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Iron-Stress Induced Redox Activity in Tomato (*Lycopersicum esculentum* Mill.) Is Localized on the Plasma Membrane[1]

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ABSTRACT

Tomato plants (*Lycopersicum esculentum* Mill.) were grown for 21-days in a complete hydroponic nutrient solution including Fe^{3+}-ethylenediamine-di(*o*-hydroxyphenylacetate) and subsequently switched to nutrient solution withholding Fe for 8 days to induce Fe stress. The roots of Fe-stressed plants reduced chelated Fe at rates sevenfold higher than roots of plants grown under Fe-sufficient conditions. The response in intact Fe-deficient roots was localized to root hairs, which developed on secondary roots during the period of Fe stress. Plasma membranes (PM) isolated by aqueous two-phase partitioning from tomato roots grown under Fe stress exhibited a 94% increase in rates of NADH-dependent Fe^{3+}-citrate reduction compared to PM isolated from roots of Fe-sufficient plants. Optimal detection of the reductase activity required the presence of detergent indicating structural latency. In contrast, NADPH-dependent Fe^{3+}-citrate reduction was not significantly different in root PM isolated from Fe-deficient *versus* Fe-sufficient plants and proceeded at substantially lower rates than NADH-dependent reduction. Mg^{2+}-ATPase activity was increased 22% in PM from roots of Fe-deficient plants compared to PM isolated from roots of Fe-sufficient plants. The results localized the increase in Fe reductase activity in roots grown under Fe stress to the PM.

Iron deficiency induces a series of adaptive reactions in roots of Fe-efficient plants (10, 26). Roots under Fe stress show a dramatic increase in root hairs and rhizodermal transfer cells (16, 17), have a 5- to 10-fold increased capacity to reduce chelated Fe (4, 11) and show increased acidification of the rhizosphere (13, 27). Both acidification and Fe reductase activities are spatially coincidental on the newly developed roots (27). In an early attempt to explain reduction of Fe by roots, Brown and Ambler (6) and Olsen *et al.* (22, 23) proposed the secretion of reductants into the rhizosphere. In support of this hypothesis, they as well as Römheld and Marschner (25) detected Fe reducing compounds, identified as *o*-diphenolics, in the rhizosphere of Fe-stressed roots. However, these compounds can reduce high-affinity Fe^{3+}-chelates only very slowly (11, 25).

An alternate mechanism of Fe-stress induced Fe reduction has been presented by Chaney *et al.* (11) and subsequently expanded upon by Bienfait *et al.* (3, 4). In their hypothesis, a transplasma membrane (PM^3) electron transport chain supplies electrons from cytoplasmic reducing equivalents to the extracytoplasmic cell surface where Fe reduction occurs. Since the proposal of an Fe-stress-responsive electron transport chain by Chaney *et al.* (11), evidence for PM redox systems and their involvement in Fe reduction has become substantial. For example, a number of reports suggest the presence of NAD(P)H dehydrogenase activity on plant PM (reviewed in ref. 12). The presence of a PM-associated redox system in plants has been demonstrated in intact roots, whole cells, protoplasts, isolated membranes, and purified PM preparations (reviewed in ref. 12). Also, the reduction of Fe at the cell surface is enzymatic (1, 4, 25), occurs on the extracytoplasmic surface of the PM (2) and is increased *circa* 10-fold during Fe stress (4, 11).

The structure of the PM Fe^{3+}-chelate reductase is largely unknown. Sijmons *et al.* (29) presented evidence indicating the involvement of cytoplasmic reduced pyridine nucleotides, possibly NADPH, as the source of reducing equivalents. Sijmons *et al.* (28) demonstrated that the addition of ferricyanide or Fe^{3+}-EDTA to intact roots caused a rapid, 30 to 40 mV depolarization of the membrane potential, suggesting a transmembrane flow of electrons. Cakmak *et al.* (9) reported the nonobligatory involvement of superoxide radicals in Fe reduction and, based on theoretical consideration, proposed a semiquinone in the 1-electron reduction of O_2. Such a mechanism differs significantly from the superoxide generating system on the PM of leucocytes, which involves a flavin-mediated transfer of electrons to a *b*-type Cyt (20).

