Iron availability affects the function of mitochondria in Fe-deficient cucumber roots

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Summary

• In Strategy-I-plants, iron (Fe) deficiency induces processes leading to increased Fe solubilization in the rhizosphere, including reduction by ferric reductases and active proton extrusion. These processes require active respiration to function. In this work we investigated the effect of Fe deficiency on respiratory activities of cucumber (*Cucumis sativus*) roots.

• We compared oxygen consumption rate and the activities of the respiratory chain complexes on purified mitochondria from roots grown in the presence or absence of Fe using biochemical and molecular approaches.

• Oxygen consumption rate in apex roots was increased under Fe deficiency that was mostly resistant to KCN and salycilichydroxamic acid (SHAM) inhibitors, indicating other oxygen-consuming reactions could be present. Indeed, enzyme assays revealed that lack of Fe induced a decrease in the activities of respiratory complexes that was proportional to the number of Fe atoms in each complex. A decrease of cyt *c*, Rieske and NAD9 proteins was also observed. Transmission electron microscopy (TEM) analysis showed that mitochondria undergo structural changes under Fe deficiency.

• Our data show that mitochondria and the electron transport chain are an important target of Fe limitation and that mitochondria modify their function to meet higher demands for organic acids while restricting the activity of enzymes with Fe cofactors.

Introduction

Iron uptake and homeostasis are tightly regulated in plants to ensure both a sufficient supply of Fe from the soil and the avoidance of a toxic excess in the cell. The importance of Fe for plants arises from the existence of two stable, but interconvertible forms, ferric [Fe3+] and ferrous [Fe2+], which take part in fundamental processes involving electron transfer reactions, mainly in the oxidative (respiratory) and biosynthetic (photosynthetic) pathways (Curie & Briat, 2003; Hell & Stephan, 2003). Iron deficiency induces various responses at the root level, aimed at increasing the availability of the metal in the rhizosphere. Strategy I plants (dicotyledonous and nongraminaceous plants) are able to respond to a lack of Fe in the soil by increasing: (i) the capacity of root tissues to reduce apoplastic Fe; (ii) the acidification of the rhizosphere to increase Fe solubility; and (iii) the Fe uptake activities in rhizodermal root cells (i.e. Fe3+-chelate reductase (FC-R), H+-ATPase and Fe-regulated transporter (IRT), respectively) (Curie & Briat, 2003). At the metabolic level, organic acid synthesis and CO2 dark fixation increase under Fe starvation, whereas phospho*enol*pyruvate carboxylase (PEPC) activity has been shown to increase several-fold in different plant species (Rabotti *et al.*, 1995; De Nisi & Zocchi, 2000; López-Millán *et al.*, 2000). The anaplerotic role of PEPC has been characterized in roots of cucumber grown under Fe deficiency (De Nisi & Zocchi, 2000) and in other Strategy I species (López-Millán *et al.*, 2000; Ollat *et al.*, 2003). When grown in Fe-limiting conditions, Strategy I plants accumulate organic acids – mainly citrate and malate – in roots (Brown, 1966; Alhendawi *et al.*, 1997) and leaves (Iljin, 1951; Landsberg, 1981). The role of these organic acids in Fe deficiency responses is not yet well established (Schmidt, 1999), although it is widely assumed that citrate plays an important role in the transport of Fe in roots (Tiffin, 1966; White *et al.*, 1981, Abadía *et al.*, 2002) and in its translocation, via xylem, to mesophyll cells (Brown, 1966; Brown *et al.*, 1971, Abadía *et al.*, 2002; Durrett *et al.*, 2007). It has been shown that significant metabolic changes occur in roots in order to promote and sustain the increased release of electrons and protons in the rhizosphere: enhancement of glycolytic and pentose phosphate pathway rates, cytosolic dehydrogenase activities and respiration rate (Rabotti *et al.*, 1995; Espen *et al.*, 2000) have been implicated in this process. The rate of oxygen consumption was shown to be increased under Fe deficiency (Espen *et al.*, 2000; López-Millán *et al.*, 2000), although it is difficult to ascribe this higher O2 utilization solely to the oxidative electron transport chain, since alternative oxidase activity is also present (Moore & Siedow, 1991). López-Millán

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et al. (2000) showed that O2 can be used by the FC-R itself when plants are grown in the complete absence of Fe. This reaction could generate H2O2, O_2^- or other reactive oxygen species (ROS); in fact, some of the enzymes involved in the detoxification of these compounds were found to be overexpressed in cucumber (Rabotti & Zocchi, 1994) and in sugar beet roots (Zaharieva & Abadía, 2003) in Fe-limiting conditions. Microarray analysis of *A. thaliana* grown in such conditions (Thimm *et al*., 2001) has revealed significant changes in the transcription of various genes, reflecting the complexity of molecular and metabolic responses to Fe deficiency. In particular, these authors highlighted the induction of some components of the mitochondrial electron transport chain (cytochrome *c* reductase and oxidase), suggesting that the observed increase in respiration activity in response to Fe deficiency involves transcriptional regulation of genes encoding for respiratory chain enzymes. However, the enzymatic complexes of the mitochondrial electron transport chain contain several Fe-S cluster and heme groups. In the absence of Fe, heme and Fe-S cluster synthesis should be impaired, and indeed the *de novo* synthesis of Fe-containing proteins occurs at a lower rate in mitochondria of Fe-deficient plants (Zocchi, 2006). However, it is not clear how the mitochondrial respiration activity participates in the increase in O2 consumption observed in roots in these growth conditions. The literature on how mitochondria are affected by Fe starvation is quite scarce – although Pascal & Douce (1993) reported that concentrations of some Fe-containing components (cytochromes and Fe-S clusters) were greatly decreased in mitochondria of Fe-deficient sycamore cells. Moreover, these authors showed that such mitochondria displayed a more dilute matrix with less pronounced cristae, consistent with the marked decline in cytochromes and Fe-S clusters. Oddly, only complex II was affected by this condition (Pascal & Douce, 1993). The aim of this work was to investigate the role and the activity of mitochondria extracted from roots of cucumber plants grown in the absence of Fe, in order to provide insights into their involvement in metabolic changes occurring during Fe limitation. The data presented in this work cast new light on the role of plant mitochondria in Fe deficiency and show that their activity is greatly compromised by a lack of Fe.

