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Strategy I responses to Fe-deficiency of two *Citrus* rootstocks differing in their tolerance to iron chlorosis

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ABSTRACT

The expression of iron (Fe) acquisition-related genes in roots was studied in roots of two different citrus seedlings, namely, Carrizo citrange (CC, Fe chlorosis-sensitive) and Cleopatra mandarin (CM, Fe chlorosis-tolerant), growing either with (control) or without (−Fe) Fe in the nutrient solution. Fe-deficiency increased expression of the gene *HA1* coding for proton-ATPase (H^+-ATPase) enzyme in both genotypes, although no differences were observed between treatments among rootstocks. Furthermore, while the gene expression levels of *FRO2* – which encodes the Ferric Chelate Reductase (FC-R) enzyme–, increased under −Fe condition in both genotypes, CM always recorded the highest activity. CC showed the greatest induction of genes *IRT1* and *IRT2* encoding two iron transporters, however only *IRT1* was significantly induced by Fe starvation. Analysis of the enzymatic activities (H^+-ATPase and FC-R) regulated by the aforementioned genes confirmed these results. Thus, in agreement with the acidification pattern registered, H^+-ATPase activities were higher in −Fe plants than in controls, although no significant differences were detected between each treatment among rootstocks. Fe starvation also induced FC-R activity; however, this was greater in CM than in CC roots. Interestingly, root ^{57}Fe uptake rates from ^{57}Fe-EDDHA solutions were increased by Fe-deficiency, especially in the CM genotype, and CM accumulated a much larger Fe pool in the root apoplast than CC. Taken together, the main trait determining Fe-chlorosis tolerance among these genotypes is the ability to boost Fe^{3+} reduction in response to Fe-deficiency through enhanced *FRO2* gene expression. Moreover, Fe chlorosis resistance in these plants could be related to the amount of Fe stored in the root apoplast.

1. Introduction

Iron (Fe) is an essential micronutrient for plants, since it participates in some life-sustaining processes, such as respiration and photosynthesis, where it is involved in electron transfer through Fe^{2+}/Fe^{3+} redox reactions.

Although Fe is relatively abundant in many cultivated soils, its acquisition by crop plants is often impaired by certain soil properties. Among the latter, high carbonate content generates alkalinity in the soil solution, which results in low Fe availability (Hell and Stephan, 2003). These conditions affect most *citrus* cultivated in the Mediterranean basin, which frequently develop Fe chlorosis symptoms, mainly interveinal yellowness in leaves, as well as stunted vegetative growth, decreased yield and poor fruit quality.

A widely applied system to prevent Fe-deficiency in fruit-tree crops, is the use of Fe chlorosis-tolerant genotypes as rootstocks (Ksouri et al., 2006, 2007; Molassiotis et al., 2006; Jiménez et al., 2008, 2011) and, with this purpose, Fe chlorosis tolerance of *citrus* rootstocks has been tested in several studies (Chouliaras et al., 2004; Pestana et al., 2005; Castle et al., 2009).

Plants have developed certain adaptive mechanisms to increase Fe-uptake capacity under Fe-deficiency conditions. Dicotyledonous and non-grass monocotyledonous species develop strategy I for Fe mobilization and acquisition (Marschner and Römheld, 1994; Schmidt, 1999; Kim and Guerinot, 2007), which includes the

Abbreviations: CC, Carrizo citrange; CM, Cleopatra mandarin; -Fe, iron-deprived; FC-R, ferric chelate reductase; H^+-ATPase, Proton-ATPase; IRT, iron transporter; RT-PCR, reverse transcription polymerase chain reaction; EDDHA, ethylenediamine-N,N′-bis(2-hydroxyphenylacetic acid); EDTA, ethylenediaminetetraacetic acid; PVPP, poly(vinylpolypyrrolidone); PMSF, phenylmethanesulfonyl fluoride; ATP, adenosine 5′-triphosphate; NADH, β-nicotinamide adenine dinucleotide; TRIS, tris(hydroxymethyl)aminomethane; MOPS, morpholinepropanesulfonic acid; BTP, 1,3-bis(tris-(hydroxymethyl)-methylamino)propane; MES, morpholineethanesulfonic acid; PEP, phosphoenolpiruvate; BSA, bovine serum albumin; BPDS, bathophenanthroline-disulfonic acid disodium salt hydrate; DW, dry weight; FW, fresh weight.