Until recently, investigations into the mechanism of Fe reduction at the PM and the mechanism of reductase induction during Fe stress adaptation have centered around intact roots. We report here evidence for the localization of the Fe-stress redox response in a tomato root PM fraction prepared by aqueous two-phase partitioning.

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[3] Abbreviations: PM, plasma membrane(s); BPDS, bathophenanthrolinedisulfonate; EDDHA, ethylenediamine-di(*o*-hydroxyphenylacetate); FCR, ferricyanide reductase; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid; PDTS, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tomato (*Lycopersicum esculentum* Mill. cv Rutgers) seeds were treated with ethanolic Arasan[4] and germinated in standard paper towels, which were moistened with Fe free macronutrient solution (see below). After 10 d germination, uniform seedlings were wrapped into bundles of 4 plants each, and 12 bundles were placed into 8 L buckets fitted with black Plexiglas covers. Plants were grown hydroponically in a growth chamber under a 16-h photoperiod at 22°C without humidity control. Cool-white fluorescent and incandescent lamps provided 250 μE m^{-2} s^{-1} at the top of the plants. The composition of the full nutrient solution was: 1.0 mM $MgSO_4$, 2.5 mM $Ca(NO_3)_2$, 2.0 mM KNO_3, 0.1 mM K_2HPO_4, 10 μM Fe-EDDHA, 10 μM B, 2 μM Mn, 1 μM Zn, 0.5 μM Cu, 0.2 μM Mo, 0.2 μM Co, 0.2 μM Ni, and 25 μM Cl. $CaCO_3$ was added at 4 g per bucket to maintain bulk solution pH at 7.5. Phosphate (10 μM K_2HPO_4) was added daily for 1 week before the Fe treatments were begun. During Fe treatment, P was increased to 20 μM per day. In order to compare roots grown with and without Fe, tomato plants were grown 8 d with either the complete solution (10 μM FeEDDHA) or with no Fe. Before transfer from Fe-containing solutions to Fe-free solutions, roots were rinsed thoroughly with Fe-free media to reduce carry-over of Fe. Nutrient solutions were replaced every 4 d.

In Vivo Fe reductase assay

Fe^{3+}-chelate reduction by roots of intact plants was assayed on the days that similarly grown roots were harvested for membrane preparation. A bundle of four plants was transferred to a 1 L assay beaker. Light was excluded by black Plexiglas lids and polyethylene wrappers. The assay solution contained full nutrient solution plus 200 μM FeHEDTA and 1 mM PDTS and 2 mM Mes buffer (pH 6.0). The solutions were continuously aerated. Aliquots were removed every 30 min. Control experiments were performed without plants to correct for possible Fe photoreduction. Fe^{2+} produced by the roots was measured spectrophotometrically as an Fe^{2+}-PDTS complex at 562 nm, and concentration calculated using a molar extinction coefficient of 27.9 mM^{-1} cm^{-1}. Rate of reduction was calculated by linear regression of Fe^{2+} produced *versus* time and expressed on a root fresh weight basis.

Isolation of PM

Roots were rinsed sequentially in tap water and distilled water to remove excess ions, blotted dry with paper towels and weighed. Secondary roots were trimmed from the primary root, chopped to about 1 mm segments with razor blades and homogenized in 5 volumes (g fresh weight ml^{-1}) of buffer consisting of 15 mM Tris-Cl (pH 7.5), 0.5 M sucrose, 1 mM EGTA, 1 mM EDTA, 6% (w/v) PVP, 0.1% (w/v) BSA, 0.1 mM DTT, and 1 mM PMSF using a Polytron homogenizer at 60% speed setting for two 30 s bursts. Microsomal pellets were obtained from the homogenate as described by Buckhout and Hrubec (8). For the phase partitioning, four 36 g phase systems were constructed containing 6.4% (w/w) dextran T500 (Pharmacia AB, Uppsala, Sweden), 6.4% (w/w) polyethylene glycol with an average molecular mass of 3350 D (Sigma Chemical Co., St. Louis, MO), 0.048% phosphate buffer titrated to pH 7.8 with KOH, 0.037% KCl and 8.56% sucrose all calculated by weight. Ten g of membranes suspended in the phosphate buffered sucrose solution were added to a phase system to give a 36 g system of the composition described above. All systems were equilibrated to 2°C and partitioned by repeated inversion for 20 s. The phases were separated by centrifugation at $2{,}000g_{max}$ for 10 min at 2°C (Sorvall HB-4 rotor, Du Pont, Wilmington, DE). The partitioning procedure was repeated for a total of 4 partitions as described (8). Upper phases were diluted with 5 volumes of 15 mM Tris-Cl (pH 7.5), 250 mM sucrose and 0.1 mM DTT (dilution medium) and pelleted at $113{,}000g_{max}$. PM pellets were resuspended in dilution medium for immediate use in ATPase or UDPase enzyme assays. PM pellets for Cyt *c* oxidase, NADH-Cyt *c* reductase, NAD(P)H-Fe^{3+} (chelate) or NAD(P)H-ferricyanide reductase were washed with 10 volumes of dilution medium without DTT, repelleted at $113{,}000g_{max}$ and resuspended in dilution medium without DTT for immediate use in enzyme assays.

