our data is that the time-dependent expression of the root turbo FC-R in response to Fe deficiency occurred in both the presence and the absence of a solid phase of $CaCO_3$ in the nutrient solution (free HCO_3^- activities of 0.5 mM and 0.4 μM, respectively, as estimated by the MINTEQA2 software). Furthermore, the FC-R in both types of materials showed qualitatively similar pH dependence. This would imply that neither Ca^{2+} nor HCO_3^- plays a determining role in triggering the specific Fe-deficiency-induced turbo FC-R expression, although the presence of a solid phase of $CaCO_3$ certainly modulates (directly or indirectly) the activity of the enzyme. This has major implications in Fe-deficiency research, since it supports the use of model systems to address the economically important problem of Fe deficiency (Fe chlorosis) in field crops.

In summary, we have found that the pH dependence of the in vivo FC-R activity in Fe-deficient plants was different from (a) that of the FC-R in control, Fe-sufficient plants and (b) those of the FCN-R in Fe-deficient and control plants. At a pH of 6.0 or below, the in vivo FC-R activity in Fe-deficient plants showed 10- to 20-fold increases (turbo), whereas the in vivo FC-R activities at a pH of 6.5 or above, the in vivo FCN-R at any pH, and the PM FC-R and FCN-R activities showed increases in the range 2- to 3.5-fold over the corresponding controls. Furthermore, we found that the K_m for EDTA of the in vivo turbo FC-R in Fe-deficient plants at a pH below 6.0 was approximately 514 μM, whereas the in vivo FC-R activities at a pH above 6.5, the in vivo FCN-R at any pH, and the PM FC-R and FCN-R activities showed K_m values for EDTA of approximately 200 to 250 μM. Based on these biochemically distinct features, we suggest that the turbo FC-R is substantially different from the constitutive FC-R. We are currently testing the hypothesis that the reason that a turbo FC-R in PM has not been found may reside in the loss, during PM preparation, of a cofactor necessary for the enzyme to be active.

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