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Iron Deficiency Decreases the Fe(III)-Chelate Reducing Activity of Leaf Protoplasts[1]

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The ferric-chelate reductase (FC-R) activity of mesophyll protoplasts isolated from Fe-sufficient (control) and Fe-deficient sugar beet (*Beta vulgaris* L.) leaves has been characterized. Measurements were made in an ionic environment similar to that in the apoplastic space of the sugar beet mesophyll cells. The FC-R activity of Fe-sufficient and Fe-deficient protoplasts was dependent on light. Fe deficiency decreased markedly the FC-R activity per protoplast surface unit. The optimal pH for the activity of the FC-R in mesophyll protoplasts was in the range 5.5 to 6.0, typical of the apoplastic space. Beyond pH 6.0, the activity of the FC-R in mesophyll protoplasts decreased markedly in both Fe-sufficient and Fe-deficient protoplasts. These data suggest that both the intrinsic decrease in FC-R activity per protoplast surface and a possible shift in the pH of the apoplastic space could lead to the accumulation of physiologically inactive Fe pools in chlorotic leaves.

Fe deficiency in plants is usually caused by low Fe availability in calcareous, high-pH soils (Lindsay, 1995). Fe deficiency produces several physiological responses at the root level (Schmidt, 1999). The existence in the roots of dicotyledonous plants of an obligatory Fe reduction step from Fe(III) to Fe(II) prior to Fe root uptake was demonstrated first by Chaney et al. (1972). The reduction of Fe(III) to Fe(II) is carried out by one or several specialized enzyme(s) located in the root cell plasma membrane (PM), the ferric-chelate reductase(s) (FC-R) (Bienfait, 1985, 1988; Cakmak et al., 1987; Rubinstein and Luster, 1993; Moog and Brüggemann, 1994; Susín et al., 1996; Robinson et al., 1998, 1999). Dicotyledonous plant species have the so-called strategy I (Marschner, 1995), that includes, among other mechanisms, the development of an increased capacity to reduce Fe. Strategy I also includes morphological changes, such as the development of root hairs and transfer cells and increased rates of excretion of protons, phenolic compounds, and flavins (Marschner et al., 1986; Welkie and Miller, 1993; Marschner and Römheld, 1994).

Once Fe is taken up by the root cells it must be transported to the different parts of the plant. Fe is probably transported as Fe(III) in the xylem complexed with citrate (Tiffin, 1970) or other organic anionic species (Cataldo et al., 1988). After Fe(III) arrives at the leaf apoplast, it must cross several membrane systems, including those of the mesophyll cells and chloroplasts. Few data are available on how Fe(III) is taken up by the mesophyll cells. Two different model systems have been used until now to estimate leaf cell FC-R activities, PM preparations (Brüggemann et al., 1993; González-Vallejo et al., 1998, 1999; Rombolà et al., 1999), and excised leaf discs (Brüggemann et al., 1993; de la Guardia and Alcántara, 1996). However, the FC-R activity of both model systems cannot be ascribed unequivocally to a PM-associated, trans-oriented electron transport activity.

Several authors have characterized the FC-R activities with leaf PM materials in the presence of the detergent Triton X-100, which opens PM vesicles and is supposed to induce maximal FC-R activities. This has been done with PM from control and Fe-deficient leaves of cowpea (Brüggemann et al., 1993) and sugar beet (González-Vallejo et al., 1998, 1999) and also with PM-enriched microsomes of Fe-sufficient kiwi (Rombolà et al., 2000). The total FC-R activity of Triton X-100-treated PM, however, has been recently suggested to include a significant contribution of cytoplasmic side cis electron transport activities in addition to the physiologically relevant trans activity (Schmidt and Bartels, 1998). Studies on FC-R activities of PM published so far have not discriminated between cis and trans activities.

A light-dependent FC-R activity has been also found in excised leaf pieces or discs of cowpea (Brüggemann et al., 1993), sunflower (de la Guardia and Alcántara, 1996), *Valerianella locusta*, peach, and sugar beet (*Beta vulgaris* L.; Grünewald, 1996). However, activities from leaf pieces may include other reducing activities not related to the PM of mesophyll cells. These include those related to leakage of reducing compounds such as organic anions at the leaf wound (Larbi, 1999). For instance, organic anions are able to induce the photochemical reduction of Fe(III) (Abadía et al., 1984). Also, organelles exposed to the media at the wound edge may have their own FC-R activity. For instance, it has been reported that chloroplasts reduce Fe from Fe(III) chelates (Bughio et al., 1997a, 1997b). Previous studies with leaf pieces (Brüggemann et al., 1993; de la Guardia and Alcántara, 1996) have not discriminated between PM-associated FC-R and other activities.

