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Iron-shortage-induced increase in citric acid content and reduction of cytosolic aconitase activity in *Citrus* fruit vesicles and calli

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Aconitase, which catalyses the conversion of citrate into isocitrate, requires Fe for its activity. The yeast and animal enzyme loses its enzymatic activity under Fe shortage and binds to RNA of genes involved in Fe homeostasis, altering their expression. Thus, the enzyme provides a regulatory link between organic acid metabolism and Fe cellular status. Roots and leaves of Fe-deficient plants show induction in organic acids, especially citrate. Although no RNA-binding activity has been so far demonstrated for the plant aconitase, whether alternations in enzyme activity by Fe could play a role in this induction remain unanswered. This question was investigated in lemon fruit [*Citrus limon* (L.) Burm var *Eureka*], characterized by the accumulation of citrate to about 0.3 *M* in the juice vesicles cells (pulp). Calli and isolated juice vesicles showed two- to three-fold induction in citrate level when subjected to Fe shortage. The mRNA level of aconitase exhibited no changes under reduced Fe concentrations. Analysis of aconitase isozymes demonstrated that out of two aconitase isozymes, typically detected in citrus fruit, only the cytosolic form displayed a reduced activity under low Fe concentrations. Our data support the notion of a limited Fe-availability-induced reduction in cytosolic aconitase, resulting in a slower rate of citrate breakdown and a concomitant increase in citrate levels.

Introduction

Aconitase (EC 4.2.1.3) catalyses the reversible conversion of citric acid and isocitric acid via the intermediate molecule *cis*-aconitic acid. The enzyme contains a single, labile [Fe–S] cluster, which is composed of]3Fe–4S[in the inactive state. Activation of the enzyme involves the binding of a fourth labile Fe (Rouault and Klausner 1996). In animal cells and yeast, the cytosolic aconitase is a regulatory link between Fe homeostasis and organic acid metabolism; when Fe supply is limited, the enzyme loses its aconitase activity and binds to the mRNAs of proteins that play a role in Fe homeostasis. For instance, it binds to the mRNA of the transferrin receptor, which induces Fe uptake and promotes its translation, leading to an increase in cellular Fe levels (reviewed in Hentze and Kuh 1996, Klausner et al. 1993, Rouault and Klausner 1996). The regulatory roles of cytosolic aconitase have been further documented by showing that a few organic-acid-metabolizing enzymes, mitochondrial aconitase, succinate dehydrogenase and glycolate oxidase, were translationally regulated by the cytosolic aconitase in animal cells under limited Fe supply (Gray et al. 1996, Kohler et al. 1995) . In contrast to animal and yeast enzymes, no RNA-binding activity was so far documented for plant aconitases, questioning the existence of a similar translational link between Fe homeostasis and organic

Abbreviations – EST, expressed sequence tag; PEPC, phosphoenolpyruvate carboxylase; TLE, 0.2 *M* Tris–HCl, pH 8.2, 0.1 *M* LiCl and 5 m*M* EDTA.

acid metabolism. However, the relationship between Fe homeostasis and organic acid levels is well established in plants (Ma 2005). Internal citrate concentrations increased in lateral and cluster roots under Fe deficiency; in the subapical root zone of non-graminaceous plants, Fe-deficient transfer cells exhibit an increased synthesis of malic and citric acids, which are used to chelate Fe from the soil (Landsberg 1986, McCluskey et al. 2004). The synthesis of citrate in roots and enhanced citrate extrusion and mobilization can be induced by both Fe and Pi deficiency (Neumann et al. 1999). Increased synthesis of citrate in root transfer cells correlates with the high number of mitochondria in these cells and with the role that citrate plays in the long distance transport of Fe in plants through the xylem (Landsberg 1986). Similarl to roots, leaves of Fe-deficient plants showed increases organic acid levels, mainly citrate and malate to a lesser extent (Clarck and Wallace 1963, DeKock and Morrison 1958, Wallace 1971).

We set on to examine whether plant aconitases could act as a metabolic link between Fe homeostasis and organic acid metabolism in citrus fruit. Citrus fruit is characterized by a massive accumulation of citric acid in the vacuole of the juice sac cells during the first half of fruit development, followed by a decline toward fruit maturation in most citrus varieties. Usually, acid concentration in the juice peaks at 0.1–0.2 *M*, but in sour lemon it reaches 0.3 *M*. An arrest in the activity of the mitochondrial aconitase could play a role in acid accumulation, as it would create a local increase in the citrate level that can be translocated for storage into the vacuole (Bogin and Wallace 1966, Sadka et al. 2000b). During the second half of fruit development, the activity of the cytosolic aconitase is induced, playing a role in acid decline (Sadka et al. 2000b). Fruit acid levels tend to increase when the tree is grown under low Fe availability (Bar Akiva 1964). We hypothesized that low Fe could reduce the cytosolic aconitase activity, leading to a lower rate of acid decline, thus affecting fruit quality (Sadka et al. 2000b). In this study, we used calli and intact juice vesicles to show that Fe shortage induces an increase in citric acid content, while inhibiting the activity of the cytosolic aconitase. In addition, we also demonstrate that the mitochondrial aconitase activity is not affected by Fe limitation, suggesting a distinct response of different aconitase forms to Fe shortage.

