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Short communication

Carboxylate metabolism changes induced by Fe deficiency in barley, a Strategy II plant species

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SUMMARY

The effects of iron (Fe) deficiency on carboxylate metabolism were investigated in barley (*Hordeum vulgare* L.) using two cultivars, Steptoe and Morex, which differ in their Fe efficiency response. In both cultivars, root extracts of plants grown in Fe-deficient conditions showed higher activities of enzymes related to organic acid metabolism, including citrate synthase, malate dehydrogenase and phospho*enol*pyruvate carboxylase, compared to activities measured in root extracts of Fe-sufficient plants. Accordingly, the concentration of total carboxylates was higher in Fe-deficient roots of both cultivars, with citrate concentration showing the greatest increase. In xylem sap, the concentration of total carboxylates was also higher with Fe deficiency in both cultivars, with citrate and malate being the major organic acids. Leaf extracts of Fe-deficient plants also showed increases in citric acid concentration and in the activities of glucose-6-phosphate dehydrogenase and fumarase activities, and decreases in aconitase activity. Our results indicate that changes in root carboxylate metabolism previously reported in Strategy I species also occur in barley, a Strategy II plant species, supporting the existence of anaplerotic carbon fixation *via* increases in the root activities of these enzymes, with citrate playing a major role. However, these changes occur less intensively than in Strategy I plants. Activities of the anaerobic metabolism enzymes pyruvate decarboxylase and lactate dehydrogenase did not change in barley roots with Fe deficiency, in contrast to what occurs in Strategy I plants, suggesting that these changes may be Strategy I-specific. No significant differences were observed in overall carboxylate metabolism between cultivars, for plants challenged with high or low Fe treatments, suggesting that carboxylate metabolism changes are not behind the Fe-efficiency differences between these cultivars. Citrate synthase was the only measured enzyme with constitutively higher activity in Steptoe relative to Morex leaf extracts.

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 to respond to Fe shortage: Strategy II, which occurs in *Poaceae* species, and Strategy I, which occurs in dicotyledonous and non-grass monocotyledonous species (Romheld and Marschner, 1986). In both strategies, Fe deficiency induces several mechanisms that facilitate Fe acquisition from the soil. In Strategy II species, there is an increase in the synthesis and secretion of phytosiderophores to the rhizosphere, parallel to the induction of an Fe(III)-phytosiderophore complex transport system (Kobayashi et al., 2006). Strategy I plants induce a multi-step mechanism for root Fe acquisition that includes the induction of an Fe(III) reductase and an Fe(II) transporter (reviewed in Schmidt, 2006; Abadía et al., 2011).

Strategy I plants, when Fe deficient, elicit several changes at the metabolic level in roots to sustain the increased Fe acquisition capacity (reviewed in Zocchi, 2006; Vigani, 2012). These changes include an accumulation of organic acids throughout the plant, mainly malate and citrate (reviewed in Abadía et al., 2002), increases in the activity of PEPC and in several enzymes of the Krebs cycle and of the glycolytic and pentose phosphate pathways (Zocchi, 2006). Some increased activities are associated with enhanced expression of the corresponding genes (Thimm et al., 2001). This metabolic reprogramming could also act as an anaplerotic source to overcome the low photosynthetic rates in Fe-deficient Strategy I plants (López-Millán et al., 2009). The increase in PEPC activity correlates with organic acid accumulation in Fe-deficient roots (López-Millán et al., 2000b) and the up-regulation

Abbreviations: CS, citrate synthase; G6PDH, glucose-6-phosphate dehydrogenase; ICDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PDC, pyruvate decarboxylase; PEPC, phospho*enol*pyruvate carboxylase.

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of the glycolytic pathway. Increases in the activities of several NAD(P)H producing enzymes can provide reducing power to keep the Fe reductase working at elevated rates (López-Millán et al., 2000a; Zocchi, 2006).

Increases in organic acid concentrations induced by Fe deficiency have also been reported in xylem sap and leaves (reviewed in Abadía et al., 2002). In xylem sap, Fe is transported as Fe(III), probably chelated by citrate (López-Millán et al., 2000a) and an Fe–citrate complex (Fe_3Cit_3) has been identified in the xylem sap of Fe-deficient tomato resupplied with Fe (Rellán-Álvarez et al., 2010). Moreover, recent studies have indicated that a citrate transporter, FRD3, found in both Strategy I and II plants, is necessary to provide a chelator for efficient Fe translocation in the xylem sap (Durrett et al., 2007; Roschzttardtz et al., 2011; Yokosho et al., 2009).

Information on the effects of Fe deficiency on carboxylate metabolism has derived mostly from studies on Strategy I plants, whereas less information on this topic is available in Strategy II plants. Therefore, the aim of this work was to investigate the Fe deficiency-induced changes in carboxylate metabolism in two cultivars of barley, shown previously to differ in their Fe efficiency response. We measured the activities of enzymes involved in these processes in roots and leaves, along with carboxylate concentrations in roots, xylem sap, and leaves.