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Data from the literature suggest that the mechanism by which Fe is acquired by leaf cells may be impaired by Fe deficiency. In many cases, low-chlorophyll (Chl) leaves from Fe-deficient plants have total leaf Fe concentrations similar to those found in Fe-sufficient leaves, the so-called "chlorosis paradox" (Abadía, 1992; Morales et al., 1998). Reasons for that could be either a strong effect of Fe deficiency on leaf growth, causing nutrient accumulation (Römheld, 2000), or the existence of immobilized, inactive Fe pools in the leaves of Fe-deficient plants (Morales et al., 1998). This latter view is supported by the fact that treatments acidifying the leaf apoplast, such as acid sprays, may in some cases diminish the chlorosis symptoms associated with Fe deficiency (Tagliavini et al., 2000). The reasons for the immobilization of Fe are still unknown. It has been hypothesized, on the basis of the possible analogies between the FC-R found in the mesophyll and that found in the roots, that a putative alkalinization of the apoplast may decrease the FC-R activity in the mesophyll cell PM (Mengel, 1995).

In this work we have isolated protoplasts from Fe-sufficient (control) and Fe-deficient sugar beet leaves, and investigated the characteristics of their FC-R activity. We measured with isolated mesophyll protoplasts the dependence of the FC-R activity on: (a) illumination, (b) pH, and (c) Fe concentration.

MATERIALS AND METHODS

Plant Material

Sugar beet (*Beta vulgaris* cv Monohil, Hilleshög AB, Landskröna, Sweden) was grown in a growth chamber with a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} photosynthetic active radiation (measured with a sensor [Li-Cor, Lincoln, NE] placed horizontally at maximum plant height) at a temperature of 25°C, 80% relative humidity, and a photoperiod of 16 h of light/8 h of dark. Seeds were germinated and grown in vermiculite for 2 weeks. Seedlings were grown for 2 more weeks in nutrient solution (in three-eighth-strength Hoagland nutrient solution) with 45 μM Fe(III)-EDTA and then transplanted (four plants per bucket) to 20-L plastic buckets lined with polyethylene bags and containing half-strength Hoagland solution (Young and Terry, 1982) with either 0 or 45 μM Fe(III)-EDTA. The pH of the Fe-free nutrient solutions was buffered at approximately 7.7 by adding 1 mM NaOH and 1 g L^{-1} of $CaCO_3$. This treatment simulates conditions usually found in the field leading to Fe deficiency (Susín et al., 1994).

One week later, young, rapidly expanding leaves were used for protoplast isolation. Leaves receiving a similar PPFD on their surface (250 μmol m^{-2} s^{-1}, measured by placing the sensor at the same angle than the leaf surface) were chosen. None of the Fe-deficient leaves sampled had green veins, and they all showed a homogeneous color throughout the leaf.

Pigment Analysis

The degree of Fe deficiency was monitored by measuring leaf Chl concentration per area non-destructively with a portable Chl meter (SPAD-502, Minolta, Osaka). In plants grown hydroponically without Fe, leaf Fe and Chl concentrations decreased concomitantly (Terry and Low, 1982). For calibration of the Chl meter, leaf discs were measured, frozen in liquid N_2, extracted with 100% acetone in the presence of Na ascorbate, and then analyzed spectrophotometrically (Abadía and Abadía, 1993). Leaves sampled for protoplast isolation had Chl concentrations of 350 ± 10 and 90 ± 10 μmol m^{-2} in Fe-sufficient and Fe-deficient plants, respectively.

The total Chl concentration in protoplasts was measured after extraction with 80% (v/v) acetone with the coefficients of MacKinney (1941). Carotenoids and Chls were analyzed by HPLC after extraction with 100% (v/v) acetone (de las Rivas et al., 1989).

Isolation and Purification of Sugar Beet Protoplasts

The procedure used to release mesophyll protoplasts from sugar beet leaves was modified from the original method of Kanai and Edwards (1973). In preliminary experiments, it was found that the introduction of polyvinylpyrrolidone (PVP) and bovine serum albumin (BSA) in the incubation buffer was necessary to obtain mesophyll protoplasts from Fe-deficient leaves. When these compounds were not included in the incubation medium, the resulting pellet obtained from the Fe-deficient leaves had a brownish color and contained few intact protoplasts. Both PVP and BSA are also necessary for the isolation of PMs from Fe-deficient sugar beet roots (Susín et al., 1996) and leaves (González-Vallejo et al., 1998, 1999). Inclusion of dithiothreitol (DTT) in the medium was crucial to obtaining good protoplast yields. For instance, a 10-fold reduction in the DTT concentration caused a reduction in yield of approximately 50%.

