Metabolic responses in iron deficient tomato plants

Ana Flor López-Millán, Fermín Morales, Yolanda Gogorcena, Anunciación Abadía, Javier Abadía

Department of Plant Nutrition, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), P.O. Box 13034, E-50080 Zaragoza, Spain

Pomology Department, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), P.O. Box 13034, E-50080 Zaragoza, Spain

Received 18 April 2008; received in revised form 13 June 2008; accepted 13 June 2008

Summary

The effects of Fe deficiency on different metabolic processes were characterized in roots, xylem sap and leaves of tomato. The total organic acid pool increased significantly with Fe deficiency in xylem sap and leaves of tomato plants, whereas it did not change in roots. However, the composition of the pool changed with Fe deficiency, with major increases in citrate concentrations in roots (20-fold), leaves (2-fold) and xylem sap (17-fold). The activity of phosphoenolpyruvate carboxylase, an enzyme leading to anaplerotic C fixation, increased 10-fold in root tip extracts with Fe deficiency, whereas no change was observed in leaf extracts. The activities of the organic acid synthesis-related enzymes malate dehydrogenase, citrate synthase, isocitrate dehydrogenase, fumarase and aconitase, as well as those of the enzymes lactate dehydrogenase and pyruvate carboxylase, increased with Fe deficiency in root extracts, whereas only citrate synthase increased significantly with Fe deficiency in leaf extracts. These results suggest that the enhanced C fixation capacity in Fe-deficient tomato roots may result in producing citrate that could be used for Fe xylem transport. Total pyridine nucleotide pools did not change significantly with Fe deficiency in roots or leaves, although NAD(P)H/NAD(P) ratios were lower in Fe-deficient roots than in controls. Rates of O2 consumption were similar in Fe-deficient and Fe-sufficient roots, but the capacity of the alternative oxidase pathway was decreased by Fe deficiency. Also, increases in Fe reductase activity with Fe deficiency were only 2-fold higher when measured in tomato root tips. These values are significantly lower than those found in other plant species, where Fe deficiency leads to larger increases in organic acid synthesis-related enzyme activities and flavin accumulation. These data support the hypothesis that...
Introduction

Iron is an essential microelement for plant growth and development. Soils normally contain high amounts of Fe, but in well aerated and alkaline soils the availability of Fe for plant uptake is very limited. Plants have developed two different strategies in response to Fe shortage: Strategy II, which occurs in Poaceae species, and Strategy I, which occurs in dicotyledonous and non-grass monocotyledonous species (Marschner et al., 1986). In both strategies, Fe deficiency induces several mechanisms aimed to increase Fe uptake from the soil. In Strategy II species, there is an increase in the synthesis and secretion of phytosiderophores to the rhizosphere, parallel to an induction of an Fe(III)-phytosiderophore complex transport system (Kobayashi et al., 2006). Strategy I plants induce a two-step mechanism for root Fe uptake that includes the induction of an Fe(II) reductase (Chaney et al., 1972) and an Fe(II) transporter (Eide et al., 1996). In addition to these changes, Strategy I plants have developed several physiological responses that aid Fe uptake from the soil by lowering soil pH and increase Fe(III) solubility. These responses can include, depending on the species, enhanced excretion of protons to the rhizosphere mediated by a plasma membrane-bound H+-ATPase (Schmidt, 1999; Zocchi et al., 2007), excretion of phenolics, and accumulation and/or secretion of flavin compounds (Susin et al., 1994) and organic acids (Abadía et al., 2002).

In the last decade, many of the key components for increased Fe uptake have been identified at the molecular level. Plasma membrane-bound Fe reductases belonging to the FRO family have been cloned in Arabidopsis thaliana, Pisum sativum and Lycopersicon esculentum (see references in Kim and Guerinot, 2007), and it has been demonstrated that Fe transporter genes involved in root uptake belong to the ZIP family of metal transporters. Members of this family have been cloned from several plant species; these include IRT1 and IRT2 from Arabidopsis thaliana, RIT1 from Pisum sativum, LeIRT1 and LeIRT2 from Lycopersicon esculentum and MtZIP6 from Medicago truncatula (Kim and Guerinot, 2007).

In xylem sap, Fe is transported as Fe(III), likely chelated by citrate (Tiffin, 1966; López-Millán et al., 2000a; Rellán-Alvarez et al., 2008). Several authors have reported an increase in xylem sap organic acid concentrations with Fe deficiency (reviewed in Abadía et al., 2002). Although the precise role of these organic acids in Fe transport and uptake by mesophyll cells is still unclear, recent advances have indicated that a citrate transporter, FRD3, is necessary for efficient Fe translocation to the xylem sap (Durrett et al., 2007), supporting a role for citrate in long-distance Fe transport. Once in the leaf apoplast, Fe(III) is reduced before uptake by the leaf cell, and this process may be mediated by a plasma membrane-bound ferric chelate reductase similar to that present in roots (González-Vallejo et al., 1999). In agreement with the biochemical data, expression of several members of the FRO family, AtFRO6, AtFRO7 and AtFRO8, has been observed in leaves (Wu et al., 2005; Mukherjee et al., 2006).

