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Characterization of Phloem Iron and Its Possible Role in the Regulation of Fe-Efficiency Reactions

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ABSTRACT

'Fe-efficiency reactions' are induced in the roots of dicotyledonous plants as a response to Fe deficiency. The role of phloem Fe in the regulation of these reactions was investigated. Iron travels in the phloem of *Ricinus communis* L. as a complex with an estimated molecular weight of 2400, as determined by gel exclusion chromatography. The complex is predominantly in the ferric form, but because of the presence of reducing compounds in the phloem sap, there must be a fast turnover *in situ* between ferric and ferrous ($k \approx 1$ min^{-1}). Iron concentrations in *R. communis* phloem were determined colorimetrically or after addition of ^{59}Fe to the nutrient solution. The iron content of the phloem in Fe-deficient plants was lower (7 micromolar) than in Fe-sufficient plants (20 micromolar). Administration of Fe-EDTA to leaves of *Phaseolus vulgaris* L. increased the iron content of the roots within 2 days, and decreased proton extrusion and ferric chelate reduction. The increase in iron content of the roots was about the same as the difference between iron contents of roots grown on two iron levels with a concomitantly different expression of Fe-efficiency reactions. We conclude that the iron content of the leaves is reflected by the iron content of the phloem sap, and that the capacity of the phloem to carry iron to the roots is sufficient to influence the development of Fe-efficiency reactions. This does not preclude other ways for the shoot to influence these reactions.

Dicotyledonous plants suffering from Fe deficiency develop a greater ability to mobilize iron from the soil. The 'Fe-efficiency reactions' may be expressed both morphologically (increased production of root hairs, formation of rhizodermal transfer cells) and biochemically (proton extrusion resulting in rhizosphere acidification, increase of the capacity to reduce dissolved ferric chelates) (20).

We showed recently that the roots can control the development and expression of Fe-efficiency reactions (3). This does not imply, however, that the regulation of these reactions is located exclusively in the roots. The leaves might send a signal to the roots which modifies the extent of development or expression of one or more of these reactions (16). The signal might be iron itself. Iron is considered to be 'intermediately mobile' in plants (27). It is present in the phloem sap of *Arenga* and *Yucca* (26, 27) and it can be exported from leaves (4, 6). A regulating influence of K and P in the phloem on the development of changes in the kinetics of K and P uptake by the roots has been proposed (8, 10).

We investigated whether Fe in the phloem modulates the expression of Fe-efficiency reactions.

MATERIALS AND METHODS

Seeds of castor oil (*Ricinus communis* L. var gibsonii rouge) and of dwarf bean (*Phaseolus vulgaris* L var Prélude) were obtained from Sluis, Enkhuizen, The Netherlands, and plants were grown on Knop nutrient solution as described (21) with varying concentrations of Fe-EDTA.

Ferric chelate reduction by the roots was determined colorimetrically with Fe-EDTA as substrate as described (1).

Xylem exudate was obtained by cutting the stem of a 7 to 14 d old plant halfway between cotyledons and roots. The exudate from the stump was collected in a 50 μl glass capillary. Phloem exudate of *R. communis* was obtained by making a superficial incision in the stem with a razor blade (13). The exudate was collected in a 50 μl glass capillary. Phloem exudates of *P. vulgaris* were collected by the method of King and Zeevaart (14) as described (9); sugar contents of phloem exudates were determined as described (9).

For determination of nitrate in xylem and phloem exudates, 10 μl exudate was diluted with 250 μl water, 750 μl 85% (w/w) H_2SO_4 was added, and, after cooling, 50 μl 1% 3,4-xylenol in glacial acetic acid. After 30 min incubation at 30°C, 500 μl water-saturated toluene was added, followed by mixing, and centrifuging in an Eppendorf centrifuge. The toluene phase was washed three times with water, after which 500 μl 0.4 N NaOH was added. After mixing and centrifugation, the water phase was separated for determination of A_{485}.