Enzyme Assays

Marker enzymes for endoplasmic reticulum, Golgi apparatus and mitochondria were, respectively, antimycin A-insensitive, NADH-Cyt *c* reductase (15), Triton X-100-stimulated UDPase (21) and Cyt *c* oxidase (15). Assays were performed by the modifications of Buckhout *et al.* (7). Vanadate-inhibited, Mg^{2+}-ATPase activity, a marker enzyme for PM, and chloride-stimulated Mg^{2+}-ATPase, a marker enzyme for tonoplast membranes, were assayed with and without 0.0125% (w/v) Triton X-100 as described by Robinson *et al.* (24). Protein was determined by the dye-binding assay of Bradford (5).

NAD(P)H-Fe^{3+}-citrate reductase was assayed in 15 mM Mes-Tris buffer (pH 6.0), containing 250 mM sucrose. The reaction mixture contained 160 μM NAD(P)H, 250 μM Fe-citrate-Tris (pH 6.0), 0.025% (w/v) Triton X-100, and 50 μM BPDS. Fe-citrate-Tris was prepared as a 10 mM $FeCl_3$ in 200 mM citrate-Tris (pH 6.0) buffer and diluted 40-fold into the assay mixture giving a citrate to Fe^{3+} ratio of 20:1. Fe-citrate reduction was assayed spectrophotometrically at 535 nm using an extinction coefficient of 22 mM^{-1} cm^{-1}. NAD(P)H-ferricyanide reductase was assayed as described (8) subtracting the sum of the rates obtained without enzyme and without NADH. An extinction coefficient of 1.02 mM^{-1} cm^{-1} was used in calculations of ferricyanide reduction.

RESULTS

Plasma Membrane Isolation

PM were isolated by the aqueous 2-phase partitioning method described by Larsson (18) and modified by Buckhout

[4] The mention of vendor or product does not imply that they are endorsed or recommended by U.S. Department of Agriculture over vendors of similar products not mentioned.

and Hrubec (8). This method provides membrane fractions composed of >90% PM vesicles of a defined, right-side-out orientation (19). Analysis of membrane marker enzymes was conducted to assess the purity of the fraction used in this study. Contamination of the PM fraction by endoplasmic reticulum (antimycin A-insensitive NADH Cyt *c* reductase) and Golgi apparatus (latent UDPase) was minimal with less than 2% of the total marker enzyme activities for these organelles recovered in the PM fraction (Table I). Similarly, mitochondria were largely absent since only 0.23% of the total Cyt *c* oxidase activity was recovered in the PM fraction. In contrast, approximately 8% of the total protein and nearly 30% of the total PM as determined by vanadate-sensitive Mg^{2+}-ATPase was recovered in the PM fraction. Inhibition of the Mg^{2+}-ATPase by nitrate or stimulation by chloride was less than 5% of the total ATPase value measured at pH 7.5 in both the microsomal fraction (8) and PM fractions and was not consistently observed (data not shown) suggesting that contamination of the PM fraction by tonoplast membranes was minimal.