Several changes occur at the metabolic level in order to sustain the increased Fe uptake capacity of Fe-deficient plants (Zocchi, 2006). These changes include an accumulation of organic acids throughout the plant, primarily malate and citrate (reviewed in Abadía et al., 2002), shifts in the redox state of the cytoplasm (Schmidt, 1999; López-Millán et al., 2000b), increases in the activity of PEPC and in several enzymes of the Krebs cycle such as citrate synthase (CS), isocitrate dehydrogenase (ICDH), fumarase and aconitase and of the glycolytic pathway such as glycereraldehyde 3-phosphate dehydrogenase (reviewed in Zocchi, 2006). Some of these increased activities are associated with enhanced expression of the corresponding genes (Thimm et al., 2001). The increase in PEPC activity correlates with the organic acid accumulation in Fe-deficient roots (López-Millán et al., 2000b) and is localized mainly in the external layers of the cortical cells of Fe-deprived root apical sections, which are very active in proton extrusion (Zocchi, 2006). An up-regulation of the glycolytic pathway, as well as increases found in the activities of several NAD(P)H-producing enzymes, can produce reducing equivalents to keep the Fe reductase working at the necessary rate (Sijmons...
and Bienfait, 1983). Previous results have suggested that flavins accumulated in Fe-deficient sugar beet roots acted as a redox bridge for electron transport to the ferric reductase (López-Millán et al., 2000b). Moreover, FRO2 belongs to a superfamily of flavocytochrome oxidoreductases, and a recent study of the topology of the FRO2 gene revealed that the protein contains NADPH, FAD and oxidoreductase sequence motifs on the inside of the membrane (Schagerlof et al., 2006).

Most information available on the effects of Fe deficiency in the physiology of leaves is relevant to either Fe acquisition from the roots and transport throughout the plant at the molecular level or to chloroplast structure and function (Abadía, 1992). Little information is available on other biochemical responses, such as organic acid metabolism changes and their role in Fe deficiency responses. The aim of this work was to investigate the extent of induction of different metabolic processes in Fe-deficient tomato plants, a species that does not accumulate flavins under Fe deficiency, to gain further understanding of the different Fe efficiency responses observed among plant species. We measured anaplerotic C fixation via PEPC activity, organic acid concentrations as well as the activities of several organic acid synthesis-related enzymes and also the size and redox poise of the leaf and root pyridine nucleotide pools. Root O2 consumption rates, including an assessment of the capacities of the cytochrome and alternative oxidase (AOX) pathways, and root Fe reductase activity were also measured.

Material and methods

Plant material

Tomato (Lycopersicon esculentum L. cv. Tres Cantos) was grown in a growth chamber with a photosynthetic flux density at leaf height of 350 μmol m−2 s−1 photosynthetic active radiation and a 16 h–8 h day/night regime. Seeds were germinated and grown in vermiculite for 2 weeks. Seedlings were grown for 3 more weeks in half-strength Hoagland nutrient solution with 45 μM Fe(III)-EDTA and then transplanted to 20 L plastic buckets (four plants per bucket) containing half-strength Hoagland nutrient solution 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 CaCO3. This treatment simulates conditions usually found in the field that lead to Fe deficiency (Susín et al., 1996). Young leaves and root tips (approximately 0–5 mm from the root apex) from plants grown for 15 d in the presence or absence of Fe were used in all experiments.

Chlorophyll determination

Leaf chlorophyll concentration was estimated non-destructively with a SPAD-502 device (Minolta Corp., Osaka, Japan). For calibration, leaf disks with different degrees of Fe deficiency were first measured with the SPAD, then extracted with 100% acetone in the presence of Na ascorbate and Chl measured spectrophotometrically. Three leaves per Chl level (each from a different plant) were taken, and four measurements were carried out in different areas of each leaf.

Xylem sap collection

Plants were detopped with a razor blade approximately 5 cm above the roots. Stumps were allowed to bleed for 1 min, the exuded fluid was carefully wiped out with paper tissue and the stem was fitted with plastic tubing. Xylem sap was then allowed to bleed into the plastic tubes for 15 min. After this period, samples were immediately collected, pH was measured, and samples were filtered with a 0.2 μM filter (Millipore, Bedford, MA, USA) and frozen until analysis.