Materials and Methods

Plant material and growth conditions

Seeds of cucumber (*Cucumis sativus* L. cv. Marketer) were surface-sterilized and sown in Agriperlite, watered with 0.1 mm CaSO4, allowed to germinate in the dark at 26°C for 3 d, and then transferred to a nutrient solution with the following composition: 2 mm Ca(NO)3, 0.75 mm K2SO4, 0.65 mm MgSO4, 0.5 mm KH2PO4, 10 μm H3BO3, 1 μm MnSO4, 0.5 μm CuSO4, 0.5 μm ZnSO4, 0.05 μm (NH4)Mo7O24 and 0.1 mm Fe(III)-EDTA (when added). The pH was adjusted to 6.0–6.2 with NaOH. Aerated hydroponic cultures were maintained in a growth chamber with a day : night regime of 16 : 8 h and a photosynthetic photon flux density (PPFD) of 200 μmol m–2 s–1 photosynthetically active radiation (PAR) at the plant level. The temperature was 18°C in the dark and 24°C in the light.

Iron-deficient plants showed the typical Fe deficiency morphological responses (development of leaf Fe deficiency chlorosis, stunted growth and appearance of lateral roots) and the increase in the specific activities (Fe3+-chelate reductase and H+-ATPase) proper of Strategy I plants (data not shown).

Isolation of mitochondria

Mitochondria were isolated from cucumber roots using a modified version of the protocol of Balk *et al*. (1999). Ten-day-old Fe-deficient and control cucumber roots were homogenized with a mortar and pestle in 0.4 m mannitol, 25 mm MOPS, pH 7.8, 1 mm EGTA, 8 mm cysteine and 0.1% (w/v) bovine serum albumin (BSA). Cell debris were pelleted by a brief centrifugation step; the rotor was stopped as soon as it reached 4000 *g*. The supernatant was re-centrifuged at 12 000 *g* for 15 min to pellet mitochondria. The crude mitochondrial pellet was resuspended in 0.4 m mannitol, 10 mm Tricine, pH 7.2, 1 mm EGTA (resuspension buffer, RB) and lightly homogenized with a potter. Mitochondria were further purified on a 40, 28 and 13.5% (v/v) Percoll (Pharmacia, Uppsala, Sweden) step gradient in RB. The buff-coloured fraction at the two

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interfaces (purified mitochondria) was collected and washed by differential centrifugation in RB. The mitochondrial pellet was frozen and stored at –80°C until use.
The purity of isolated mitochondria was determined by the abundance of porin (see Fig. 2); porin was greatly enriched in mitochondrial fractions, indicating that they were sufficiently purified (data not shown).
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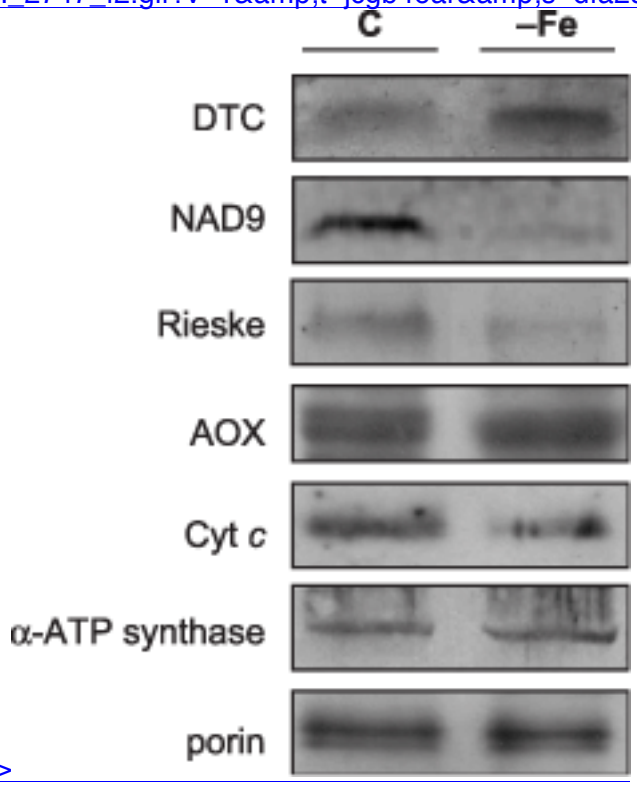

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Figure 2.

Western blot analysis performed on purified mitochondria from control (C) and Fe-deficient (–Fe) cucumber (*Cucumis sativus*) roots. For each sample, 10 μg of protein was used. The antibodies used were for: DTC (di-tricarboxylic acid carrier), NAD9 (a subunit of complex I), Rieske (a subunit of complex III), α-subunit of ATP-synthase (a subunit of complex V), cyt *c* (cytochrome *c*), AOX (alternative oxidase) and porin.

Enzyme assays

All enzymatic activities were assayed spectrophotometrically using purified mitochondria from Fe-deficient and control roots.
Citrate synthase (CS, EC 4.1.3.7) activity was assayed according to Srere (1969) by reducing acetyl-CoA to CoA with 5-5′-dithio-bis-2-nitrobenzoic acid (DTNB); thionitrobenzoic acid (TNB) formation was monitored at 412 nm. The reaction was carried out by adding purified mitochondria (50 μg protein) in 75 mm Tris-HCl, pH 8.0, 0.1 mm DTNB, 0.1% Triton X-100, 0.4 mm acetyl-CoA. After a preincubation at 30°C for 2 min, the reaction was started by addition of oxalacetate to 0.5 mm final concentration.
Complex I (EC 1.6.5.3) activity was assayed by monitoring the oxidation of NADH at 340 nm at 30°C. Purified mitochondria (50 μg protein), in 20 mm phosphate-buffer, pH 8.0, 0.2 mm NADH, 1 mm NaN3 and 100 μl of 1% (w/v) BSA in 10 mm EDTA, pH 7.4, were preincubated at 30°C for 2 min. Coenzyme Q (CoQ1) was added to 50 μm final concentration and 5 μm Rotenone was added after 30 s to block the reaction. CoQ1 was prepared according to Ragan *et al*. (1987).
Complex II (EC 1.3.5.1) activity was assayed by monitoring the decrease in the concentration of dichloroindophenol (DCIP) at 600 nm at 30°C. Purified mitochondria (50 μg protein) in 50 mm

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phosphate buffer, pH 7.0, 0.1 mm DICP, and 1.5 mm NaCN were preincubated at 30°C for 2 min. The reaction was followed by addition of 16 μm succinate and 50 μm CoQ1 final concentration. Absorbance was registered for 2 min after an incubation time of 30 s. CoQ1 was prepared as indicated earlier.