For reasons of PM availability, marker enzyme analyses were conducted only on membrane fractions from Fe-sufficient roots. We present the following qualitative and quantitative evidence for a similar purity in PM fractions from Fe-sufficient and Fe-deficient roots. First, pellets of PM isolated by the two-phase method appear opalescent and homogeneous (8). PM pellets from Fe-sufficient and Fe-deficient roots were similar in appearance. Second, the specific activity of the Mg^{2+}-ATPase was not significantly different in PM fraction isolated from Fe-sufficient and Fe-deficient roots (*i.e.* >1 standard deviation from the mean, Table IV) suggesting that the relative abundance of this endogenous PM enzyme was equal in both membrane fractions. Third, the ratio of nmol phospholipid Pi to mg membrane protein was 170.26 ± 22.55 (± SE) and 179.89 ± 18.14 for PM isolated from Fe-sufficient and Fe-deficient roots, respectively (n = 3), suggesting a similar membrane composition in PM from both sources. These data support a similar high purity of PM vesicles in fractions from both Fe-sufficient and Fe-deficient roots.

Iron Stress Response in Whole Roots

Tomato seedlings were grown for 10 d and transferred to full nutrient solution for a 7-d preculture growth period before culture without Fe was begun. Seedlings were grown for 8 d under with or without Fe conditions and were subsequently analyzed for root-associated Fe reductase activity. Plants became chlorotic after approximately 3.5 d when grown in the absence of Fe and the severity of chlorosis increased with time, while control plants remained green. Yields on a per plant dry weight basis for roots were 4.1 g for Fe-sufficient and 2.2 g for Fe-deficient plants. After 2 to 2.5 d of culture without Fe, initiation of extensive lateral root development was apparent only in roots cultured without Fe. From this time on, all new root growths were densely covered with root hairs. To clarify terminology, roots harvested from plants grown in the presence or absence of Fe are subsequently referred to as Fe-sufficient or Fe-deficient roots, respectively.

Reduction of FeHEDTA by the intact plants was significantly increased in Fe-deficient plants (Table II). Fe-deficient roots reduced the Fe^{3+}-chelate 7.4-times faster than Fe-sufficient roots. For the 3.5 h of the assay, the rates of reduction remained linear for both control and Fe-deficient plants with linear correlation coefficients (r^2) of 0.84 and 0.83, respectively. Although fresh weights of the Fe-deficient roots were lower than those for Fe-sufficient roots, the total Fe reduced was greater for the Fe-deficient roots.

Localization of Fe reduction along the roots was tested in a parallel study using the Prussian blue stain (2). As great as the differences in reduction rate for FeHEDTA were, staining of localized Fe reduction indicated an even greater difference due to culture without Fe (data not shown; 2). Fe-deficient roots showed intense Fe reduction only on the young root hairs covering primary and lateral roots. Fe-sufficient roots showed little or no localized reduction. For the Fe-deficient roots, Fe reduction was localized near the primary and lateral root tips and on root hairs, while the Fe-sufficient plants showed very light and diffuse staining over most of the root system (2). Electron microscopic examination indicated that

Table I. *Analysis of Marker Enzymes in Membrane Fractions Isolated by the Aqueous Two-Phase Partitioning Procedure*

PM was isolated from Fe-sufficient roots grown as described in "Materials and Methods." Numbers in parentheses are specific activities. NADH-Cyt *c* reductase was assayed in the presence of 10 $\mu g \cdot ml^{-1}$ antimycin A. No significant (*i.e.* >5%) chloride-stimulated or nitrate-inhibited ATPase activity was detected in the microsomal fraction or PM fractions. ATPase was assayed with and without 0.0125% (w/v) Triton X-100. The values reported were assayed with Triton X-100. The PM vanadate-sensitive ATPase was stimulated about 11-fold by detergent.

Fraction	Protein	Triton X-100-stimulated, UDPase[a]	NADH-Cyt *c* reductase[b]	Cyt *c* oxidase[b]	Vanadate-sensitive Mg^{2+}ATPase[a]
	mg				
Microsome fraction	45.83	872.80 (19.04)	9.20 (0.20)	17.61 (0.38)	448.57 (9.79)
% recovered	100.00	100.00	100.00	100.00	100.00
PM fraction	3.56	13.99 (3.93)	0.16 (0.04)	0.04 (0.01)	131.92 (37.09)
% recovered	7.80	1.60	1.75	0.23	29.41

[a] $nmol \cdot min^{-1}$ ($nmol \cdot [min \cdot mg\ protein]^{-1}$). [b] $mmol \cdot min^{-1}$ ($mmol \cdot [min \cdot mg\ protein]^{-1}$).