In experiments with labeled Fe, ^{55}Fe or ^{59}Fe was applied to the abaxial surface of both cotyledons of *R. communis*, or added to the nutrient solution as the ferric-EDTA complex.

For gel filtration chromatography of phloem sap labeled with ^{55}Fe or ^{59}Fe, columns were prepared in 5-ml glass pipettes with Biogel P2, Biogel P10, or Sephadex G25. The columns were equilibrated and eluted with water, 10 mM Hepes (pH 7.5) or 1 M NaCl, which all yielded comparable results. Void volume (V_0) and total volume (V_t) were determined with Dextran Blue 2000 and $CuCl_2$, $K_3Fe(CN)_6$ or $NiCl_2$. Samples of 15 μl were layered on the column and 2-drop fractions were collected with a fraction collector. Radioactivities were determined with a scintillation counter.

Electrophoresis of ^{59}Fe-compounds was performed in 1% agar gels in 10 mM Hepes (pH 7.5). The gels (76 × 25 × 1 mm) were prepared on microscope slides. A slot of 8 × 2 × 1 mm was punched

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out at 1 cm from one of the short sides. The bottom of the slot was sealed with a thin layer of agar. Samples of 5 μl were applied, the anodic and cathodic compartments were filled with 10 mM Hepes (pH 7.5) and strips of filtration paper were used to contact the gel with the compartments. The voltage across the gel was 15 V. After 3 h electrophoresis the gels were cut into 5 mm-slices for determination of radioactivity.

Tissue Fe was determined colorimetrically after drying of the plant material at 70°C and dry ashing (2).

RESULTS

Effects of Fe Deficiency on *Ricinus communis* and *Phaseolus vulgaris*. Both *P. vulgaris* and *R. communis* developed a greatly increased ferric reduction and proton extrusion by the roots in response to iron deficiency, as shown in Table I. Onset of the reactions appeared after about 1 week of growth on Fe-free medium and coincided with the appearance of chlorosis symptoms in the first trifoliate, respectively palmitate leaf. The reactions of the two species were comparable quantitatively also.

Analysis of Xylem and Phloem Exudates. Phloem and xylem saps of *R. communis* were analyzed for nitrate to determine whether phloem sap was contaminated with xylem sap. In three separate experiments, xylem nitrate was 18, 20, and 20 mM and phloem nitrate 0.6, 0.8, and 0.2 mM, which shows that the contamination of phloem sap by xylem sap was less than 5%. The pH of phloem sap was about 7, the pH of xylem sap was 5, as determined with pH paper.

Fe concentrations in phloem exudate of Fe-sufficient and Fe-deficient plants were determined colorimetrically, and, with Fe-sufficient plants, also 64 and 88 h after transfer to nutrient solution containing ^{59}Fe-labeled Fe-EDTA. We assumed that the specific activity of ^{59}Fe in the phloem was by that time the same as that in the nutrient solution. The values obtained showed a significant difference between the Fe contents of phloem from Fe-deficient plants (7 μM) and that from Fe-sufficient plants (20–33μM) (Table IIA).

When labeled Fe-EDTA was administered to the cotyledons, radioactivity in the phloem exudate appeared within 2 h (Table IIB).

Characterization of Fe Compounds in Xylem and Phloem Sap of *R. communis*. When an iron-deficient *R. communis* plant was placed with its roots in nutrient medium containing 40 μM [^{59}Fe]EDTA, iron was taken up rapidly. Xylem sap was collected 1 d later and analyzed by electrophoresis. The peak of radioactivity co-migrated with [^{59}Fe]citrate.

Filtration of ^{59}Fe-labeled phloem exudate over Bio-Gel P-2, Bio-Gel P-10, and Sephadex revealed that the bulk of phloem iron was in one distinct peak (Fig. 1, A–C). A small peak or shoulder at the elution volume of Fe-EDTA from the Bio-Gel B-2 column may indicate that some EDTA or [^{59}Fe]EDTA had been taken up as such in the phloem. An estimated mol w of about 2400 was calculated from the K value of the eluted radioactive fractions from Bio-Gel P-10 and Sephadex G-25.