Mesophyll tissue was exposed in leaf pieces by stripping away the lower epidermis. Mesophyll tissue (2 g fresh weight) was cut in 1-cm^2 pieces, which were placed immediately in a Petri dish containing 100 mL of buffer A containing 0.5 M sorbitol, 5% (w/v) PVP, 0.2% (w/v) BSA, 1 mM CaCl2, 2 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES)-KOH, pH 5.6, and 2 mM DTT. The buffer also contained 1.2% (w/v) cellulysin and 0.08% (w/v) pectolyase when using control plants. In the case of Fe-deficient plants the buffer was supplemented with 2% (w/v) cellulysin and 0.1% (w/v) pectolyase. Incubation was carried out overnight (15 h) at 25°C in a dark room with gentle shaking. After incubation, protoplasts were separated from undigested tissue by filtration through a nylon sieve (Scrynel NYHC, 150-μm size, Polylabo, Strasbourg, France). Undigested mesophyll tissue was washed with fresh buffer in the Petri dish, and the resulting suspension was filtered with the sieve. The filtrate was centrifuged at 100*g* for 10 min (SS-34 rotor, Sorvall Products, Newtown, CT) to pellet protoplasts. Protoplasts were washed three times with

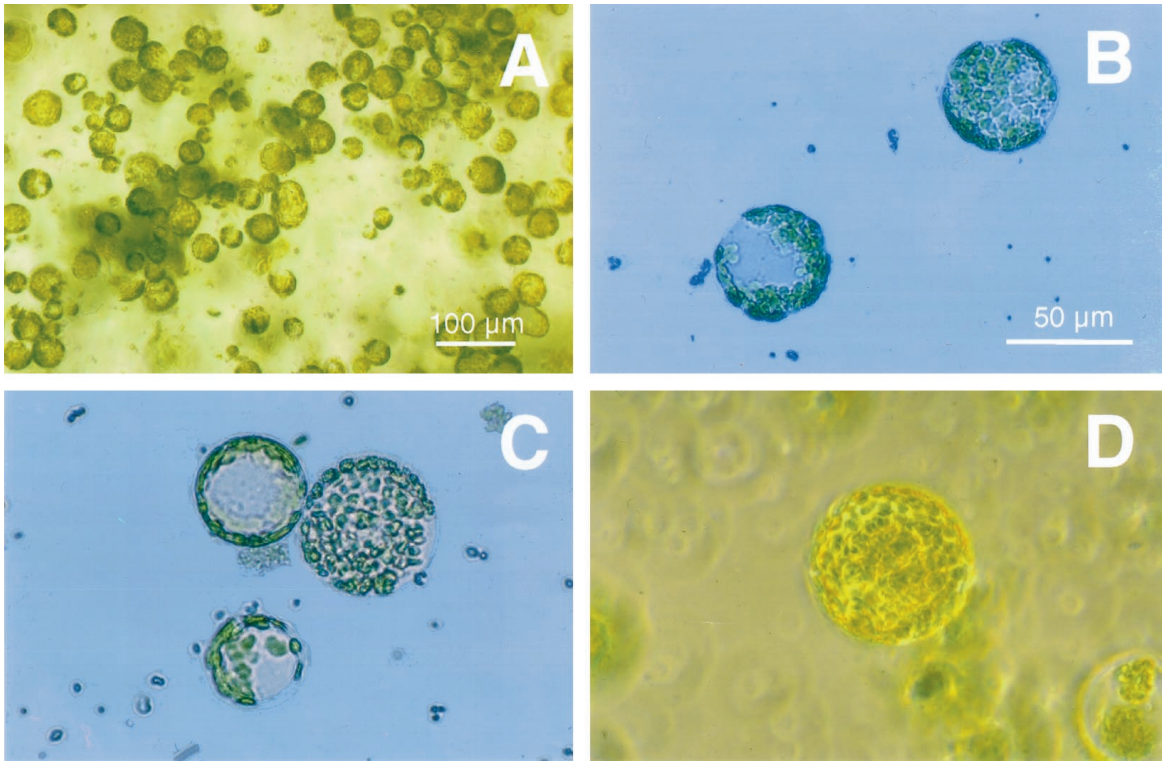

Figure 1. A, Freshly isolated sugar beet protoplasts observed in a chamber-type slide without a cover. B, Sugar beet protoplasts from control, Fe-sufficient leaves. C, Sugar beet protoplasts from Fe-deficient leaves. D, Protoplasts from Fe-sufficient leaves after reduction of Fe(III)-EDTA to Fe(II) in the presence of PDTS observed with phase contrast in a chamber-type slide without a cover.

fresh buffer without DTT to eliminate this compound, because it is capable of reducing Fe(III)-EDTA (not shown).