Complex III (EC 1.10.2.2) activity was assayed by monitoring the reduction of cytochrome *c* at 550 nm at 30°C. The reaction was carried out adding purified mitochondria (50 μg protein) in 50 mm phosphate buffer, pH 7.4, 2 mm NaN3, 50 μm bovine cytochrome c (12.4 mg ml–1), 100 μl 1% BSA in 10 mm EDTA, pH 7.4, plus and minus antimycin A (1 mg ml–1 in 50% EtOH) and 50 μm decylubiquinol (DBH2). Decylubiquinol was prepared according to Zheng *et al*. (1990).

Complex IV (EC 1.9.3.1) activity was assayed by monitoring the oxidation of reduced cytochrome *c* at 550 nm at 38°C. The reaction was carried out with purified mitochondria (50 μg protein) in 10 mm phosphate buffer, pH 7.0, 80 μm reduced cytochrome *c*, and 0.1% (w/v) BSA in 10 mm EDTA, pH 7.4. The reduced cytochrome *c* was prepared according to Di Mauro *et al.* (1987).

Complex V (EC 3.6.1.3) activity was assayed by monitoring the decrease in NADH at 340 nm at 30°C. The reaction was carried out with purified mitochondria (50 μg protein) in 500 μl of 10 mm MgSO4, 100 mm Hepes-KOH, pH 8.0, 0.3 mm NADH, 2.5 mm phospho*enol*pyruvate, 10 μl antimycin A (0.2 mg ml–1 in EtOH 50%), 5 μl piruvate kinase (10 mg ml–1), and 10 μl lactate dehydrogenase (5 mg ml–1). After 2 min at 30°C the reaction was started by adding 2.5 mm ATP. Absorbance was registered for 2 min after an incubation of 30 s. Successively, 10 μl oligomycin (0.2 mg ml–1 in EtOH 50%) was added and absorbance monitored for 2 min.

All the enzymatic activities of the respiratory chain are expressed as a ratio with respect to CS (mitochondrial marker enzyme) to compensate for differing mitochondrial enrichment in different preparations (Heales *et al*., 1996).

Western blot analysis

Purified mitochondrial isolated from roots of plants grown in the presence and absence of Fe were loaded on a discontinuous SDS-polyacrylamide gel (3.75% (w/v) acrylamide stacking gel, and 10–15% (w/v) acrylamide separating gel) (according to the method of Laemmli, 1970). Electrophoretic transfer to nitrocellulose membrane filters (Sigma, Milan, Italy) was performed in 0.048 m Tris, 0.039 m glycine, 0.0375% SDS and 20% (v/v) methanol for 1.5 h at room temperature at 0.8 mA cm–2. After blotting, the membrane was incubated for 1 h in PBS-TB block buffer (phosphate-buffered saline, 0.1% Tween-20, 1% BSA, (for monoclonal antibodies)) or TBS-TM block buffer (Tris-buffered saline, 0.1% Tween-20, 5% commercial dried skimmed milk, (for polyclonal antibodies)). Six different antibodies were used, corresponding to: the di-tricarboxylate carrier (DTC) (polyclonal antibody) from tobacco (a kind gift from Prof. L. Palmieri, University of Bari); the NAD9 (polyclonal antibody) from wheat (from J. M. Grienenberger); the alternative oxidase (AOX), the α-ATP synthase and the porin, maize proteins (monoclonal antibodies) (Balk & Leaver, 2001), and the Rieske and the cytochrome *c* from yeast (polyclonal antibodies) (from R. Lill). The incubation in primary antibody, diluted in block buffer, was carried out for 2 h at room temperature. After rinsing with TBS-TM or PBS-TB, nitrocellulose membranes were incubated at room temperature for 2 h with a 1 : 10 000 diluted secondary antibody (alkaline phosphatase-conjugated anti-rabbit (for polyclonals) or anti-mouse (for monoclonals) IgG, Sigma). After rinsing in TBS-T (Tris-buffered saline, 0.1% Tween-20) or PBS-T (phosphate-buffered saline, 0.1% Tween-20), filters were incubated in 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (FAST BCIP/NBT, Sigma) for detection.

Oxygen consumption

Apical root segments (*c*. 2 cm) were excised under water at room temperature from plants illuminated for several hours. Root O2 consumption rates were measured from the decrease in O2 concentration in an aqueous phase with a Clark-type O2 electrode (YS1 Analytical Control) at 25°C. Calibration was made from the difference in signal between aerated water and Na-dithionite saturated water. The effect of the respiration inhibitors KCN and salycilichydroxamic acid (SHAM) were studied. Addition of 2 mm KCN was made directly to roots in the

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measurement chamber. Roots were preincubated with 2 mm SHAM for 20 min before measurement. O2 consumption rates were also determined in the presence of 2 mm Fe(III)-EDTA alone or in combination with 2 mm KCN, 2 mm SHAM, or 2 mm KCN plus 2 mm SHAM.

Transmission electron microscopy

Samples of Fe-deficient and Fe-sufficient apical root segments (0–1 cm) were fixed in a mixture of 3% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 m phosphate buffer, pH 7, overnight at room temperature. Samples were subsequently postfixed with 1% (w/v) osmium tetroxide in the same buffer for 1 h at 4°C and dehydrated in a graded ethanol series before being embedded in SPURR resin (Electron Microscopy Sciences, Washington, PA, USA). Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a Jeol JEM-100 SX TEM at 80 kV.

Iron content determination

Iron content in purified mitochondria was determined by ICP-OES AX Liberty (Varian, Fort Collins, CO, USA) after mineralization in HNO3 at 100–120°C.

Protein content

Protein content of purified mitochondria was determined according to the Bradford (1976) procedure, using BSA as a standard.