Table II. *In Vivo Fe^{3+} Reduction by Tomato Roots Grown under Fe-Sufficient or -Defficient Conditions*

Seedlings were grown 8 d under Fe-sufficient or Fe-deficient conditions, and Fe^{3+}-chelate reductase activity was determined by spectrophotometric analysis of Fe^{2+} as the PDTS complex when roots were supplied 100 μM FeHEDTA at pH 6.0 (see "Materials and Methods" for details).

Treatment	Rate ± SE
	$\mu mol \cdot h^{-1} \cdot g$ root fresh wt^{-1}
Fe-sufficient (+ FeEDDHA)	0.87 ± 0.20
Fe-deficient (− Fe)	6.41 ± 1.27

Table III. *Analysis of Fe^{3+} Reductase Activities in PM Isolated from Tomato Roots Grown under Fe-Sufficient or -Deficient Conditions*

Activity was determined spectrophotometrically in the presence of 0.025% (w/v) Triton X-100. Results are reported as means of four independent experiments. Latency was calculated by the following equation: ([rate with Triton X-100] − [rate without Triton X-100])·(rate with Triton X-100)$^{-1}$·100. Results for enzyme latency are from a single experiment.

Acceptor	Donor	Treatment	Rate ± SE	Increase	Latency
			$nmol \cdot (mg\ protein^{-1}) \cdot min^{-1}$	%	
Fe^{3+}-citrate	NADH	+ Fe	121 ± 17		97
		− Fe	234 ± 49	93	96
	NADPH	+ Fe	3.7 ± 2.5		ND[a]
		− Fe	4.2 ± 2.6	14	ND
$Fe(CN)_6^{3-}$	NADH	+ Fe	697 ± 53		93
		− Fe	1474 ± 89	116	92
	NADPH	+ Fe	162 ± 42		ND
		− Fe	197 ± 57	22	ND

[a] Not determined.

Prussian blue accumulated between the PM and the cell wall of root hairs and epidermal cells (31).

Iron-Stress Response in Isolated PM Vesicles

In spite of the evidence for the induction of Fe^{3+}-chelate reductase activity on the PM of root cells in response to Fe stress (3, 10), such an electron transport system has not been demonstrated on isolated PM. Therefore, Fe^{3+}-chelate reduction was investigated in purified PM vesicles isolated from Fe-sufficient and Fe-deficient roots. In PM isolated from Fe-sufficient roots, Fe^{3+}-citrate and ferricyanide were reduced at rates of 121 and 697 nmol·(mg protein)$^{-1}$·min^{-1}, respectively, with NADH as the electron donor (Table III). The rate of NADPH-dependent reduction of Fe^{3+} was 3 and 23% of the rate supported by NADH for Fe^{3+}-citrate and ferricyanide, respectively (Table III). Pyridine nucleotide-dependent Fe^{3+}-chelate reduction occurred at significantly higher rates in PM vesicles isolated from Fe-deficient roots compared to controls (Table III). The specific activity of NADH-dependent Fe^{3+}-citrate reduction for the Fe-deficient roots was increased 93% over controls, while ferricyanide reduction was increased 116%. The specific activity of NADPH-dependent reduction of both Fe^{3+}-citrate and ferricyanide was only minimally increased in PM isolated from Fe stressed roots compared to controls. Although the increases in NADPH-dependent Fe reduction were not statistically significant (*i.e.* greater than 2 standard deviations from the mean), the NADPH-dependent Fe reductase activity in PM from Fe-deficient roots was greater than that in controls in all experiments conducted (n = 4). Detection of optimal activity required the presence of 0.025% (w/v) Triton X-100. This dependence on detergent indicated that either the electron donor, acceptor, or both required access to sites on the enzyme not exposed to the external solution in the reductase assay. These results clearly demonstrate the induction of a PM-bound Fe^{3+}-chelate reductase activity in response to Fe stress.