Table I. *Development of Fe-Efficiency Reactions in P. vulgaris and R. communis after Transfer to Iron-Free Nutrient Solution*

Conditions	*P. vulgaris*	*R. communis*
Days before appearance of first chlorotic leaf	7	8
Days before first significant acidification of the nutrient solution	6	8
Fe^{3+} reduction by the roots with Fe-EDTA (μmol · h^{-1} · g fresh wt^{-1}): before transfer	0.5	0.6
The same, 8 d after transfer	5	7.5

Phloem Fe, Fe-EDTA, and Fe-citrate all behaved as negative ions electrophoretically. The relative migration rates were Fe-citrate > Fe-EDTA > phloem-Fe (Fig. 2). The electrophoretic behavior patterns of the phloem-Fe fractions labeled by administration of [^{59}Fe]EDTA to the leaves or to the roots were similar (Fig. 2, A and B), which indicates that iron was transported in the same form, irrespective of the way in which the plant was given ^{59}Fe.

Oxidation-Reduction Turnover of Phloem Iron. Addition of the ferrous scavenger 2,2′-bipyridyl to phloem sap of Fe-sufficient *R. communis* resulted in a gradual formation of the red Fe(II)-bipyridyl complex which was complete within 1 min. The strong reductant dithionite, which reduced iron much faster, did not yield more Fe(II)bipyridyl when added after completion of the endogenous reaction. Fe-EDTA, added to phloem sap of Fe-deficient or Fe-sufficient *R. communis*, was reduced at a comparable rate.

Phloem sap, from which low mol wt compounds were removed by drop dialysis (17), did not develop the red color upon addition of bipyridyl; but upon addition of dithionite, the same amount of Fe(II)bipyridyl was formed as in undialyzed phloem sap. Apparently, iron in the phloem sap is bound as a ferric complex, which is continuously reduced by endogenous low mol wt compounds, and the resulting ferrous ion is so loosely bound that it can be scavenged by bipyridyl. However, in the sap of *R. communis* phloem which is about pH 8 (12), O_2 oxidizes the ferrous ions to ferric simultaneously and at a higher rate: no red color is formed immediately after addition of bipyridyl to phloem sap, which indicates that iron in the steady state is mainly ferric. The continuous reduction-oxidation cycle of phloem iron, in combination with the ease at which bipyridyl scavenges the ferrous ions, implies that amounts of strong ferrous scavengers in the phloem sap itself are not significant.

Suppression of Fe-efficiency Reactions by Administration of Fe to Roots or Leaves of *P. vulgaris*. When *P. vulgaris* plants grown for 8 d on Fe-free medium were transferred to medium containing 40 μ*M* Fe-EDTA, ferric reduction increased slightly for 1 d, and the pH of the medium decreased markedly (9, 22). Thereafter, pH lowering stopped and ferric reductase activity decreased. After 3 or 4 d, ferric reductase activity was at the level of that in control plants.

Under the experimental conditions, the ferric reductase of *P. vulgris* increased at Fe-EDTA levels in the nutrient solution lower than 2 μM, and proton extrusion was measurable at levels under 1 μM (Fig. 3). In order to determine whether endogenous Fe levels in the roots correlated with the regulation of Fe-efficiency reactions, plants were grown in nutrient solution containing 0.1 or 3 μM Fe-EDTA, and a lowered phosphate concentration (0.1 mM) to minimize ferric phosphate deposition in the xylem. On the day when the plants growing on 0.1 μM Fe showed their first proton extrusion cycle, the root systems of all plants were harvested for the determination of ferric reductase or cellular Fe content. After removal of apoplasmic iron (2), the iron contents in the two lots of plants differed by 1.5 nmol·mg dry $weight^{-1}$ (Table III).