Protoplast purification was carried out as described by Vanková and Bornman (1987). Protoplast suspensions (4 mL) were layered on the top of centrifuge tubes (Corex, Corning, NY) containing 9 mL of 1 M Suc and centrifuged at 50*g* for 5 min in the rotor. After centrifugation, intact protoplasts were localized at the interphase between the 0.5 M sorbitol and 1 M Suc layers. Protoplasts were collected with a pipette and maintained on ice.

Protoplasts were used immediately after isolation. The final Chl concentrations were 100 to 150 μg Chl mL^{-1}. The fraction of intact protoplasts in the samples was determined before the FC-R measurements through their ability to exclude Evan's blue (Kanai and Edwards, 1973). Protoplasts were photographed with an inverted microscope (TE300, Nikon, Tokyo) with a chamber-type slide without a cover. Using normal slides and covers produced significant protoplast rupture. Protoplast size was estimated with a laser particle size analyzer (LS230, Coulter, Miami). The number of protoplasts per unit volume was estimated with a microscope (Zeiss, Jena, Germany) and a hemocytometer.

Fe-Reducing Activity

The rate of Fe(III) reduction by isolated protoplasts was determined by measuring spectrophotometrically the formation of the Fe(II)-3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (PDTS) complex from Fe(III)-EDTA at 562 nm with an extinction coefficient of 27.9 mM^{-1} cm^{-1} (Cowart et al., 1993). All measurements were performed at room temperature (20°C–25°C) with a personal-computer-controlled double-beam spectrophotometer (model 2101-PC, Shimadzu, Kyoto) using a 1-nm slit. The basic buffer used in the reduction experiment (buffer B) mimicked the ionic composition found in the apoplast of sugar beet (A.F.

Table I. *Leaf and protoplast Chl concentration, protoplast yields, and intactness as affected by Fe deficiency*

The fraction of intact protoplasts in the samples was determined with Evans Blue. Values are the means ± SE of between two and six experiments.

Fe Status	Leaf Chl		Protoplast Chl		Percentage Intactness
	μmol m^{-2}	μg g^{-1} fresh wt		pg^{-1} protoplast	
Fe-sufficient	350 ± 10	606 ± 17	230 ± 6 (38%)	137 ± 2	91 ± 2
Fe-deficient	90 ± 10	156 ± 17	78 ± 8 (50%)	23 ± 2	96 ± 1

Table II. *Relative photosynthetic pigment composition of protoplasts from Fe-sufficient and Fe-deficient sugar beet leaves*

Values are in millimoles of pigment per mole Chl *a*. Data are the means ± SE of three replications.

Pigment	Fe-Sufficient	Fe-Deficient
Violaxanthin/Chl *a*	59 ± 1	70 ± 1
Antheraxanthin/Chl *a*	7 ± 1	20 ± 1
Lutein/Chl *a*	158 ± 5	207 ± 5
Zeaxanthin/Chl *a*	1 ± 1	8 ± 1
β-Carotene/Chl *a*	110 ± 1	105 ± 5
Chl *a*/Chl *b*	3.4 ± 0.1	3.4 ± 0.2

López-Millán and J. Abadía, unpublished data), and contained 2.5 mM Mg^{+2}, 6.5 mM Cl^{-}, 1 mM Na^{+}, 1 mM NH_4^{+}, 3.5 mM NO_3^{-}, 15 mM K^{+}, 0.5 mM SO_4^{-2}, 5 mM PO_4^{-3}, and 10 mM MES-KOH at pH 5.5. For FC-R measurements, protoplasts were added (final concentration of 1–3 μg Chl mL^{-1}) to buffer B supplemented with 0.5 M sorbitol, 0.5 mM $CaCl_2$, 1.5 mM $KHCO_3$, 400 μM PDTS, and 400 μM Fe(III)-EDTA. Experiments were conducted in Eppendorf tubes with a final volume of 1 mL. Protoplasts were removed by centrifugation at 100*g* for 2 min (1,000 rpm, Microcentrifuge Force 7, Denver Instrument, Denver) and the supernatant was used for the absorbance measurements. Measurements were made with protoplast samples either dark-adapted or illuminated with red light (PPFD 90 μmol m^{-2} s^{-1}), and also in the absence of protoplasts to correct for any unspecific Fe reduction. Experiments were generally run for 30 to 60 min, but the reaction was linear for at least 90 min. For obtaining the kinetics, the concentration of Fe(III)-EDTA was varied in the range of 50 μM to 1 mM. In other experiments the pH was varied in the range 5.0 to 7.5, using 10 mM phosphate buffer (pH 5.0), 10 mM MES-KOH (pH 5.5–6.5), or 10 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS)-KOH (pH 7.0–7.5). The rates of Fe(III) reduction by isolated protoplasts are given on a protoplast surface area basis (nanomoles of Fe reduced per square micrometer per second) or on a Chl basis (nanomoles of Fe reduced per milligram of Chl per second).