Organic anion analysis

Root tip material (ca. 100 mg FW) or leaf samples (three leaf disks of 0.96 cm2 each, taken with a calibrated cork borer) were frozen in liquid N2 and ground in a mortar with 8 mM H2SO4. Homogenates were boiled for 30 min, filtered with a 0.2 μM PVDF filter (LiDA, Kenosha, WI, USA), taken to a final volume of 2 mL with 8 mM H2SO4 and kept at −80 °C until analysis.

Organic anions were analyzed by HPLC with a 300 × 7.8 mm Aminex ion-exchange column (HPX-87 H from Bio-Rad, Hercules, CA, USA) with a HPLC Waters system, including a 600E pump, a 996 photodiode array detector and the Millenium 2010 software. Samples were injected with a Rheodyne injector (20 μL loop). Mobile phase (8 mM H2SO4) was pumped with a 0.6 mL min−1 flow rate. Organic anions were detected at 210 nm. Peaks corresponding to oxalate, cis-aconitate, citrate, 2-oxoglutarate, malate and fumarate were identified by comparison of their retention times with those of known standards from Bio-Rad and Sigma. Identification was confirmed by their peaks in the UV spectra and/or HPLC–MS (in the latter case formic acid was used as the mobile phase instead of H2SO4). Quantification was carried out with known amounts of each organic anion using peak areas.

Enzyme assays

Extracts for measuring enzyme activities were made by grinding approximately 100 mg FW of root tip material (or three leaf disks of 0.96 cm2 each) in a mortar with 1 mL of extraction buffer containing 30 mM sorbitol, 1% BSA and 1% PVP in 100 mM HEPES-KOH, pH 8.0. The slurry was centrifuged for 15 min at 10,000g and 4 °C, and the supernatant was collected and analyzed immediately.
The activities of all enzymes were analyzed in 1 mL (final volume) of the media indicated below. An initial assay was performed to assess the stability of enzyme activities in the extracts over time, and blanks were run without enzyme substrate to correct for non-enzymatic reactions.

Malate dehydrogenase (MDH; EC 1.1.1.37) activity was determined with oxalacetate as substrate by measuring the decrease in $A_{340}$ due to the enzymatic oxidation of NADH. The reaction was carried out with 5 μL of extract in 0.1 mM NADH, 0.4 mM oxalacetate and 46.5 mM Tris–HCl, pH 9.5. CS (EC 4.1.3.7) was assayed spectrophotometrically by monitoring the reduction of acetyl CoA to CoA with 5-5'-dithio-bis-2-nitrobenzoic (DTNB) acid at 412 nm. The reaction was carried out with 50 μL of extract in 0.1 mM DTNB, 0.36 mM acetyl CoA, 0.5 mM oxalacetate and 100 mM Tris–HCl, pH 8.1. Aconitase (EC 4.2.1.3) activity was measured from the formation of cis-aconitate, monitored at 240 nm with 60 μL of extract in 500 mM sucrose, 50 mM isocitrate and 100 mM Tris–HCl (pH 8.5). ICDH (EC 1.1.1.42) activity was determined with 50 μL of extract by monitoring the reduction of NADP$^+$ at 340 nm in a reaction mixture containing 3.5 mM MgCl$_2$, 0.41 mM NADP$, 0.55$ mM isocitrate and 88 mM imidazole buffer, pH 8.0. Fumarase (EC 4.2.1.2) was assayed with 50 μL of extract following the increase in $A_{240}$ due to the formation of fumarate. The reaction buffer was 50 mM malate and 100 mM phosphate buffer, pH 7.4.

Phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) activity was measured in a coupled enzymatic assay with MDH with 75 μL of extract in 2 mM phosphoenolpyruvate (PEP), 10 mM NaHCO$_3$, 5 mM MgCl$_2$, 0.16 mM NADH and 100 mM Bicine–HCl, pH 8.5. For the determination of lactate dehydrogenase (LDH; EC 1.1.1.27) and pyruvate decarboxylase (PDC; EC 4.1.1.1), the oxidation of pyruvate was monitored at 340 nm with 50 μL of extract. LDH was assayed in a reaction buffer containing 94.5 mM phosphate buffer (pH 9.5), 0.77 mM pyruvate and 0.2 mM NADH. PDC activity was determined in 190 mM citrate–KOH buffer (pH 6.0), 30 mM pyruvate, 0.32 mM NADH and 33 μg mL$^{-1}$ alcohol dehydrogenase.