So-called Fe-efficient plants respond to Fe stress by acidification of the rhizosphere and induction of Fe^{3+}-chelate reductase activity. An increase in Fe^{3+}-chelate reductase activity has been clearly demonstrated in isolated PM from Fe-stressed roots (Table III). Parallel to the analysis of reductase activity, PMs from Fe-sufficient and -deficient roots were analyzed for Mg^{2+}-ATPase activity. Since the PM Mg^{2+}-ATPase is a proton-transporting enzyme involved in establishing the protonmotive force, acid outside, across the PM, it is possible that this ATPase is involved in the increased acidification of the rhizosphere in response to Fe stress. However, analysis of the ATPase activity in PMs from Fe-sufficient and -deficient roots showed only a slight increase in ATPase specific activity in membranes derived from Fe-deficient roots (Table IV). Since the physiological response to Fe stress in intact roots is a dramatic acidification of the rhizosphere (13, 27), one must conclude that either the increase in ATPase occurs only on the PM of epidermal cells and that these vesicles are only a minor population of the PM preparation, or that this relatively small increase in specific activity of the ATPase in PM isolated from Fe-deficient roots is not related to the mechanism of acidification observed in intact roots growing under Fe stress.

DISCUSSION

In response to Fe deficiency, roots of dicotyledonous plants induce a series of reactions which allow absorption of Fe from the rhizosphere with greater efficiency. These adaptive reactions have been shown to occur on the root surface and include an increased reduction of Fe^{3+}-chelate, acidification of the rhizosphere to increase Fe mobility and persistence of Fe^{2+} and alterations in root morphology. The data in this report demonstrate for the first time an increase in Fe^{3+}-

Table IV. *Analysis of Mg^{2+}-ATPase Activity in PM Isolated from Tomato Roots Grown under Fe-Sufficient or Fe-Deficient Conditions*

Activity was determined spectrophotometrically as the increase in P_i in the presence of Mg^{2+} and 0.025% (w/v) Triton X-100. Units of activity are nmol·(mg protein)$^{-1}$·min^{-1} and are means ± SE (n = 4). Latency was calculated by the following equation: ([rate with Triton X-100] − [rate without Triton X-100])·(rate with Triton X-100)$^{-1}$·100.

Treatment	Activity	Increase	Latency
		%	
+ Fe	162 ± 42		97
− Fe	197 ± 57	22	96

chelate reductase activity in the PM isolated from Fe-deficient roots. This finding is significant because it conclusively links cytochemical localization (2) with direct enzymological determination of reductase activity at the PM.

An early theory for the mechanism of Fe reduction in Fe-stressed roots implicated phenolic compounds secreted by the roots as the source of reducing potential (ref. 3 and references cited therein). In support of this theory, Römheld and Marschner (25) demonstrated that acidification of the root free space in response to Fe-stress promoted the release of reducing substances identified as *o*-diphenolics. However, the facts that the pH optima for Fe reduction by roots and phenolics were widely disparate (5.0 *versus* >7.0, respectively) and the rate of reduction of high affinity Fe^{3+} chelates under physiological conditions could not be accounted for by phenolics, do not support this hypothesis (25). Recently, Tipton and Thowsen (30) proposed a modification of the secretion hypothesis in which malate would be secreted into the cell wall with the subsequent production of NADH by malate dehydrogenase and NADH supplying the reducing equivalents necessary to reduce Fe. Although malate dehydrogenase has been localized in isolated cell walls (9, 14), two lines of evidence argue against its role in Fe reduction. First, Fe stress was coincident with a modest increase in soybean seedling malate content (30); however, the concentration of malate dehydrogenase in the cell wall is not adequate to support the observed rate of Fe reduction by whole roots (9). Second, the addition of malate to intact roots actually inhibited Fe reduction (9) inconsistent with the proposed role of malate as the reductant for NADH. Taken as a whole, secretion of Fe reducing substances seems not to be the major source of Fe reducing potential in Fe-stressed roots.

An alternate mechanism for the reduction of Fe^{3+}-chelates by Fe-deficient roots is by enzymic reduction at the cell surface. Fe^{3+}-chelate reduction activity was first proposed by Chaney *et al.* (11), and this theory has been subsequently confirmed and extended by Bienfait (3) and Römheld and Marschner (25, 26). Results presented here clearly show that the primary mechanism of Fe-deficiency induced Fe reduction is through the increase in activity of a PM electron transport system.