RESULTS

Isolation and Purification of Protoplasts from Sugar Beet Leaves

Protoplasts were isolated from Fe-deficient and Fe-sufficient sugar beet leaves with 90 and 350 μmol Chl cm^{-2}, respectively. Protoplasts were green and spheroid with chloroplasts distributed around their periphery (Fig. 1, A–C). Approximately half of the leaf Chl was recovered in the protoplasts. Average protoplast yields were 230 and 78 μg Chl g^{-1} leaf fresh weight, accounting in the Fe-sufficient and Fe-deficient protoplasts for 38% and 50% of the total leaf Chl, respectively (Table I). These recoveries are similar to those reported previously (Huber and Edwards, 1975; Rathnam and Edwards, 1976).

Isolated protoplasts were largely intact and physiologically active, with most of them (91%–96%) being able to exclude the dye Evan's blue (Table I). Approximately 50% of the Fe-deficient and control protoplasts had diameters in the range of 22 to 41 and 24 to 50 μm, respectively. The average diameter was 32 μm for the Fe-deficient protoplasts and 38 μm for the controls. The number of protoplasts and the amount of Chl in protoplast preparations were measured on a volume basis. From these data, the estimated mean Chl per protoplast was 23 and 137 pg in Fe-deficient and control protoplasts, respectively (Table I). Therefore, Chl decreases associated with Fe deficiency were similar when calculated on a leaf area basis (74%) and on a protoplast basis (83%).

Changes in the relative pigment composition of sugar beet leaves with Fe deficiency are well known (Morales et al., 1990). To ascertain whether the isolated protoplasts were representative of the source leaves, their relative photosynthetic pigment composition was examined by HPLC (Table II). Compared with the Fe-sufficient controls, Fe-deficient protoplasts had 19% and 31% increases in the violaxanthin/Chl *a* and lutein/Chl *a* molar ratios, respectively (Table II). These pigment differences are similar to those expected for those leaves used to obtain the protoplasts, which had 350 and 90 μmol Chl m^{-2} (Morales et al., 1990). These data suggest that the protoplast preparations were representative of the cells present in the mesophyll of the source leaves.

Reduction of Fe Chelates by Intact Mesophyll Protoplasts

Mesophyll protoplasts isolated from Fe-sufficient and Fe-deficient sugar beet leaves were able to reduce Fe(III)-EDTA in a light-dependent manner (Table III). In the dark and on a protoplast surface basis, Fe-sufficient and Fe-deficient protoplasts reduced only 1.3×10^{-12} and less than 0.1×10^{-12} nmol Fe μm^{-2} s^{-1}, respectively (Table III). When illuminated with red light (PPFD of 90 μmol m^{-2}

Table III. *Light dependence of the FC-R measured in vivo in protoplasts isolated from Fe-sufficient and Fe-deficient sugar beet leaves*

Measurements were made at pH 5.5 with 400 μM Fe(III)-EDTA as a substrate in dark-adapted samples and in samples illuminated with red light (PPFD 90 μmol m^{-2} s^{-1}). Values are given on a protoplast surface basis (nanomoles of Fe reduced per square micrometer per second) and they are the means ± SE of two experiments with three replications each.

Fe Status	Dark-Adapted	Illuminated	Increase
			fold
Fe-sufficient	1.33 ± 0.66 ($\times 10^{-12}$)	47.68 ± 2.88 ($\times 10^{-12}$)	35
Fe-deficient	<0.10 ($\times 10^{-12}$)	13.82 ± 0.92 ($\times 10^{-12}$)	>100

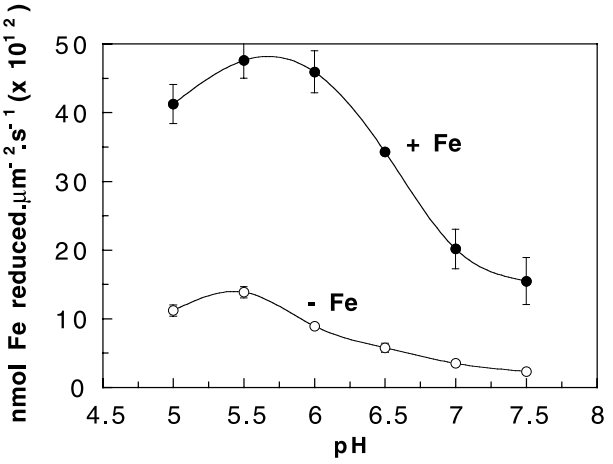

Figure 2. pH dependence of the FC-R measured in vivo in protoplasts isolated from Fe-sufficient and Fe-deficient sugar beet leaves. Measurements were made with 400 μM Fe(III)-EDTA as a substrate. Samples were pre-illuminated with red light (PPFD 90 μmol m^{-2} s^{-1}). Data are means ± SE of two experiments with three replications each.

s^{-1}), the Fe-reducing activity of Fe-sufficient and Fe-deficient protoplasts increased to 47.7 and 13.8 × 10^{-12} nmol Fe μm^{-2} s^{-1}, respectively (Table III). It should be noted that all experiments were carried out with a buffer that mimics the ionic composition of the sugar beet leaf apoplast, thus minimizing possible non-physiological processes that may affect FC-R activities.