Results

O2 consumption rate measurement at root level

In order to obtain an initial overview of mitochondrial activity, we measured the O2 consumption rate of apical root segments: those from Fe-deficient plants showed a 56% increase in O2 consumption rates with respect to those from Fe-sufficient plants (463 nmol O2 min−1 g−1 FW versus 297 nmol O2 min−1 g−1 FW; Table 1). After KCN addition, the total O2 consumption decreased to *c*. 50 and 37% in control and Fe-deficient roots, respectively. The presence of SHAM, which inhibits the alternative oxidase, caused a 15% decrease in the consumption of O2 in Fe-deficient roots, while in control roots this effect was *c*. 30%. The residual O2 consumption (i.e. the fraction of oxygen uptake that is resistant to the combination of KCN and SHAM (Ribas-Carbò*et al*., 1997)) was *c*. 27% of the initial rates in Fe-deficient roots and practically zero in the control. Addition of 2 mm Fe(III)-EDTA decreased the initial O2 consumption rate in Fe-deficient apical root segments by *c*. 12%, whereas in Fe-sufficient roots the rate was unaffected (Table 1). Since Fe(III)-EDTA cannot cross the plasma membrane (López-Millán *et al*., 2000), the addition of this ferric chelate is consistent with the hypothesis that a FC-R enzyme induced by Fe deficiency could transfer electrons to oxygen when Fe is not present. O2 consumption was inhibited by 87% upon addition of 2 mm Fe(III)-EDTA in Fe-deficient roots treated with KCN plus SHAM, while it did not show any effect in the control roots. These data show that the measurement of O2 consumption in root segments is complex, since more than one relevant enzyme activity is present.

Table 1. Oxygen consumption rates in excised apical root segments from Fe-sufficient (C) and Fe-deficient (–Fe) cucumber (*Cucumis sativus*) plants

	O2 consumption rate (nmol O2 min−1 g−1 FW)	
	C	–Fe
Initial rate	297.4 ± 18	463.3 ± 23
KCN	151.7 ± 11 (–49%)	291.9 ± 13 (–37%)
SHAM	208.2 ± 13 (–30%)	393.8 ± 19 (–15%)
KCN+ SHAM	3.87 ± 0.1 (–98.7%)	125.1 ± 9.1 (–73%)
FeIIIEDTA	295.8 ± 12 (0%)	407.7 ± 10 (–12%)
KCN + SHAM + FeIIIEDTA	3.8 ± 0.1 (–98.7%)	60.2 ± 2.4 (–87%)

To verify the specific contribution of mitochondria on O2 consumption, we performed a preliminary experiment with isolated mitochondria. This experiment revealed that O2 consumption in Fe-deficient mitochondria was decreased by *c*. 40% (data not shown). This difference leads us to investigate further the respiratory chain activity of mitochondria in Fe-deficient roots.

Enzymatic assays

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To determine the specific contribution of mitochondrial respiratory chain activities to O2 consumption, we performed enzymatic assays on purified mitochondria from roots of plants grown in the presence and absence of Fe.

First, CS activity in isolated mitochondria was determined. Several authors have suggested the this activity is a typical mitochondrial marker (Holloszy *et al.*, 1970; Williams *et al.*, 1986; Hood *et al.*, 1989) and also that CS activity is generally proportional to the number of mitochondria. While this relationship has been questioned in developmental (Drahota *et al.*, 2004) and age-related studies (Marin-Garcia *et al.*, 1998), it is generally accepted that mitochondrial and cellular respiration activity may be expressed in terms of CS activity (Kuznetsov *et al.*, 2002; Renner *et al.*, 2003; Hütter *et al.*, 2004).

In purified mitochondria from Fe-deficient cucumber roots, CS activity was more than doubled with respect to controls (Fig. 1). The enzymatic assays of complexes I, II, III, IV and V showed that only the activities of complexes I and II were decreased under Fe-limiting conditions (Table 2a). In particular, complex I showed the most dramatic decrease (*c.* 90%), while complex II activity was reduced by *c.* 52%. Using CS activity to normalize specific activities for each respiratory chain complex (Table 2b), those of complexes III, IV and V were decrease by *c.* 56, 50 and 52%, respectively, with Fe deficiency.

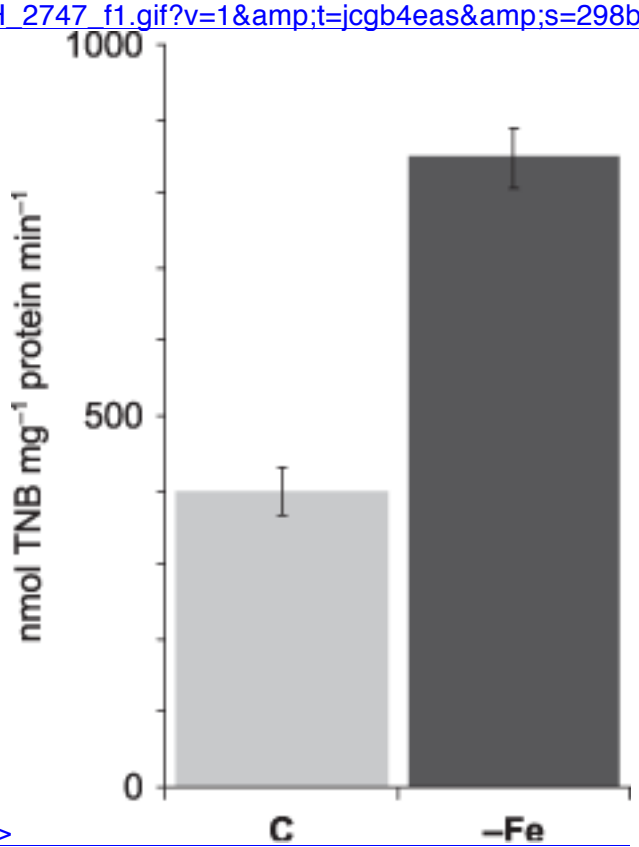

Figure 1.

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Citrate synthase activity determined in purified mitochondria from Fe-sufficient (C) and Fe-deficient (–Fe) cucumber (*Cucumis sativus*) roots. Data are expressed as nmol thionitrobenzoic acid (TNB) mg−1 protein min−1 and represent the mean ± SE of four independent experiments run in triplicate. SE never exceeded 7%.

Table 2. Enzymatic activity of respiratory chain complexes

Enzymes	a			b		
	(U mg−1 prot)			Specific activity ((complex activity/CS activity) × 100)		
	C	–Fe	%	C	–Fe	%

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Complex I	1.21 ± 0.04 a	0.12 ± 0.01 b	–90	303.1 ± 11.2 a	14.2 ± 5.9 b	–95
Complex II	0.85 ± 0.04 a	0.41 ± 0.05 b	–52	214.6 ± 9.6 a	48.3 ± 6.3 b	–77
Complex III	0.31 ± 0.02 a	0.29 ± 0.01 a	–	78.3 ± 6.3 a	34.7 ± 1.3 b	–56
Complex IV	0.67 ± 0.03 a	0.69 ± 0.05 a	–	165.7 ± 6.5 a	82.2 ± 7.7 b	–50
Complex V	0.76 ± 0.04 a	0.77 ± 0.04 a	–	190.7 ± 9.7 a	90.9 ± 4.9 b	–52

Protein concentrations in Fe-deficient mitochondria

To corroborate changes in activities of the respiratory complexes, the expression of selected mitochondrial proteins was investigated by Western blotting.