Nucleotide analysis

Pyridine nucleotides were extracted from liquid N$_2$-frozen root material (approximately 30 mg FW) and from leaf disks (0.96 cm$^2$) in 1 mL of 100 mM NaOH (for NAD(P)H) or 5% TCA (for NAD(P)$^+$). The extracts were boiled for 6 min, cooled on ice and centrifuged at 12,000g for 6 min. Samples were adjusted to pH 8.0 with HCl or NaOH and 100 mM Bicine (pH 8.0). Nucleotides were quantified by the enzyme-cycling method (Matsumura and Miyachi, 1980).

Root tip O$_2$ consumption

Root tips were excised under water at room temperature from plants illuminated for several h. Root O$_2$ consumption rates were measured from the decrease in O$_2$ concentration in an aqueous phase with a Clark-type O$_2$ electrode (Hansatech, Kings Lynn, UK). Calibration was made from the difference in signal between air and N$_2$-saturated water. The effects of the respiration inhibitors KCN and hydroxy-salicylic acid (SHAM) were studied at different concentrations. Sequential additions of KCN were made directly to roots in the measuring cuvette. Roots were pre-incubated with different concentrations of SHAM for 30 min prior to measurement. A new batch of root material was used for each SHAM concentration.

Iron reductase activity

Iron reductase activity in excised tomato root tips and whole root systems was monitored by measuring the formation of the Fe(II)–BPDS$_3$ complex from Fe(III)–EDTA in the assay solution (Zouari et al., 2001). Whole plants and root tips were placed in the assay medium after 2–3 h of light onset and absorbance readings of the assay solution at 535 nm after 30 min of reaction time were taken after centrifugation. Controls were also carried out in the absence of plants or root tips to correct for non-enzymatic Fe reduction.

Results

Changes in organic anion concentrations with iron deficiency

Total organic anion concentrations in tomato root tips did not change significantly with Fe deficiency, but the composition of the organic anion pool was markedly changed (Table 1). In control root tips, the major organic anions (more than 90% of the total organic anion concentrations) were oxalate, 2-oxoglutarate and malate, whereas in Fe-deficient root tips citrate and malate accounted for 90% of the total organic anion concentrations (Table 1). Iron deficiency led to a major (20-fold) increase in citrate concentration and to large decreases in the concentrations of 2-oxoglutarate, oxalate and malate (96%, 90% and 67%, respectively) (Table 1). Low concentrations of cis-aconitate and fumarate were also detected in tomato root tips, but concentrations did not change significantly as a result of Fe shortage (Table 1).

In tomato leaves, Fe deficiency caused a 2-fold increase in the total organic anion pool, although the major organic acids found in leaves were citrate and malate, regardless of the Fe regime in which plants were grown. This increase was associated with 2-fold increases in the concentrations of both citrate and malate (Table 1). Fumarate, 2-oxoglutarate and oxalate were also found in both Fe-deficient and Fe-sufficient leaves,
but their concentrations did not change significantly with Fe deficiency (Table 1).

In tomato xylem sap, Fe deficiency led to a 7-fold increase in the total organic anion pool (Table 1), with the major organic acids found being citrate and malate in all cases. Major increases in the concentrations of citrate, malate, oxalate and fumarate in sap (17-, 6-, 4- and 4-fold, respectively) were found in Fe-deficient plants as compared to Fe-sufficient plants (Table 1). Also, cis-aconitate was detected in trace amounts in control xylem sap and reached concentrations of approximately 21 \mu M in Fe-deficient xylem sap samples (Table 1).

### Changes in enzymatic activities with iron deficiency

We measured five enzymatic activities involved in organic acid metabolism in tissue extracts. All of these increased significantly at the \( p < 0.05 \) level (Student’s \( t \)-test) in the extracts of Fe-deficient root tips when compared with those obtained from Fe-sufficient root tips (Table 2). Increases were 10.5-fold for ICDH, 7.2-fold for aconitate, 4.9-fold for MDH, 3.5-fold for fumarase and 2.3-fold for CS (Table 2).

In leaf extracts, the activities of the five measured enzymes were in the same range as those found in root tips. CS activity was 2.3-fold higher in Fe-deficient than in Fe-sufficient leaf extracts, whereas the activities of MDH, ICDH and aconitate did not change significantly at \( p < 0.05 \). However, MDH activity changes were significant at \( p < 0.10 \). Fumarase activity was lower in extracts from Fe-deficient than from Fe-sufficient leaves (Table 2).