Protoplasts were not disrupted by the Fe(III)-reduction assays (Fig. 1D). Average protoplast diameters after reduction were 26 and 35 μm in Fe-deficient and control protoplasts, respectively, values that were not significantly different from those measured in the protoplasts before the assays. Also, protoplasts were still able to exclude Evan's blue after the Fe reduction process (not shown).

The Fe-reducing activity of protoplasts isolated from both Fe-sufficient and Fe-deficient leaves was dependent on pH (Fig. 2). Maximal FC-R activities were found at pH 5.5 for protoplasts from Fe-deficient leaves, whereas protoplasts from control leaves had maximal activities between 5.5 and 6.0 (Fig. 2). When expressed on a protoplast surface basis the FC-R activities of Fe-deficient protoplasts at pH 5.5 were approximately 30% of the control values (Fig. 2). However, at pH values between 6.0 and 6.5, the FC-R activities of Fe-deficient protoplasts were only 19% and 17% of the control values on the same basis, respectively.

The protoplast FC-R activities with Fe(III)-EDTA at pH 5.5 had apparent Michaelis-Menten kinetics (Fig. 3). The Eadie-Hofstee plots revealed kinetic characteristics slightly different for Fe-sufficient and Fe-deficient protoplasts (Fig. 3). The apparent K_m for Fe(III)-EDTA was 153 and 239 μM in Fe-sufficient and Fe-deficient protoplasts, respectively (Table IV). On a protoplast surface basis Fe deficiency decreased the FC-R activity by 65%, from 62 × 10^{-12} in the controls to 21 × 10^{-12} nmol Fe reduced μm^{-2} s^{-1} in the Fe-deficient protoplasts (Table IV). On a per protoplast basis the decrease in FC-R with Fe-deficiency was 75%, from 0.28 to 0.07 nmol Fe reduced s^{-1} per 10^6 protoplasts (Table IV). When expressed on a Chl basis, however, the FC-R activities of Fe-deficient protoplasts at pH 5.5 were 1.5-fold higher than the controls, with V_{max} values of approximately 2.0 and 3.1 nmol Fe reduced mg^{-1} Chl s^{-1}, respectively (Table IV).

DISCUSSION

Protoplasts from Fe-deficient and control, Fe-sufficient leaves have a light-dependent FC-R enzymatic activity. The FC-R activity observed could be ascribed to a true PM-associated, trans-oriented electron transport activity, since the protoplasts were found to be undisrupted and physiologically active after Fe(III) reduction. Measurements of the leaf FC-R activity made until now were generally obtained with leaf pieces (Brüggemann et al., 1993; de la Guardia and Alcántara, 1996) or isolated PM (Brüggemann et al., 1993; González-Vallejo et al., 1998, 1999), two materials that may include other FC-R sources different from the physiologically relevant trans-oriented activity. Leaf protoplasts have been used to study the FC-R activities of the tomato mutant chloronerva and its wild type, Bonner Beste, although experiments were done only in the dark (Pich and

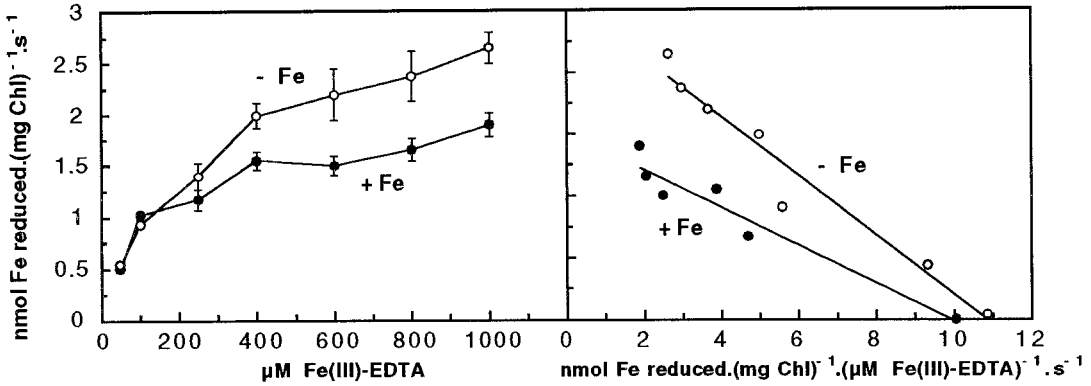

Figure 3. Dependence on the Fe(III)-EDTA concentration and corresponding Eadie-Hofstee plots of the FC-R measured in vivo at pH 5.5 in protoplasts isolated from Fe-sufficient and Fe-deficient sugar beet leaves. Samples were pre-illuminated with red light (PPFD 90 μmol m^{-2} s^{-1}). Data are the means ± SE of two experiments with three replications each.