Treatment of the leaves of Fe-deficient plants with Fe-EDTA had the same effect on proton extrusion and ferric reductase activity as transfer of whole plants to Fe-containing nutrient solution. Administration of 1.5 μmol Fe-EDTA was sufficient to suppress proton extrusion within 1 d, and to lower the ferric reductase to the control level within 2 d (Fig. 4) (*cf.* Fig. 5.3. in Ref. 20).

Plants were grown on Fe-free nutrient solution for a titration experiment, and the under and upper sides of the leaves were sprayed at d 3, 6, and 7 from the start of Fe-free culture with varying concentrations of Fe-EDTA in 0.01% Triton X-100 (11). The result (Fig. 5) shows that after 8 d the proton extrusion

Table II. *Fe in Phloem of R. communis*

A, Determination by colorimetry and after addition of [^{55}Fe] or [^{59}Fe]EDTA to the nutrient solution; B, uptake of labeled Fe administered to the cotyledons.

Experiment	Preculture of Plant	Method Used	Specific Activity of Label	Time Elapsed Before Tapping	Fe in Exudate	Calculated Fe in Phloem
			cpm per μmol Fe	*h*	*cpm per μl*	*μM*
			A			
1	−Fe	Colorimetry				7
2	+Fe	Colorimetry				20
3	+Fe	Label	$3.0.10^5$	64	7	23
4	+Fe	Label	$3.0.10^5$	88	10	33
			B			
5	−Fe	Label	$6.0.10^7$	2	660	
6	−Fe	Label	$6.0.10^7$	2	330	
7	+Fe	Label	$0.9.10^7$	2	530	
8	+Fe	Label	$0.9.10^7$	24	350	

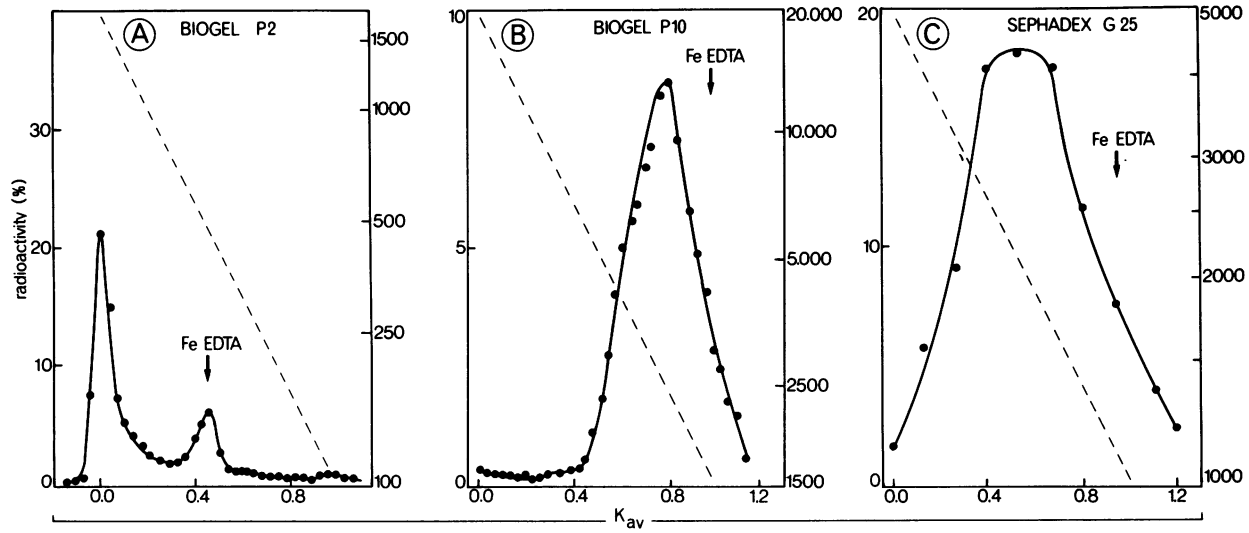

FIG. 1. Gel filtration of phloem sap from Fe-deficient *R. communis* labeled with ^{59}Fe over (A) Bio-Gel P-2, (B) Bio-Gel P-10, and (C) Sephadex G-25.