Activities of PEPC and two enzymes related to the anaerobic metabolism, LDH and PDC, were also measured in extracts from root tips and leaves. Iron deficiency caused a 10-fold increase in PEPC activity in root extracts, whereas it did not

---

**Table 1.** Organic anion concentrations in root tips and leaves (in nmol g\(^{-1}\) FW) and xylem sap (in \( \mu M \)) of Fe-sufficient (+Fe, 300 \( \mu M \) Chl m\(^{-2} \)) and Fe-deficient (-Fe, 12 \( \mu M \) Chl m\(^{-2} \)) tomato plants

<table>
<thead>
<tr>
<th></th>
<th>Root tips</th>
<th>Leaves</th>
<th>Xylem sap</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Fe</td>
<td>−Fe</td>
<td>+Fe</td>
</tr>
<tr>
<td>Oxalate</td>
<td>2.51 ± 0.10 a</td>
<td>0.25 ± 0.12 b</td>
<td>0.93 ± 0.19 a</td>
</tr>
<tr>
<td>Cis-aconitate</td>
<td>0.37 ± 0.03 a</td>
<td>0.69 ± 0.18 a</td>
<td>1.01 ± 0.25 a</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.52 ± 0.08 b</td>
<td>10.18 ± 2.14 a</td>
<td>15.90 ± 0.03 b</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>5.57 ± 0.92 a</td>
<td>0.21 ± 0.03 b</td>
<td>0.15 ± 0.03 a</td>
</tr>
<tr>
<td>Malate</td>
<td>4.48 ± 0.02 a</td>
<td>1.49 ± 0.39 b</td>
<td>13.11 ± 2.65 b</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.03 ± 0.01 a</td>
<td>0.09 ± 0.01 a</td>
<td>0.16 ± 0.02 a</td>
</tr>
<tr>
<td>Total</td>
<td>13.5</td>
<td>12.9</td>
<td>31.3</td>
</tr>
</tbody>
</table>

Data are means ± SE of five replicates. Data followed by the same letter within the same row are not significantly different (Student’s \( t \)-test) at the \( p < 0.05 \) level.

<table>
<thead>
<tr>
<th></th>
<th>Root tips</th>
<th>Leaves</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Fe</td>
<td>−Fe</td>
<td></td>
</tr>
<tr>
<td>MDH</td>
<td>16.8 ± 1.0 b</td>
<td>82.9 ± 8.5 a</td>
<td>11.5 ± 1.6 a</td>
</tr>
<tr>
<td>CS</td>
<td>0.50 ± 0.07 b</td>
<td>1.15 ± 0.09 a</td>
<td>5.15 ± 0.77 b</td>
</tr>
<tr>
<td>ICDH</td>
<td>0.13 ± 0.05 b</td>
<td>1.36 ± 0.21 a</td>
<td>0.07 ± 0.01 a</td>
</tr>
<tr>
<td>Aconitate</td>
<td>26 ± 15 b</td>
<td>184 ± 32 a</td>
<td>186 ± 28 a</td>
</tr>
<tr>
<td>Fumarase</td>
<td>336 ± 150 b</td>
<td>1183 ± 305 a</td>
<td>584 ± 92 a</td>
</tr>
<tr>
<td>PEPC</td>
<td>0.24 ± 0.06 b</td>
<td>2.44 ± 0.25 a</td>
<td>0.17 ± 0.02 a</td>
</tr>
<tr>
<td>LDH</td>
<td>0.04 ± 0.01 b</td>
<td>0.23 ± 0.04 a</td>
<td>0.05 ± 0.01 a</td>
</tr>
<tr>
<td>PDC</td>
<td>0.07 ± 0.01 b</td>
<td>0.20 ± 0.05 a</td>
<td>0.09 ± 0.01 a</td>
</tr>
</tbody>
</table>

Data are means ± SE of five replicates. Data followed by the same letter within the same row were not significantly different (Student’s \( t \)-test) at the \( p < 0.05 \) level.
significantly affect PEPC activity in leaf extracts (Table 2). The enzymatic activities of LDH and PDC increased by 6- and 3-fold, respectively, with Fe deficiency in root tip extracts, whereas in leaves they did not change significantly (Table 2).

Changes in nucleotide concentrations with iron deficiency

The total pool of pyridine nucleotides did not change significantly with Fe deficiency (Table 3). Concentrations of the NAD(H) pair did not change significantly with Fe deficiency, whereas NADPH and NADP⁺ concentrations were 10% lower and 60% higher, respectively, in Fe-deficient than in Fe-sufficient leaves (Table 3). As a result of these changes, the NADPH/NADP⁺ ratio in leaves decreased by 45% with Fe deficiency, whereas the NADH/NAD⁺ ratio increased by 83%.