Table IV. *Enzymatic characteristics of the FC-R measured in vivo in protoplasts isolated from Fe-sufficient and Fe-deficient sugar beet leaves*

Measurements were made at pH 5.5 in samples illuminated with red light (PPFD 90 μmol m^{s-1} s^{-1}). K_m values are in micromolar, and V_{max} values are expressed per protoplast and also on protoplast surface and chlorophyll bases. Values were obtained from the Eadie-Hofstee plots (see Fig. 3) obtained from two protoplast isolations with three replications each.

Fe Status	K_m	V_{max}		
		nmol Fe reduced protoplast^{-1} s^{-1}	*nmol Fe reduced μm^{-2} s^{-1}*	*nmol Fe reduced mg^{-1} Chl s^{-1}*
Fe-sufficient	153	0.28×10^{-6}	62.1×10^{-12}	2.02
Fe-deficient	239	0.07×10^{-6}	21.5×10^{-12}	3.09
−Fe/+Fe	1.6	0.25	0.35	1.5

Schölz, 1991). The light-dependent increase in FC-R activity found with sugar beet protoplasts was much larger (at least 35-fold) than those found previously in leaf pieces of cowpea (3-fold; Brüggemann et al., 1993) and sunflower (10-fold; de la Guardia and Alcántara, 1996).

Fe deficiency decreased markedly the protoplast capacity for reducing external Fe(III). When expressed on a protoplast surface basis, FC-R activity decreased with Fe deficiency by approximately 65%. This indicates that Fe-deficient leaf cells have intrinsic difficulties in acquiring Fe from the apoplastic space. Therefore, the development of other spatially separated Fe-efficiency response mechanisms, such as Fe acquisition by the root and transport to the shoot, may be not sufficient to improve Fe availability by leaves.

The optimal pH values for the activity of the FC-R were 5.5 and 5.5 to 6.0 in the mesophyll protoplasts isolated from Fe-deficient and Fe-sufficient plants, respectively. These values are in the pH range found in the apoplasts of sugar beet leaves (A.F. López-Millán and Abadía, unpublished data), and are approximately 1 unit lower than those found previously for optimal FC-R activity in Triton X-100-treated leaf PM from sugar beet (González-Vallejo et al., 1999) and cowpea leaves (Brüggemann et al., 1993). It has been previously hypothesized that the leaf FC-R enzyme could have a strict pH regulation (Mengel, 1995). Following this rationale, an increase in apoplastic pH caused by Fe deficiency could lead to inactivation of the enzyme, preventing Fe uptake by mesophyll cells and in turn favoring the accumulation of Fe in inactive pools in the apoplast. Our data support that pH values of 7.0 or higher in the apoplast would cause the activity of the mesophyll cell FC-R to decrease markedly. However, such high apoplast pH values have not yet been detected in Fe-deficient leaves (data not shown).

In this paper we used Fe(III)-EDTA as a model for Fe(III) reduction to allow for comparison with data found previously with other systems. The natural acceptor in the mesophyll, however, is possibly Fe(III)-citrate, although there is some recent evidence that Fe(III)-malate could be also involved (González-Vallejo et al., 1999; Larbi, 1999; Rombolà et al., 2000). The characteristics of Fe(III)-EDTA and Fe(III)-citrate reduction (with a 5:1 citrate:Fe ratio) by leaf mesophyll are similar. Both the pH optima and the K_m values are similar for both Fe(III)-chelates in PM preparations isolated from Fe-deficient and control sugar beet leaves (González-Vallejo et al., 1999). Furthermore, in leaf mesophyll discs, both the pH optima and the K_m values were similar for both Fe(III)-chelates (Larbi, 1999; A. Larbi and J. Abadía, unpublished data). Using Fe(III)-citrate, however, poses some methodological difficulties that are not completely resolved so far. The chemical form of Fe(III)-citrate in the apoplast and the possibility that Fe(III)-citrate polymers could be formed are still matters of debate (see Schmidt, 1999, and refs. therein). Also, Fe(III)-citrate is photoreduced by light (Bienfait and Scheffers, 1991; Larbi, 1999), making it difficult to ascertain which part of the reduction is due to the light-dependent FC-R enzyme and which is due to photoreduction. We believe that having more information on the changes in apoplast composition mediated by Fe deficiency could be a prerequisite to carrying out meaningful experiments, including Fe(III)-citrate as the acceptor for the FC-R.