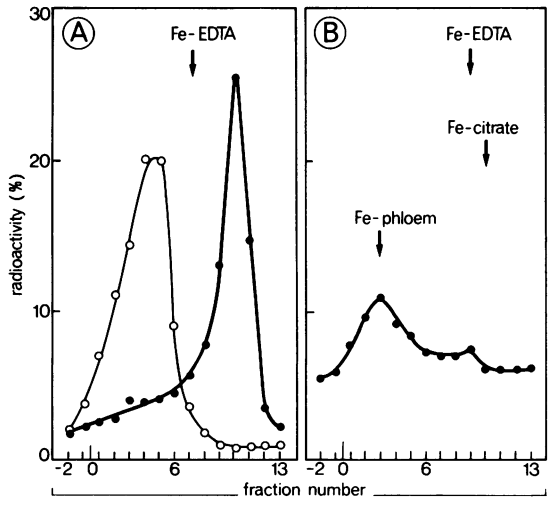

FIG. 2. Agar-electrophoresis of ^{59}Fe-labeled phloem and xylem sap from *R. communis*. A, (○) ^{59}Fe-phloem; (●), ^{59}Fe-xylem. Phloem was labeled via the leaves, xylem through the nutrient solution. B, ^{59}Fe-phloem, labeled through the nutrient solution.

activity was correlated negatively to the Fe content of the roots, within a range of Fe contents of less than 1 nmol·mg dry weight^{-1}. In one experiment the ferric reductase activity decreased as well upon administration of high amounts of iron.

Correlation between Ferric Reduction, Proton Extrusion, and Extractable Phloem Sugars of Bean Plants with Different Fe Status. Bean plants growing on different levels of Fe-EDTA showed increased levels of ferric chelate reduction at Fe concentrations in the nutrient solution lower than 3 μM (Fig. 3B). These plants also lowered the pH of the nutrient solution (Fig. 3C). The expression of these two Fe-efficiency reactions parallelled the amounts of phloem sugars that were obtained in a 2-h bleeding period (Fig. 3A).

DISCUSSION

Characterization of Phloem Iron. In order to study the role of phloem-Fe in regulating Fe-efficiency responses, we characterized the form in which Fe is present in the phloem. The method of King and Zeevaart (14) yielded very small amounts of phloem sap with *P. vulgaris*. Moreover, as the method depends on the use of a chelator to prevent sieve tube clogging, it would not permit identification of transport forms of iron, as the chelator might act as a scavenger. We therefore used *R. communis* in

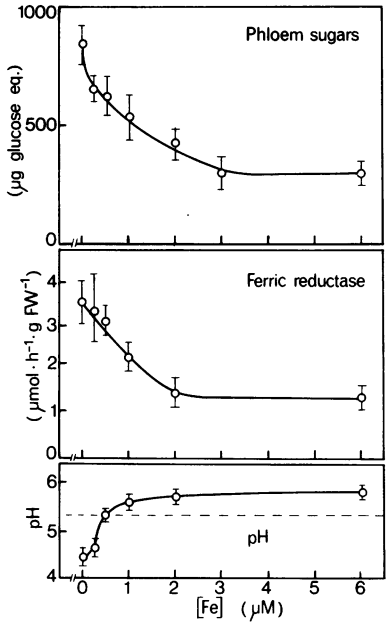

FIG. 3. Phloem sugars collected from the sprout, and ferric reductase capacities and proton extrusion activities in roots of *P. vulgaris* grown on different Fe concentrations in the nutrient solution. Phloem sugars were collected in 10 mM K-EDTA (pH 7.5) during 2 h. The pH values were measured 1 d after replenishment of the media. The stippled line indicates the pH of fresh medium. All values means of 3 measurements ± SD.