Changes in root tip oxygen consumption rates induced by iron deficiency

Root tips from Fe-deficient and Fe-sufficient tomato plants had similar O₂ consumption rates (240 ± 63 and 260 ± 49 nmol O₂ g⁻¹ FW min⁻¹, respectively). Cyanide (2 mM)-resistant O₂ consumption was approximately 40% of the total consumption in Fe-deficient roots and 75% in Fe-sufficient roots (Figure 1A and B, respectively). The presence of SHAM at concentrations of 2 mM decreased root tip O₂ consumption by 40% and 50% in Fe-deficient (Figure 1A) and Fe-sufficient roots (Figure 1B), respectively. Residual O₂ consumption, the fraction of oxygen uptake resistant
to the combination of KCN and SHAM, was below 10% of the initial value in both Fe-deficient and sufficient roots.

Changes in root tip ferric chelate reductase activity with iron deficiency

Iron reductase activities were higher when measured in tomato root tips than when measured in whole root systems. Iron reductase activity measured in whole root systems increased 7-fold with Fe deficiency (Figure 2A), whereas in root tips, activity increased 2.4-fold (Figure 2B).

Discussion

When Fe is in low supply, some Strategy I plant species are able to elicit, in addition to the Fe(III) uptake system, several other biochemical mechanisms such as root proton extrusion and exudation and/or accumulation of organic compounds including flavins, organic acids and phenolics, as well as to undergo morphological changes such as the development of root transfer cells (Marschner, 1995). All of these processes require an energetic effort that plants deal with by modulating different metabolic pathways, including anaplerotic C fixation, Krebs cycle and O2 respiration (Zocchi, 2006). Thus, differences observed among species with respect to Fe efficiency may rely on the fine regulation of all these metabolic pathways. In the present work, we analyzed several of these responses in tomato plants in order to gain insight into the relationships among these processes and Fe efficiency.

Iron-deficient tomato root tips had an enhanced capacity to fix C from bicarbonate, with a large (10-fold) increase in PEPC activity over the control values. Increases with Fe deficiency in C fixation and/or PEPC activity have been reported previously to occur in roots of Strategy I plant species (Abadía et al., 2002). Increases reported have ranged from 2-fold in pepper to 30-fold in sugar beet (Landsberg, 1986; Andaluz et al., 2002) and have been described to coincide spatially with areas having increased Fe reductase activity in several species, including pepper, cucumber and sugar beet (Landsberg, 1986; de Nisi and Zocchi, 2000; López-Millán et al., 2000b). Consistent with these results, Fe deficiency caused increases in the root Fe reductase activity of tomato (2.4- and 7-fold in root tips and whole root systems, respectively). This induction is within the range found previously in tomato by several authors (Zouari et al., 2001), but is much lower than the Fe reductase activity induction found in sugar beet (Susín et al., 1996). These data suggest that tomato plants are less efficient at overcoming Fe deficiency than other species such as sugar beet, and this may be associated with the moderate PEPC activity induction found in tomato roots with Fe deficiency.

The total organic acid pool did not increase in Fe-deficient tomato root tips, whereas the increase in citrate, 20-fold, was only slightly lower than the 26-fold reported previously in sugar beet. In contrast to results in sugar beet, malate concentration was 3 times lower in Fe-deficient than in Fe-sufficient root tips. The decrease in malate – as well as 2-oxoglutarate – concentrations in Fe-deficient tomato root tips can be explained by an export of these acids to the leaves via xylem sap. This is in agreement with the increases measured in xylem sap malate and 2-oxoglutarate concentrations with Fe deficiency. A significant influx of organic acids from roots to leaves has been proposed before in Fe-deficient tomato (Bialczyk and Lechowski, 1995) and sugar beet (López-Millán et al., 2000b). A second factor that can contribute to the decreased malate concentration in Fe-deficient tomato root tips is root exudation of organic acids, which can chelate Fe in the rhizosphere and increase its availability (Schmidt, 1999). Changes found in the organic acid pool in tomato roots with Fe deficiency are likely associated with the increase in PEPC activity, which is accompanied by increases in the activities of several enzymes involved in organic acid synthesis, including MDH, CS, ICDH, aconitase and fumarase (4.9-, 2.3-, 10.5-, 7- and 3.5-fold, respectively). PEPC catalyses the carboxylation of PEP to oxaloacetate, which could subsequently be reduced to malate via cytosolic MDH. Malate could then be transported to mitochondria via malate-oxalacetate shuttle and enter the Krebs cycle. An increased
input of malate in the Krebs cycle would generate NADH and FADH$_2$ that, if exported to the cytoplasm, may be used by the root Fe reductase. The fact that the total organic acid pool did not change in Fe-deficient tomato root tips, in spite of having increased activities of organic acid synthesis-related enzymes, is associated with the decrease in oxalate concentration in Fe-deficient tomato root tips, since the carboxylate pool increased when oxalate was not taken into account. The implications of oxalate concentration decreases with Fe deficiency are still not known, since the role of oxalic acid in plants is quite different from that of the other organic acids, and for a long time it has been considered as a toxin or a metabolic end product (Franceschi and Nakata, 2005). On the other hand, the induction of all enzymes measured was markedly lower than in sugar beet (López-Millán et al., 2000b). These results indicate that these species have metabolic differences that may account for their different Fe efficiency.