Mitochondrial DTC (Picault *et al*., 2002) showed a large accumulation under Fe deficiency with respect to the control. This result is in agreement with the increases in the CS activity (Fig. 1) and with cytosolic citrate accumulation in Fe-deficient cucumber roots (Rabotti *et al*., 1995). Expression of NAD9, a subunit of plant complex I homologous to NuoC and NDUFS3 of *E. coli* and *H. sapiens*, respectively (Rasmusson *et al*., 1998), was almost undetectable in mitochondria isolated from Fe-deficient roots (Fig. 2). These data are in accordance with the activity of complex I determined in Fe-deficient mitochondria (Table 2): the decrease in this activity could be linked to a reduced protein synthesis of relevant subunits. Complex I contains more than 20 atoms of Fe (see Fig. 5) and it is probable that, under Fe-limiting conditions, the synthesis of such subunits might be greatly impaired. Moreover, the amount of Rieske proteins (Fe-S clusters containing proteins) and cytochrome *c* (Fe-heme containing proteins) also decreased in Fe-deficient mitochondria (Fig. 2). This result is consistent with decreased cytochrome synthesis observed in Fe-deficient sycamore cells (Pascal & Douce, 1993). The concentration of AOX protein in mitochondria from Fe-deficient cucumber roots was not changed with respect to controls (Fig. 2). AOX belongs to the di-iron-carboxylate protein family and contains neither Fe-heme nor Fe-S clusters. Iron deficiency did not alter the concentration of the protein (Fig. 2).

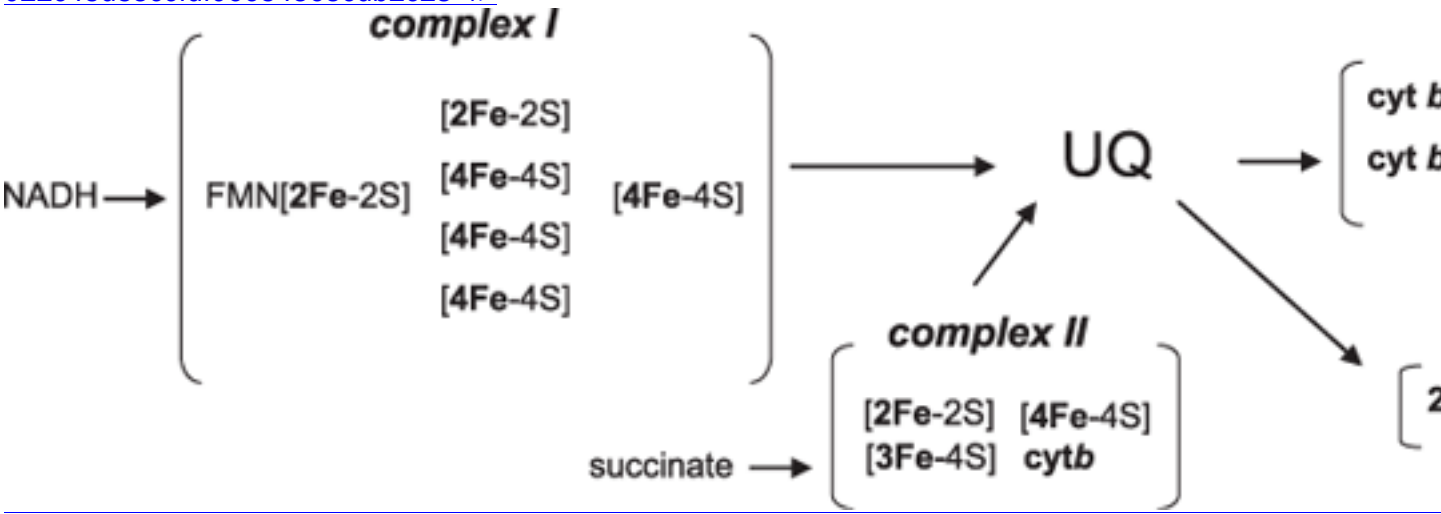

Figure 5.

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Schematic representation of Fe distribution in the respiratory chain. The Fe content of each complex was obtained from Ohnishi (1998), Nicholls & Ferguson (2002), Siedow *et al*. (1995)

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and Berthold & Stenmark (2003). UQ, ubiquinone pool.
The α-subunit of the complex V (ATP synthase) did not show any difference in the protein expression between Fe-deficient and control mitochondria (Fig. 2). Complex V does not contain Fe, in accordance with the hypothesis that Fe deficiency should affect only the synthesis of heme or Fe-S cluster-containing proteins.
Iron content in isolated mitochondria
To investigate whether a lack of Fe might be responsible for the decrease in respiratory chain complex activity, we determined the amount of Fe present in isolated mitochondria (Fig. 3). While, under standard conditions, mitochondria had *c*. 375 nmol Fe mg−1 protein, Fe-deficient mitochondria showed only 14. Thus, it seems that Fe deficiency status in cucumber plants induces a strong decrease in mitochondrial Fe content, associated with the loss of electron transport chain activity.
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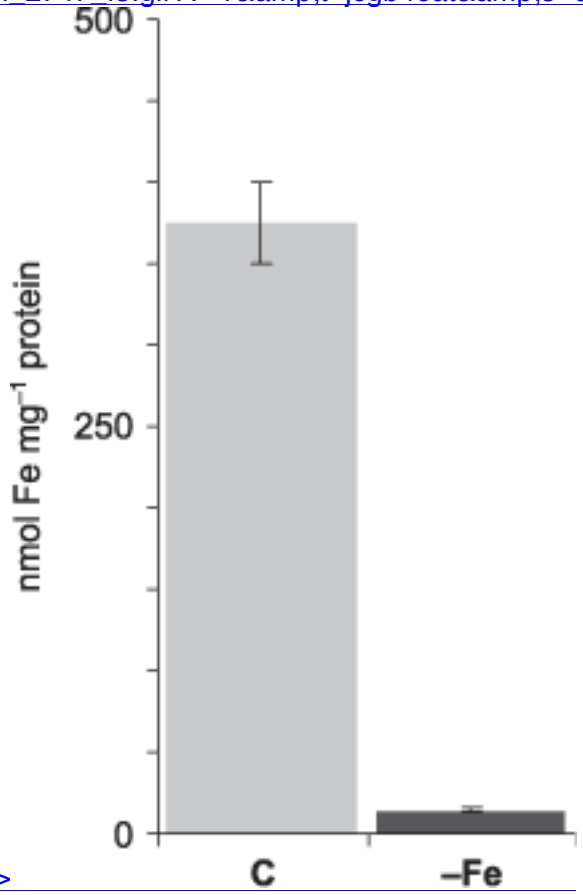

Figure 3.