Table III. *Fe-efficiency reactions and Fe Content in Roots of P. vulgaris Grown at Two Fe Levels*

Conditions	Fe in Nutrient Solution	
	0.1 µM	3 µM
Ferric reduction rate (µmol · h⁻¹ · g fresh wt⁻¹)	3.0	2.4
	3.8	2.6
pH of the nutrient solution at d 7	3.8–4.2	5.6–5.8
Fe content of the roots (nmol · mg dry wt⁻¹) after removal of apoplasmic Fe	1.8 ± 0.2	3.3 ± 0.5

those experiments where the phloem form of iron was to be qualitatively and quantitatively determined. *R. communis* was similar to *P. vulgaris* with respect to the development and expression of Fe-efficiency reactions (Table I).

Phloem exudate generally contains much less nitrate than xylem exudate (13, 18, 25). Consequently, we used nitrate as an indicator for xylem sap contamination in phloem exudate. Since the xylem exudate contained 20-fold more nitrate than the phloem exudate, we concluded that the phloem exudate was not contaminated appreciably by xylem sap. The Fe concentration in the phloem of Fe-deficient plants, measured by colorimetry (7 µM), was substantially lower than that in Fe-sufficient plants, measured either by colorimetry or by isotope feeding after more than 2 d equilibration (20 and 33 µM).

The transport form of iron in *R. communis* xylem was Fe-citrate, as determined by electrophoresis (data not shown), which agrees with the results of Tiffin (23, 24). For the characterization of Fe in the phloem we routinely used phloem labeled by application of [^{59}Fe]EDTA to the cotyledons, since this labeling yielded the highest radioactivities per µl exudate (Table II B). In one control electrophoresis experiment, phloem exudate was used from a plant labeled via the nutrient solution. Fe from

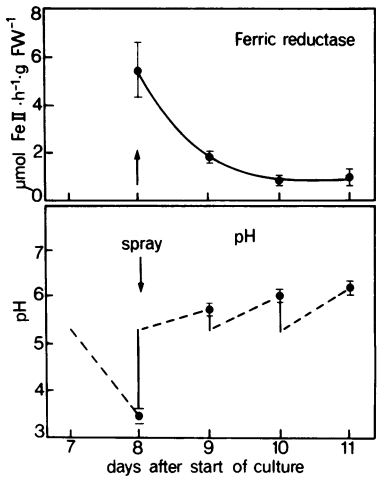

FIG. 4. Time course of suppression of Fe-efficiency reactions in roots of *P. vulgaris* by administration of Fe-EDTA to the leaves. Plants were grown for 7 d on Fe-free nutrient solution; on d 8, 0.3 ml 5 mM Fe-EDTA was applied to the underside of the primary leaves. The nutrient solution (pH 5.3) was replenished at d 4, 7, 8, 9, and 10. Top, ferric reduction activity of the roots; bottom, the pH of the nutrient solutions. All values are means of 8 measurements ± SD.