Both pyridine nucleotide pool sizes and O$_2$ consumption rates were not changed by Fe deficiency in tomato root tips, whereas in sugar beet they increased 4 and 3 times, respectively, with Fe deficiency (López-Millán et al., 2000b). The pyridine nucleotide pool was more oxidized in the Fe-deficient tomato root tips than in controls, similar to what occurs in sugar beet (López-Millán et al., 2000b). The opposite was reported to occur in bean (Sijmons et al., 1984), whereas in Plantago lanceolata the total pool was more oxidized with Fe deficiency, although the NADPH/NADP$^+$ pool was slightly more reduced (Schmidt and Schuck, 1996). The increased activities of several NADH-consuming enzymes, such as LDH, ICDH and MDH, could contribute to oxidize the nucleotide pool in tomato roots.

The CN$^-$-resistant O$_2$ consumption was higher in Fe-sufficient than in Fe-deficient tomato root tips, indicating that the capacity of the AOX CN-resistant pathway (that reflects the maximum possible flux of electrons to AOX, and generally correlates with AOX protein levels; Lennon et al., 1997; McDonald et al., 2002) decreases with Fe deficiency. A decrease in AOX protein level would be expected under Fe deficiency, since it contains Fe (Moore and Siedow, 1991). It should be kept in mind that inhibitor experiments provide only a semi-quantitative electron allocation estimate of the two pathways (Ribas-Carbó et al., 1995; Day et al., 1996). Another factor that may influence AOX activity is the presence of compounds such as $\alpha$-ketoacids and malate, which are known to enhance AOX activity (Siedow and Day, 2000). Iron-sufficient root tips had higher concentrations of 2-oxoglutarate and malate when compared to Fe-deficient roots (26- and 3-fold increases, respectively), and this would also favor higher AOX activity in Fe-sufficient root tips. Interestingly, this phenomenon was not observed in sugar beet root tips, where Fe deficiency led to an increase in the concentrations of both 2-oxoglutarate and malate (López-Millán et al., 2000b). Further, electron flow through AOX is also enhanced by the reduction of an intermolecular disulfide bond that links covalently the two AOX monomers (Siedow and Day, 2000), and the reduced state is also more sensitive to stimulation by $\alpha$-ketoacids (Umbach et al., 1994).

Another feature that makes tomato plants interesting for this study is the lack of Fe deficiency-induced flavin accumulation in root tips. It has been hypothesized that Fe efficiency could be related to flavin accumulation in roots, since these compounds may act as a redox bridge helping electron transport from the pyridine nucleotides to the Fe reductase in the plasma membrane (Sijmons et al., 1984; López-Millán et al., 2000b). Increases in NAD(P)H-generating enzymes and reductase activity were less marked in Fe-deficient tomato roots, which do not accumulate flavins when grown under Fe shortage, than in flavin-accumulating plants such as sugar beet. A recent study of the transmembrane topology of FRO2 from Arabidopsis has revealed that the structural homology of this protein with the flavocytochrome b family suggests that NAD(P)H is oxidized on the cytoplasmic side and the electrons are transferred via flavin and two heme groups to a site on the outside surface of the plasma membrane, where reduction of O$_2$ or Fe(III) takes place (Schagerlof et al., 2006). Thus, the induction of flavin synthesis in Fe deficiency may be a species-dependent advantage, which could contribute to the Fe efficiency response.

In tomato xylem sap, the carboxylate pool also increased with Fe deficiency, confirming previous reports (White et al., 1981; Miller et al., 1990), and in agreement with what occurs in other species (López-Millán et al., 2000a, 2001). This further supports the important role for citrate in Fe metabolism, especially in long-distance xylem sap Fe transport, as it has been previously suggested (Tiffin, 1966; López-Millán et al., 2000a). In addition, a recent study at the molecular level described a protein, FRD3, that loads citrate into the xylem and is necessary for the correct localization of Fe throughout the plant (Durrett et al., 2007). In tomato leaves, the organic acid pool also increased with Fe deficiency, and this increase was restricted to citrate and malate. Both acids showed 2-fold concentration increases, in
contrast with other species in which increases were
more marked and included other organic anions.
Malate and citrate increases in leaves from Fe-
deficient tomato can be explained by the contribu-
tion of two factors: first, the increases measured in
the activities of MDH and CS in the same leaves
(1.4- and 3.4-fold, respectively, significant at
p<0.10 and p<0.05), and second, an influx of
these acids from the root via xylem sap, as
proposed to occur in sugar beet and pear Fe-
deficient leaves (López-Millán et al., 2000a, 2001).