Iron content in mitochondria extracted from control (C) and Fe-deficient (–Fe) cucumber (*Cucumis sativus*) roots. Data are expressed as nmol Fe mg−1 protein and represent the mean ± SE of three independent experiments run in triplicate. SE never exceeded 7%.
TEM analysis
Under TEM analysis, ultrathin sections of root apical segments from Fe-deprived plants presented a handlebar-like configuration, while in control cells, mitochondria were well separated (Fig. 4). This configuration was seen both in the epidermal cells and in the cortex cells throughout the section.
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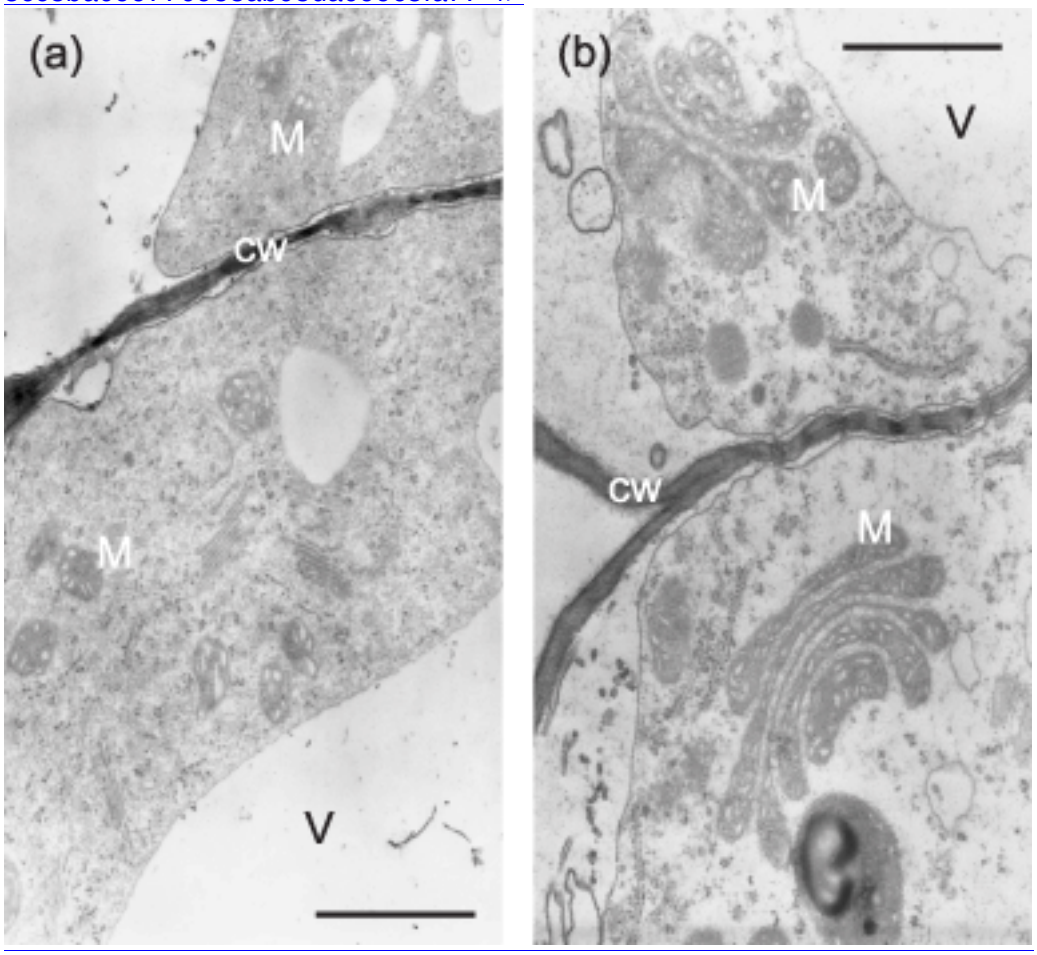

Figure 4.

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Transmission electron micrographs of cross-sections from apical root segments of control (a) and Fe-deficient (b) cucumber (*Cucumis sativus*) plants. V, vacuole; M, mitochondria; CW, cell wall. Bars, 1 μm.

To date, to our knowledge, this kind of change in the mitochondrial morphology under Fe deficiency or other physiological condition has never been shown. Logan *et al*. (2004) observed the formation of matrixules, elongations in the central part of mitochondria, in Arabidopsis *adl2* mutants. A similar phenotype can be observed in Fe-deficient mitochondria.

Discussion

In Strategy I plants, Fe deficiency induces Fe3+ reduction and increased proton extrusion, both increasing demand for NAD(P)H and ATP and accelerating respiration (and O2 consumption) (Espen *et al*., 2000 and López-Millán *et al*., 2000).

Ten-day-old Fe-deficient plants showed the typical Fe-deficiency symptoms (leaf Fe-deficiency chlorosis, stunted growth and appearance of lateral roots) and the increase in the specific activities (Fe3+-chelate reductase and H+-ATPase) proper of Strategy I plants (data not shown). Indeed, under Fe deprivation, we observed an increase of *c*. 56% in O2 consumption by cucumber root segments. Only a small proportion of the additional oxygen consumption by Fe-deprived root segments can be ascribed to mitochondrial respiration (Table 1). In fact, a further decrease in O2 consumption rate was observed after the addition of Fe(III)-EDTA to KCN plus SHAM-treated Fe-deficient root segments, consistent with the hypothesis of López-Millán *et al*. (2000) by which the PM FC-R enzyme, induced by Fe deficiency, could use O2 as an electron acceptor in the absence of ferric chelate compounds. However, in contrast to these authors, we observed a residual O2 consumption in Fe-deficient roots after addition of Fe(III)-EDTA. Oxygen can also accept electrons from NADH via a semi-reduced flavin or quinone (Misra & Fridovich,

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1972) forming the O_2^- radical. Thus, it is probable that the residual O2 consumption (*c*. 10–15%) may be linked to increased oxidative stress. Indeed, under Fe deficiency, the activities of some enzymes involved in detoxification of ROS compounds were increased (Rabotti & Zocchi, 1994; Zaharieva & Abadía, 2003).