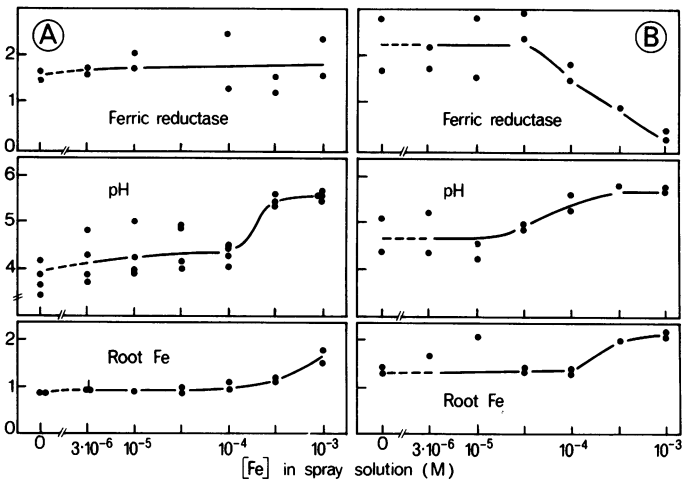

FIG. 5. Suppression of Fe-efficiency reactions in roots of *P. vulgaris* by administration of Fe-EDTA to the leaves. Plants were grown on Fe-free nutrient solution for 7 or 8 d. On d 3, 6 (experiments A and B) and 7 (experiment B), the media were refreshed and the leaves were sprayed with 0.01% Triton X-100 containing Fe-EDTA as indicated. The pH in the nutrient solution, ferric reductase, and Fe content of the roots were measured on d 7 (experiment A) or 8 (experiment B). The units on the ordinates are, from top to bottom: Ferric reductase, µmol Fe(II)·h⁻¹·g fresh weight⁻¹; pH, pH units, Root Fe, nmol Fe·mg dry weight⁻¹.

phloem sap migrated as a negative ion, at lower rates than ferric citrate and ferric EDTA. The elution patterns from Biogel and Sephadex indicated a mol wt of about 2400. The real value will be smaller inasmuch as the molecule deviates from the globular form.

The experiments with the ferrous chelator 2,2′-bipyridyl show that, if the sieve tubes are not anaerobic, phloem iron is bound in the ferric form, and that it must undergo continuous reduction-oxidation *in situ*, the rate being determined by the rate of reduction (about 1 min⁻¹). Although the ferric form is predominant, the fast reduction-oxidation cycle must result in a low but significant steady-state ferrous concentration. This implies that

the ferric chelate at the place of phloem unloading does not need to be reduced by an additional mechanism previous to iron export (5, 7). It will suffice if the translocator has a high enough affinity for the ferrous ion.

Correlations between Fe Levels in the Roots and Development of Fe-Efficiency Reactions. *P. vulgaris* plants grown on 0.1 and 3 μM Fe differed little in leaf Chl contents, but those grown on the lower level developed a marked proton extrusion and had significantly greater ferric reduction in the roots (Table III). The levels of Fe in the roots, after removal of ferric deposits in the apoplasmic space, differed about 1.5 nmol·mg dry weight^{-1} (Table III). Spraying of Fe-EDTA on leaves of plants grown on Fe-free nutrient solution suppressed Fe-efficiency reactions, in parallel with an increase of root Fe content of about 0.7 nmol·mg dry weight^{-1}. Since the Fe status of the roots is a sufficient factor in the regulation of Fe efficiency reactions (3), it follows that the leaves can exert control on their development by means of phloem Fe.

Manipulation of the Fe concentration in the phloem is not the only way for the sprout to control Fe-efficiency reactions in the roots. The work of Landsberg (15) shows that proton excretion requires the continuous presence of an intact connection of tissue with the leaves. On the other hand, the development of transfer cells, which are essential for proton extrusion (19), and the synthesis of the ferric chelate reduction system occur also on isolated roots growing on Fe-free sterile sucrose solution (3).

These observations indicate that the synthesis of the components for the Fe-efficiency reactions, such as transfer cells and the ferric chelate reduction system, is controlled by the Fe status of the roots (3). The leaves could modulate this synthetic activity via the phloem-Fe concentration, according to this work. On the other hand, the extent to which these reactions are expressed might depend on the immediate supply of sugars in the phloem. This is most likely the case for proton excretion. But the expression of ferric chelate reduction must depend also on a supply of phloem sugar, since the reduction attains its maximal capacity during and shortly after a proton extrusion cycle (22). A positive correlation between phloem sugar supply and expression of Fe-efficiency reactions is indicated by Figure 3. The leaves therefore are able to modulate both the development (via phloem Fe) and the expression (via phloem sugars) of Fe-efficiency reactions.

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