The role of the reductase enzyme in Fe uptake has been recently questioned by Schmidt (1999), based on the experiments of Pich and Schölz (1991). Similar rates of Fe reduction but different uptake rates were found by these authors in the tomato mutant chloronerva and its wild type (Pich and Schölz, 1991). These experiments, however, should be interpreted with caution because they reported only measurements with protoplasts kept in darkness, whereas the FC-R activity, as shown in the present paper, is markedly light dependent. A role for the FC-R in Fe uptake by mesophyll tissue was clearly demonstrated in the experiments of Brüggemann et al. (1993), since an excess of the Fe(II) chelator markedly decreased Fe uptake from Fe(III)-chelates by mesophyll tissue. However, the effects of Fe deficiency on the Fe(II) PM transporter have not been studied and deserve further research.

The enzymatic characteristics of the protoplast FC-R activity are similar to those of other PM-associated oxidoreductases. The apparent K_m values of the FC-R activity for Fe(III)-EDTA were 153 and 239 μM in Fe-sufficient and Fe-deficient sugar beet leaf protoplasts, respectively. These values are similar to those reported for the FC-R activities of PM preparations from Fe-sufficient leaves of cowpea (173 μM; Brüggemann et al., 1993), PM from Fe-sufficient and Fe-deficient leaves of sugar beet (103 and 122 μM, respectively; González-Vallejo et al., 1999), and leaf pieces of Fe-sufficient cowpea (226 μM; Brüggemann et al., 1993).

Until now, FC-R values have been generally expressed on a Chl basis. On this basis, the FC-R rates found in

illuminated sugar beet protoplasts (2–3 nmol Fe reduced mg^{-1} Chl s^{-1}) are much larger than those reported for cowpea leaf pieces (0.01 nmol Fe reduced mg^{-1} Chl s^{-1}; Brüggemann et al., 1993), but similar to those found in sunflower (0.40 nmol Fe reduced mg^{-1} Chl s^{-1}; de la Guardia and Alcántara, 1996), *V. locusta*, peach, and sugar beet leaf pieces (1.2, 3.1, and 2.4 nmol Fe reduced mg^{-1} Chl s^{-1}; Grünewald, 1996). On a Chl basis, Fe deficiency increased markedly (by 30%–50%) the FC-R activity of sugar beet protoplasts. Fe deficiency has been reported to cause 3-, 6-, 16-, and 4-fold increases on the same basis in the FC-R activity of cowpea, *V. locusta*, peach, and sugar beet leaf pieces (Brüggemann et al., 1993; Grünewald, 1996), but only small (5%) increases in sunflower leaf discs (de la Guardia and Alcántara, 1996).

It is also possible from our data to make, for the first time to our knowledge, a comparison between the FC-R activities of isolated protoplasts and those of isolated PM. The FC-R maximal values for leaf PM of control and Fe-deficient sugar beet were 114 and 175 nmol Fe reduced mg^{-1} protein min^{-1} (González-Vallejo et al., 1999), values within the range found for other plant materials (Moog and Brüggemann, 1994; Susín et al., 1996). If one assumes for PM an average protein content of 30% to 40% (mass/mass) and a density of 1.1 to 1.2 g mL^{-1} (Larsson and Moller, 1990), these FC-R values would be equivalent on a protoplast surface basis to approximately 5 to 10 and 9 to 15 $\times$ 10^{-12} nmol Fe reduced μm^{-2} s^{-1} for control and Fe-deficient sugar beet PM, respectively. Therefore, the FC-R values seen in vitro with isolated PM (in the presence of Triton X-100 and NADH) are approximately 10% and 50% of those found in intact mesophyll protoplasts of control and Fe-deficient plants, respectively. Taking into account that isolated PM would also include a cis FC-R activity not present in protoplasts, our data suggest that a co-factor necessary for optimal physiological FC-R activity is lost during the isolation of PMs.

In summary, we have characterized the FC-R activity of mesophyll protoplasts isolated from Fe-sufficient and Fe-deficient sugar beet leaves. The FC-R activity of Fe-sufficient and Fe-deficient protoplasts was light dependent and was maximal in the 5.5 to 6.0 pH range, typical of the apoplastic space. Fe deficiency decreased markedly the FC-R activity per protoplast surface unit. These data indicate that both the intrinsic decrease in FC-R activity per protoplast surface and a possible shift in the pH of the apoplastic space could lead to the accumulation of physiologically inactive Fe pools in chlorotic leaves.

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