The strong increase in O2 consumption rate under Fe deficiency seems to be attributable not to an increase in the mitochondrial respiration, but mainly to the induction of other O2-consuming reactions (i.e. FC-R and some ROS detoxification activities). Since the complexes of respiratory chain contain many Fe atoms (Fig. 5), we should expected to find a decrease in mitochondrial O2 consumption. What really happens at the respiratory chain under Fe deficiency? Heme and Fe-S cluster synthesis should be expected to be impaired and the *de novo* synthesis of Fe-containing proteins (i.e. cytochromes) should occur at a lower rate in mitochondria of Fe-deficient plants. In fact, Pascal & Douce (1993) showed that in mitochondria of Fe-deficient sycamore cells, concentrations of Fe-containing compounds, mainly cytochromes and some Fe-S clusters, were greatly decreased. However, these authors report that only the activity of complex II was affected, whereas complexes I, III, and IV, as well as the ATP synthase, were unimpaired (Pascal & Douce, 1993).

By way of contrast, our data show a generalized impairment of the respiratory chain activity under Fe deficiency. Table 2(b) shows that in isolated mitochondria from Fe-deficient cucumber roots, the activities of all respiratory chain complexes were strongly decreased. To compare the activities between control and Fe-deficient mitochondria, we normalized these activities to the CS activity. Normalization has been adopted since the number of mitochondria was shown to change between these two conditions, as reported in the literature (Landsberg, 1986, 1994; Pascal & Douce, 1993; Dell'Orto *et al*., 2002). This might explain the variation in the response of the electron transport chain complexes, where for complexes I and II, which contain the greatest amount of Fe, the decrease was evident, even without normalization. For complex III and IV (with only five and two iron atoms, respectively, as reported in Fig. 5), the decrease was only detectable after normalization. This apparent lack of decrease might be the result of a greater number of mitochondria in the Fe-deficient conditions, which hampers the real decrease that can be seen with normalization. Western blot analysis shows that, in Fe-deficient purified mitochondria, only the protein concentrations of NAD9 (a component of complex I that contains Fe-S clusters), Rieske proteins (Fe-S clusters proteins) and cyt *c* (heme protein) were affected, supporting the data obtained with CS normalization, while neither AOX nor the α-subunit of complex V were affected at the protein level (Fig. 2). However, the activity of complex V in Fe-deficient roots decreased (Table 2), and the decrease observed *in vitro* should be the consequence of the increase in the number of mitochondria under Fe deficiency. The activities of control and Fe-deficient mitochondria were similar, but after normalization to CS, the activity in the Fe-deprived condition was lower. *In vivo*, the expected decrease in the complex V activity seems to be correlated with the decrease in the electrochemical H+ gradient generated by the reduced activity of the respiratory chain (see Table 2). Furthermore, mitochondria from Fe-deficient cucumber roots showed a dramatic loss of Fe content with respect to the control (Fig. 3). Iron-deficient mitochondria from sycamore cells showed a moderate decrease in Fe content compared with the control, and the difference between our results and the data shown by Pascal & Douce (1993) could be the result of the strongly Fe-deficient growing conditions used in the current study.

The number of Fe ions required for correct assembly of each respiratory chain complex (Fig. 5) and their relative activities (Table 2) in Fe-deficient conditions are strongly correlated: complex IV, which has only two Fe atoms, shows a decrease of *c*. 50%; while complexes I and II, which require at least 20 and 10 Fe atoms, respectively, show a greater decrease (95 and 77%, respectively). Taken together, these data suggest that in Fe-limiting conditions, mitochondria could preferentially allocate Fe to complexes III, IV rather than complexes I and II. This could be rationalized as follows: (a) the UQ pool reduction in plant mitochondria can be catalysed by the alternative rotenone-insensitive type II NAD(P)H dehydrogenases located on the external and

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internal surfaces of the inner membrane (Møller, 1997), and which are not Fe-dependent (Fig. 6); (b) the oxidation of UQH2 through the Q-cycle by complex IV and AOX would allow both the generation of the proton gradient across the membrane and the reduction of O2 to H2O, thus avoiding a ROS overproduction and consequent oxidative stress.