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following responses: (1) The excretion of protons (H^+) into the rhizosphere through the activation of specific plasma membrane-bound Proton-ATPases (H^+-ATPases, EC 3.6.3.6) from the epidermal cells of the roots (Rabotti and Zocchi, 1994; Dell'Orto et al., 2000b), which lowers the pH of the soil solution and increases Fe^{3+} solubilisation (Rabotti et al., 1995); (2) An increased Ferric Chelate Reductase (FC-R, EC 1.16.1.7) activity through the induction of a plasma membrane-bound enzyme, which reduces Fe^{3+} to Fe^{2+} at the root surface (Yi and Guerinot, 1996; Susín et al., 1996; Cohen et al., 1997; Robinson et al., 1999); and (3) The stimulation of Fe^{2+}-transport across root cell membranes through the activation of a specific iron-regulated transporter, IRT (Eide et al., 1996).

The development of genomic tools has also contributed to a better understanding of the molecular and metabolic processes leading to Fe uptake in plants. With respect to proton release, some genes coding for Fe-regulated H^+-ATPases have been characterized (Santi and Schmidt, 2009). Thus, in cucumber roots, Cs*HA1* was induced in Fe-deficient roots, while Cs*HA2* did not respond to Fe-deficiency (Santi et al., 2005).

On the other hand, plasma membrane FC-R enzymes are encoded by the *FRO* gene family (Jeong and Connolly, 2009). The main genes have been identified in several species, including *Arabidopsis* (Robinson et al., 1999), pea (Waters et al., 2002), tomato (Li et al., 2004), cucumber (Waters et al., 2007) and *Medicago truncatula* (Andaluz et al., 2009). *FRO2*, which is expressed in the epidermal cells of roots, is believed to be primarily responsible for enhancing FC-R activity due to Fe-deficiency, (Robinson et al., 1999) and overexpression confers tolerance to low Fe conditions (Connolly et al., 2003).

Finally, *IRT* genes code for family members of zinc transporter proteins (ZIP) in *Arabidopsis* (Eide et al., 1996). Among them, the *IRT1* gene is localized to the plasma membrane of epidermal cells in roots and its expression is induced by Fe-deficiency, generating the major transporter responsible for Fe uptake from the soil (Connolly et al., 2002; Vert et al., 2002). *IRT2* is also expressed in the external layers of Fe-deficient roots, although it seems to play a different role to that played by *IRT1* (Vert et al., 2001, 2009). IRT orthologues have been found in many other species such as tomato (Eckhardt et al., 2001), pea (Cohen et al., 2004), cucumber (Waters et al., 2007), rice (Bughio et al., 2002), peanut (Ding et al., 2010) and tobacco (Enomoto et al., 2007).

Another aspect to be considered is the localization of absorbed Fe in the plant. In this sense, it has been proposed that the root apoplast may operate as an Fe storage pool (Bienfait et al., 1985), and the amount of extra-plasmatic Fe accumulated and retranslocated from roots could be the cause of genotypical differences in the Fe-deficiency resistance of some plants such as soybean (Longnecker and Welch, 1990).

Since the relative importance of Fe-acquisition system components seems to differ considerably between plant species and genotypes, some studies have focused on the differences among citrus species in root responses to Fe-deficiency (Treeby and Uren, 1993; Manthey et al., 1994; Chouliaras et al., 2004). The main objectives of this work were to evaluate the activity of the key enzymes and transporters involved in the process, and the expression level of the genes which codified them. Moreover, this study increases knowledge about the regulation of the Fe-acquisition system based on the iron nutrition state of the plant and the extent to which the tolerance to iron deficiency is determined. With this purpose, assays were carried out in Fe-deficient and Fe-sufficient seedlings of Carrizo citrange (CC, iron chlorosis sensitive genotype) and Cleopatra mandarin (CM, iron chlorosis-tolerant genotype), two different citrus genotypes widely used as *citrus* rootstocks and known for their different tolerance to lime-induced Fe chlorosis (Castle et al., 2009).