Materials and methods

Plant material, growth, and harvest

Seeds of two barley cultivars (*Hordeum vulgare* L.; cvs. Steptoe and Morex) previously shown to differ in Fe efficiency (Crowley et al., 2002) were obtained from the National Small Grains Collection (USDA-ARS, Aberdeen, ID, USA). Plants were grown hydroponically in a controlled environment chamber with a photosynthetic photon flux density at leaf height of 350 μmol m^{-2} s^{-1} photosynthetically active radiation, 50% relative humidity and at 15 h 22.5 °C/9 h 17.5 °C day/night regime. Seeds were imbibed in water and placed in CYG germination pouches (Mega International, St. Paul, MN) with approximately 15 mL water for 5 d in the dark at 22 °C. Seedlings were transplanted to seedling cups supported in a lid, placed over a 20 L tub (10 plants per tub), with roots placed in a nutrient solution containing: (in mM) 3.0 KNO_3, 1.0 $Ca(NO_3)_2$, 0.5 KH_2PO_4, 0.75 K_2SO_4, 0.5 $MgSO_4$, and (in μM) 25 $CaCl_2$, 25 H_3BO_3, 2 $MnSO_4$, 2 $ZnSO_4$, 0.5 $CuSO_4$, 0.5 H_2MoO_4, 0.1 $NiSO_4$, 0.1 K_2SiO_3, 1 mM MES buffer (pH 5.5) and 20 μM Fe(III)-HEDTA [hydroxyethyl ethylenediamine triacetic acid]. After 10 d, plants were transferred to 4.5 L containers (5 plants per container) and treatments (0 and 20 μM Fe(III)-HEDTA) were initiated using the same base nutrient solution described above. SPAD readings (SPAD-502P, Konica Minolta, Tokyo, Japan) were taken 10 d after the start of treatments, using all leaves. On the same day, xylem sap (see below), root, or leaf samples were collected, frozen in liquid N_2 and stored at −80 °C. The experiment was repeated with three different batches of plants. Within each batch, roots and leaves were collected from three plants per treatment and xylem samples were pooled from four plants per treatment.

Xylem sap collection

Plants were detopped with a razor blade approximately 1 cm above the roots. Stumps were allowed to exude for 1 min, the exuded fluid was carefully wiped off with tissue paper, and then exudates were collected for 30 min. After this period, samples were filtered with a 0.2 μM filter (Millipore, Bedford, MA, USA) and frozen in N_2 (l) until analysis. Malate dehydrogenase activity was used as a cytosolic contamination marker (López-Millán et al., 2000a).

Carboxylate analysis

Leaf and root materials (*ca.* 150 mg of fresh weight) were extracted in a Retsch MM301 mill (Retsch, Düsseldorf, Germany) with 1 mL of 5 mM sulfuric acid. Homogenates were boiled for 15 min, centrifuged at 10,000 × *g* for 10 min, filtered with a 0.2 μM PTFE filter (PALL Corp., Port Washington, NY), taken to a final volume of 1.5 mL with 5 mM sulfuric acid, and kept at −80 °C until analysis. Xylem sap samples were filtered (0.2 μM PTFE), taken to a final volume of 200 μL with 5 mM sulfuric acid, and kept at −80 °C until analysis. Carboxylates were analyzed by HPLC with an Aminex ion-exchange column (300 mm × 7.8 mm, HPX-87H, Bio-Rad, Hercules, CA), using a Dionex ICS-3000 system (Sunnyvale, CA) equipped with an AD25 absorbance detector, following the protocol described elsewhere (López-Millán et al., 2000a).

Enzyme assays

Extracts were made by grinding frozen roots or leaves (100 mg of fresh weight) in a mortar with 1.5 mL of extraction buffer (López-Millán et al., 2009). Activities were measured for: seven enzymes involved in carboxylate metabolism: malate dehydrogenase (MDH, EC 1.1.1.37), citrate synthase (CS, EC 2.3.3.1), isocitrate dehydrogenase (ICDH, EC 1.1.1.42), fumarase (EC 4.2.1.2), aconitase (EC 4.2.1.3), phospho*en*olpyruvate carboxylase (PEPC, EC 4.1.1.31), and glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49); and two enzymes involved in anaerobic metabolism: lactate dehydrogenase (LDH, EC 1.1.1.27) and pyruvate decarboxylase (PDC; EC 4.1.1.1). All activities were measured in 1 mL final volume of the corresponding reaction buffer (López-Millán et al., 2000a).

Results and discussion

In the present study, we analyzed several enzymatic activities and carboxylate concentrations related to anaplerotic carbon fixation that have previously been described to increase in Fe-deficient-Strategy I plants (López-Millán et al., 2000b, 2009; Zocchi, 2006), using two cultivars of barley (a Strategy II species), that were previously shown to differ in Fe efficiency (Crowley et al., 2002). Plants were grown for 10 d in two Fe treatments (0 or 20 μM Fe[III]-HEDTA), and SPAD values were measured to ensure that the low Fe-treated plants were responding to the Fe deficiency stress. The more Fe-efficient cultivar, Steptoe, exhibited a leaf SPAD value with Fe deficiency (29.2 ± 1.1) that was 20% lower than its Fe-sufficient control (SPAD value: 36.9 ± 1.3). The less Fe-efficient cultivar, Morex, exhibited a leaf SPAD value with Fe deficiency (18.0 ± 1.9) that was 50% lower than the Fe-sufficient control (SPAD value: 35.9 ± 1.3). These results are in agreement with a previous report that assessed the link between differences in Fe efficiency among barley cultivars to the amount of phytosiderophores released by roots (Crowley et al., 2002).

Upon Fe shortage, the more efficient cultivar, Steptoe, showed significantly higher enzymatic activities in root extracts for PEPC (180%), MDH (70%), CS (60%), ICDH (60%), aconitase (40%) and fumarase (90%), when compared to the Fe-sufficient controls (Table 1). The less Fe-efficient cultivar, Morex, also showed significantly higher enzymatic activities for PEPC (130%), MDH (60%), CS (80%), ICDH (40%), and fumarase (100%) in extracts of Fe-deficient roots when compared to the controls, whereas aconitase activity did not increase significantly (Table 1). The activity of G6PDH, a pentose phosphate pathway enzyme, was 70% and 60% higher in Fe-deficient root extracts than in the controls in Steptoe and Morex, respectively (Table 1). The activity of LDH was approximately 30%