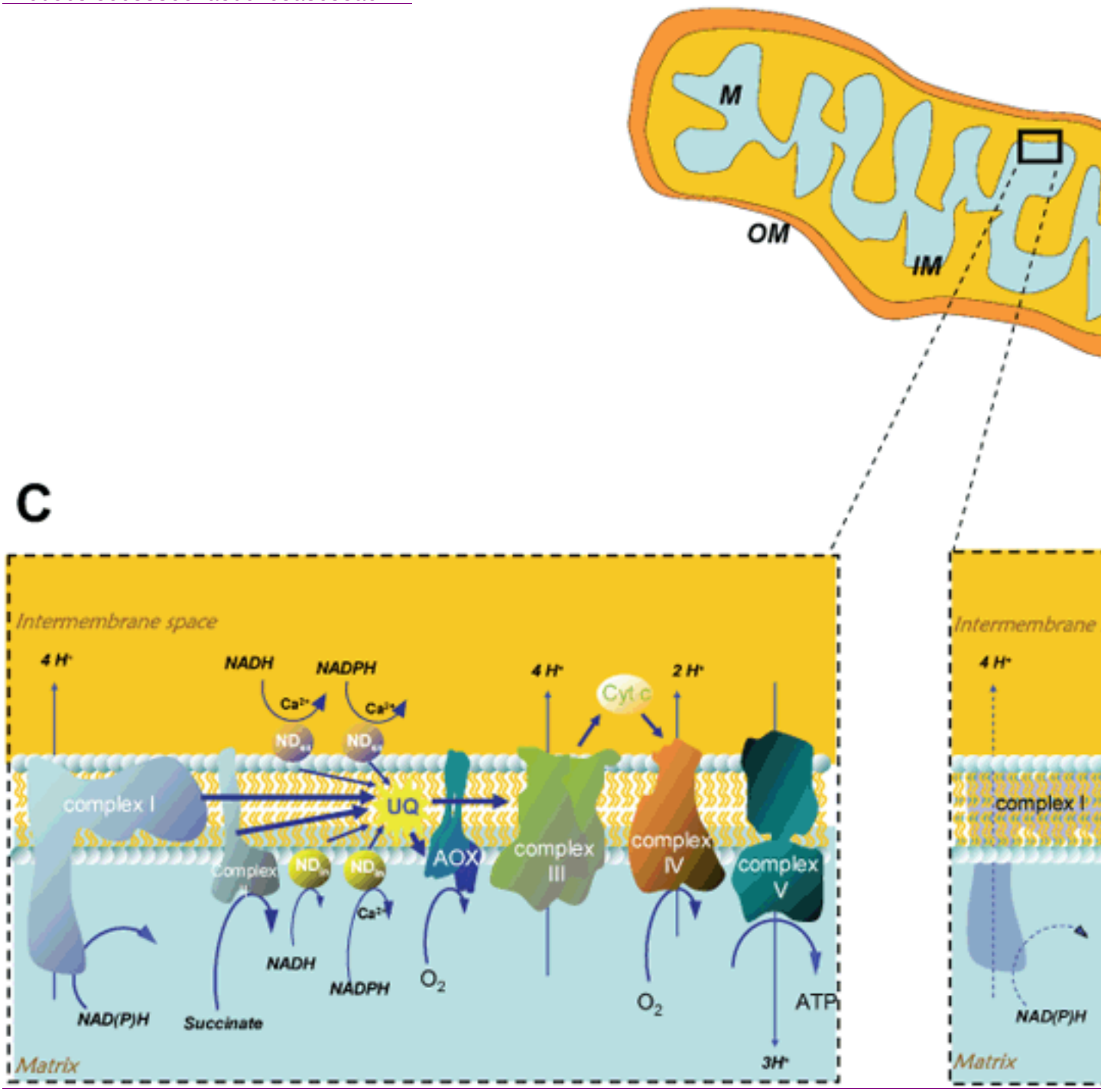

Figure 6.

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Schematic representation of mitochondrial activities in roots. What occurs in mitochondria from control roots (C) is reported in the left panel. Right panel, what we hypothesized to occur in Fe-

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deficient mitochondria (–Fe): transparency of the complexes is related to the decrease in their activity; the different arrow thickness in –Fe conditions would indicate the change in the electron flux, where the main loss of the complex I and II activities could be replaced by the alternative external and internal NAD(P)H dehydrogenases (NDex and NDin, respectively). M, matrix; IM, inner membrane; IS, intermembrane space; OM, outer membrane.

Ultrastructural changes also occur under Fe deficiency, which were never shown before (Fig. 4). Comparing our ultrastructural data with those obtained, for instance, by Landsberg (1986), the difference in the presence of the handlebar-like mitochondrial structure could be ascribed to the different growing culture. In fact, while our plants were grown from the beginning in the complete absence of Fe, which would lead to the very low Fe concentration seen in Fig. 3, in the Landsberg paper, plants were precultured for 2 wk in the presence of Fe and Fe deficiency induced by transferring plants for 3–4 d in the absence of the ion. We believe that in these conditions mitochondria will retain enough Fe and so not behave as Fe-free mitochondria. As Logan *et al.* (2004) observed, *adl2* mutant mitochondria from *Arabidopsis* showed the formation of matrixules. A similar elongated structure has been also observed in mitochondria of Fe-deficient cucumber roots (Fig. 4). Fe deficiency is therefore presumed to impair the fission process, suggesting that the adl2 (or, as recently named, the DPR3A (Logan, 2006)) pathway might be altered in Fe-deficient cells. Actually, it was shown that *adl2* mutation decreased the number of mitochondria (Logan, 2006); however, at the moment we do not know if this also occurs in mitochondria from Fe-deficient cucumber roots.

The increase in CS activity corresponds to the increase in the concentration of citric acid in Fe-deficient roots (Rabotti *et al*., 1995) and might explain the accumulation of DTC protein observed by Western blot analysis (Fig. 2). Considering CS activity as a mitochondrial marker (see the Results section), the accumulation of DTC protein could be simply linked to an increase in the membrane surface and/or to an increase in the number of mitochondria.

Why should the cell need to increase mitochondrial number under Fe deficiency? The DTC protein accumulation confirms that there is increased communication between the cytosolic and the mitochondrial pool of organic acids. The increase in organic acid flux between cytosol and mitochondria could be a strategy of the cell to allow a higher turnover of reducing equivalents. Both the cytosolic and the mitochondrial NADH need to be oxidized. An inefficient respiratory chain activity could impair this turnover, leading to a high ratio of reduced : oxidized compounds. A lower turnover might, in turn, decrease the rate of other metabolic pathways (for instance, glycolysis), causing an imbalance of energy and metabolite production. By enhancing the number of mitochondria, the cell increases the number of compartments that can sustain the turnover of reducing equivalents. Therefore, mitochondria from Fe-deficient roots are limited in the electron transport chain and ATP synthesis (see Table 2), but they still maintain the capacity to reduce NADH. On the other hand, Fe-deficient cells could enable mechanisms to sustain the metabolic rise both at the levels of carbon flux and the turnover of reducing equivalents (Fig. 6). Previous results from our laboratory have shown that glycolysis and the pentose phosphate pathway were increased during Fe deficiency (Rabotti *et al*., 1995; Espen *et al*., 2000), and López-Millán *et al*. (2000) found that activities of enzymes of anaerobic metabolism were also increased under Fe deficiency. These findings are well supported by microarray analysis (Thimm *et al*. 2001) carried out on Fe-deficient *Arabidopsis* showing induction of genes encoding enzymes of these metabolic pathways.

We suggest that Fe deficiency could simulate a kind of ‘cellular effort’ in which to overcome a respiratory chain impairment; the cell needs alternative pathways to sustain both energetic requirements and NAD(P)H turnover. The cell could then enable glycolytic and anaerobic pathways, to sustain ATP synthesis; and organic acid exchange between mitochondria and cytosol to sustain NAD(P)H turnover and carbon flux. The combination of exchange of malate and citrate between mitochondria and cytosol, as determined by the increase in the DTC protein, along with the increase in the PEPC activity allows the acceleration of the glycolytic pathway.

Nevertheless, the respiratory chain in mitochondria from Fe-deficient cucumber roots is not

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completely blocked and can partially contribute to oxygen consumption, ATP production and, above all, NAD(P)H turnover in the Fe-deficient roots. Hence, from the data obtained in this work, we could suggest that under Fe deficiency the loss in efficiency of the respiratory chain is compensated by an increase in the number of mitochondria. The increased O2 consumption rate measured in Fe-deficient root segments should not be interpreted as an increase in mitochondrial activity but rather as the result of an increase in the number of less efficient mitochondria and in the induction of different O2-consuming reactions (i.e. FC-R and some ROS detoxification activities).

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