In conclusion, this work adds further support to
the hypothesis that the extent of activation of
several metabolic pathways, including carbon fixa-
tion via PEPC, organic acid synthesis-related
enzymes and O2 consumption is different among
species, and could determine Fe efficiency. Citrate
seems to be a central point in the responses of
plants to Fe deficiency, since large increases of
citrate concentrations in all plant tissues appear
to be conserved among species, whereas the
degree of elicitation of other responses could differ
considerably.

Acknowledgements

This study was supported by the Spanish Ministry
of Science and Education (projects AGL2006-1416
and AGL2007-61948, co-financed with FEDER), the
European Community (Thematic Priority 5—Food
Quality and Safety, 6th Framework RTD Programme,
Contract no. FP6-FOOD-CT-2006-016279) and the
Aragón Government (group A03). We thank M.
Ribas-Carbó for his help in interpreting O2
consumption data.

References

Abadía J. Leaf responses to Fe deficiency – a review.
Abadía J, López-Millán AF, Rombolá A, Abadía A.
Organic acids and Fe deficiency: a review. Plant Soil 2002;
241:75–86.
Andaluz S, López-Millán AF, Peleato ML, Abadía J, Abadía
A. Increases in phosphoenolpyruvate carboxylase
activity in iron-deficient sugar beet roots: analysis of
spatial localization and post-translational modifica-
Bialczyk J, Lechowski Z. Chemical-composition of xylem
sap of tomato grown on bicarbonate containing
Chaney RL, Brown JC, Tiffin LO. Obligatory reduction of
ferric chelates in iron uptake by soybeans. Plant

Day DA, Krab K, Lambers H, Moore AL, Siedow JN, Wagner
AM, et al. The cyanide-resistant oxidase: to inhibit or
De Nisi P, Zocchi G. Phosphoenolpyruvate carboxylase in
cucumber (Cucumis sativus L.) roots under iron
deficiency: activity and kinetic characterization.
Durrett TP, Gassmann W, Rogers EE. The FRD3-mediated
efflux of citrate into the root vasculature is necessary
for efficient iron translocation. Plant Physiol 2007;177:
197–205.
Eide D, Broderius M, Fett J, Guerinot ML. A novel iron-
regulated metal transporter from plants identified by
functional expression in yeast. P Natl Acad Sci USA
Franceschi VR, Nakata PA. Calcium oxalate in plants:
formation and function. Annu Rev Plant Biol 2005;56:
41–71.
González-Vallejo EB, González-Reyes JA, Abadía A,
López-Millán AF, Yunta F, Lucena JJ, et al. Reduction
of ferric chelates by leaf plasma membrane prepara-
tions from Fe-deficient and Fe-sufficient sugar beet.
Kim SA, Guerinot ML. Mining iron: iron uptake and
Kobayashi T, Nishizawa NK, Mori S. Molecular analysis of
iron-deficient graminaceous plants. In: Barton LL,
Abadía A, editors. Iron nutrition in plants and rhizo-
Landsberg EC. Function of rhizodermal transfer cells in
the iron stress response mechanisms of Capsicum
Lennon AM, Neuenschwander UH, Ribas-Carbó M,
Giles L, Ryals JA, Siedow JN. The effects of salicylic
acid and tobacco mosaic virus infection on the
alternative oxidase of tobacco. Plant Physiol 1997;
115:783–91.
López-Millán AF, Morales F, Abadía A, Abadía J. Effects of
iron deficiency on the composition of the leaf
apoplastic fluid and xylem sap in sugar beet. Implica-
tions for iron and carbon transport. Plant Physiol 2000a;
124:873–84.
López-Millán AF, Morales F, Andaluz S, Gogorcena Y,
Abadía A, De las Rivas J, et al. Responses of sugar beet
roots to iron deficiency. Changes in carbon assimila-
López-Millán AF, Morales F, Abadía A, Abadía J. Iron-
deficiency-associated changes in the composition of
the leaf apoplastic fluid from field-grown pear (Pyrus
Marschner H. Mineral nutrition of higher plants. London:
Marschner H, Römheld V, Kissel M. Different strategies
in higher-plants in mobilization and uptake of iron.
Matsumura H, Miyachi S. Cycling assay for nicotinamide
adenine dinucleotides. Methods Enzymol 1980;69:
465–70.