2. Materials and methods

2.1. Plant material and growth conditions

CC (hybrid of *Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.) and CM (*Citrus reshni* Hort. ex Tan.) seeds were germinated in a glasshouse using a sterile substrate comprising peat, coconut fibre, sand and perlite (50:25:20:5) supplemented with 1.38 g kg^{-1} calcium superphosphate and irrigated twice weekly with the following nutrient solution: 1.5 mM $Ca(NO_3)_2$, 1.5 mM KNO_3, 1 mM $MgSO_4$, 1.2 mM H_3PO_4, 20 μM Fe-EDDHA, 23.2 μM H_3BO_3, 27.2 μM $MnSO_4{\cdot}H_2O$, 3.8 μM $ZnSO_4{\cdot}7H_2O$, 0.27 μM MoO_3 and 0.25 μM $CuSO_4{\cdot}5H_2O$. Nutrient solution pH was adjusted to 6.0 with 1 M KOH or 1 M H_2SO_4.

After 8 weeks, seedlings were selected based on uniformity of size and transplanted individually to opaque plastic 500 mL pots filled with coarse sand. Seedlings of each genotype were then separated into two groups and fed with the previous nutrient solution (pH 6), 2-fold strength either with or without 20 μM Fe-EDDHA, control and −Fe plants respectively. Seedlings (n = 30 per genotype and treatment) were randomized over the experimental area and a row of plants not included in the experiment was placed around the perimeter as a buffer row.

Seedlings were grown under glasshouse conditions with supplementary light (250 μmol m^{-2} s^{-1}, 400–700 nm) to extend the photoperiod to 16 h. Temperatures ranged between 16–18 °C at night and 26–28 °C during the day. Relative humidity (RH) was maintained at approximately 80%. Pots were irrigated twice weekly, using 200 mL of solution per pot in each watering event. Excess solution drained out of the pot, thereby avoiding salt accumulation in the sand. Plants were maintained in these conditions for 8 weeks and then seedlings from each genotype and treatment were carefully removed from pots and the roots washed with water to eliminate sand. Thereafter, whole seedlings were rinsed with de-ionized water before processing for further measurements.

2.2. Root RNA extraction and real-time RT-PCR analysis

Total RNA was extracted from approximately 1.5 g of frozen root tissue using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA samples were treated with RNase-free DNase (Qiagen) through column purification following the manufacturer's instructions. RNA quality (OD_{260}/OD_{280} ratio) and concentration were determined spectrophotometrically (Nanodrop Technologies, Thermo Fisher Scientific, Delaware, USA). Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was performed with a LightCycler 2.0 Instrument (Roche, Mannheim, Germany) equipped with Light Cycler Software version 4.0. Reactions contained 2.5 units of MultiScribe Reverse Transcriptase (Applied Biosystems, Roche Molecular Systems, New Jersey, USA), 1 unit of RNase Inhibitor (Applied Biosystems), 2 μL LC Fast Start DNA Master PLUS SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany), 25 ng total RNA and 0.250 μM of the specific forward and reverse primers of each gene in a total volume of 10 μL. Incubations were carried out at 48 °C for 30 min, 95 °C for 10 min followed by 45 cycles at 95 °C for 2 s, 58 °C for 8 s and 72 °C for 8 s. Fluorescent intensity data were acquired during the 72 °C-extension step and transformed into relative mRNA values using a 10-fold dilution series of an RNA sample as a standard curve. Relative mRNA levels were then normalized to total RNA amounts as previously described (Bustin, 2002; Hashimoto et al., 2004) and an expression value of 1 was arbitrarily assigned to the values of the CM control seedlings. Actin was used as the reference gene (Yan et al., 2012); the specificity of the amplification reactions was assessed by post-amplification dissociation curves and by sequencing the