In intact roots, Fe stress results in a greater than 7-fold increase in Fe^{3+}-chelate reductase activity in tomato (Table II), a 6-fold increase in bean roots (4), and up to 20-fold in peanut (11). In isolated PM from tomato roots, Fe^{3+}-chelate reduction was increased approximately 2-fold in Fe-deficient roots compared to controls (Table III). This quantitative difference between the increased reductase activity in intact roots and isolated PM likely reflects the fact that the Fe-stress response is restricted to the epidermal region of the root (2, 4) while epidermally derived PM vesicles in the membrane preparation represent only a small fraction of the total PM vesicle population. Thus, the quantitative disparity between *in vivo* and *in vitro* results may reflect more the spatial localization of the Fe stress response to the root surface than implying an alternate mechanism for supplying Fe reducing potential.

Bienfait (3) has suggested that roots contain two Fe reductase activities in dicotyledonous and nongraminaceous, monocotyledonous roots; one that is capable of reducing chelated Fe and ferricyanide and another that reduces only ferricyanide. The Fe^{3+}-chelate reductase was thought to be largely expressed in epidermal cells of young lateral roots grown under Fe-deficient conditions (Turbo system) while the ferricyanide reductase activity was thought to be constitutively expressed (standard system) in all root cells. If this hypothesis is correct, then one would expect that the increase in Fe^{3+}-chelate reductase observed with PM from Fe-deficient roots would be much greater than the increase in ferricyanide reductase activity under similar Fe-stress conditions. This was not observed (Table III). Both ferricyanide and Fe^{3+}-citrate reductases were increased by approximately twofold. We have not attempted to subfractionate root tissues to further test this hypothesis. An explanation consistent with the data in Table III would suggest the presence of both ferricyanide and Fe^{3+}-chelate reductases on the PM of all root cells. Previous results suggest that the response of the root to Fe stress is restricted to the epidermis and does not extend into the root cortex (2, 17, 27). Thus, the response of the root to Fe stress is to increase Fe^{3+}-chelate reductase in young epidermal cells. No evidence to date or presented here suggest that Fe^{3+}-chelate and ferricyanide reductase activities are different enzymes.

Detection of optimal ferricyanide and Fe^{3+}-chelate reductase activity required the presence of detergent. Detergents function in exposing catalytic oxidation/reduction sites not exposed to the external solution. These results suggest that either NADH and/or the Fe reducing component(s) of the enzyme are located on the internal surface of the membrane. Although the data are not conclusive from these experiments, they are consistent with a *trans*-PM flow of electrons from cytoplasmic NADH to extracytoplasmic ferricyanide or Fe^{3+}-chelate.

The preferred electron donor in the Fe-stress induced ferricyanide and Fe^{3+}-chelate reductases was NADH. Little or no increase in NADPH-dependent Fe-chelate or ferricyanide reductase activities was detected (Table III). The results of Sijmons *et al.* (29), which report a two-fold increase in total NADP and the NADPH/NADP ratio in Fe-deficient bean roots and a marked decrease in the NADPH/NADP ratio in Fe-deficient lateral roots following ferricyanide addition, have been interpreted as evidence for NADPH as the primary electron donor for Fe^{3+}-chelate reduction. However, direct enzymatic analysis of Fe^{3+}-chelate reductase activity in PM demonstrates the involvement of NADH as the primary electron donor. Thus, the increase in NADP levels in Fe-stressed roots may be the result of inhibition of NADP utilization as a result of Fe stress as suggested by Sijmons *et al.* (29) rather than indicating a role of NADPH in Fe^{3+}-chelate reduction. Furthermore, the decrease in NADPH following addition of ferricyanide to Fe-deficient roots might suggest a conversion of NADP to NAD by a pyridine nucleotide kinase/phosphatase in response to decreasing NADH levels, or alternately the decrease may reflect utilization of NADPH unrelated to the reduction of Fe^{3+} chelates. These results should provide a foundation for further analysis of the molecular aspects of the Fe^{3+}-chelate reductase in relation to adaptation by plants to Fe stress.

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