Materials and methods

Plant material

Fruits of sour lemon [*Citrus limon* (L.) Brum var. *Eureka*] were collected from orchards in the central coastal region of Israel. Tissue cultures originated from the juice sacs were prepared essentially as described before (Sadka et al. 2000a). The lemon fruits (30–50 mm in diameter) were surface sterilized with 70% ethanol and cut into halves, and the juice sacs, including the stalks, were placed in 50–ml glass tubes containing 10 ml of a Murashige and Skoog medium (Sigma-Aldrich, St Louis, MO), as described by Erner and Reuveni (1981), except for omission of orange juice. The growth medium contained 0.1 m*M* Fe as FeNaEDTA. The explants were incubated in the dark at 28°C, and calli were formed within 4–6 weeks. Hundred-milligram pieces were cut from the callus under aseptic conditions, transferred to fresh medium and allowed to grow for 4 weeks. At least two cycles of growth were performed before calli were used in experiments. The same procedure was applied in the experiments, and the calli pieces were transferred into solid media containing various Fe concentrations, as indicated. When intact juice vesicles were used, they were isolated from 40 to 50 mm diameter lemon fruits, washed few times in a Fe–free liquid medium, containing the same components as the solid medium, omitting the hormones, followed by incubation in the same medium.

Total titratable acid and organic acid analyses

Total titratable acid content of isolated juice sacs was determined by titration (Sinclair 1984). Citrate was assayed in calli with a 330 Gas Chromatograph (Varian, Walnut Creek, CA) according to Erner and Reuveni 1981. We used a Megabore DB17 column (J & W Scientific, Folsom, CA) with the following conditions: N_2 flow-through, 10 ml min^{-1}; N_2 makeup, 20 ml min^{-1}; H_2, 30 ml min^{-1}; air, 400 ml min^{-1}. Detection was performed with a 4290 Integrator (Varian).

Antibody preparation

The peptide YLLERGVDRKDFNSYGSR was chemically synthesized based on the putative amino acid sequence of the lemon aconitase clone, (GenBank accession no. AF073507) in the Department of Organic Chemistry, The Weizmann Institute of Science (Rehovot, Israel) and conjugated with Keyhole Limpet Hemocyanin (Pierce, Rockford, IL) according to the manufacturer's instructions. About 280 μg of protein were emulsified with Freund's complete adjuvant and injected into rabbits (Harlan Corp, Rehovot, Israel), followed by three boost injections in 3-week intervals. Bleedings were carried on 1 week following the second and third boost injections.

Protein extraction, Western blot and aconitase isozymes analysis

Total proteins were extracted from calli and juice sacs at different developmental stages using essentially the same procedure but at different tissue to buffer ratios, depending on the tissue used. Frozen calli and juice sacs from fruits up to 25 mm in diameter were ground in liquid nitrogen using mortar and pestle. About 1.2 g of the powder was transferred into a tube containing 2 ml of extraction buffer composed of 100 m*M* Tris–HCl pH 7.5, 10 m*M* KCl, 10 m*M* $MgCl_2$, 4% [w/v] poly-vinyl-pyrrolidone (MW = 40 000), 14.3 m*M* β-mercaptoethanol and 1 m*M* EDTA and homogenized with a Polytron (Kinematica, Bazel, Switzerland) at setting 3–4 for 2–5 s on ice. When fruits larger than 25 mm diameter were used, about 3 g of tissue were ground in 3 ml of 2× extraction buffer. Following centrifugation at 10 000 *g* for 10 min at 4°C, the supernatant was collected and the protein contents were determined using Bradford reagent (BioRad, Hercules, CA), according to the manufacturer's instructions. Ten to twenty milligram of protein extracts were resolved by SDS–PAGE in a 7.5% acrylamide gel (Laemmli 1970). The gels were electroblotted into nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), and the proteins were detected with the 1:1000–1:2000 diluted antibodies using ECL system (Amersham, Buckinghamshire, UK). For aconitase isozyme analyses, about 100 μg protein extracts were resolved by non-denaturing PAGE gels. Following protein separation, the gel was stained for aconitase activity, as described before (Sadka et al. 2000a).

Calli fractionation

Calli were fractionated into soluble, organelle and membrane fractions, and the mitochondria were purified essentially as described (Klein et al. 1998). All the solutions contained 100 μg ml^{-1} protease inhibitor cocktail (Sigma, St. Louis, MO), and all procedures were carried out at 4°C. About 15 g of calli were homogenized in 45 ml of a buffer containing 500 m*M* sucrose, 0.1 *M* MOPS–KOH (pH 7.5), 5 m*M* EGTA, 0.1% β-mercaptoethanol and 0.6% poly-vinyl-pyrrolidone in a blender (Waring Commercial, Torrington, CT) using the following settings: one pulse of 15 s at high speed and two pulses of 15 s at low speed with 30 s intervals in between. Cell debris were removed by filtration through four layers of cheesecloth, followed by filtration through two layers of Miracloth (Calbochem, San Diego, CA) and centrifuged at 2000 *g* for 5 min. The resulting supernatant was filtrated through two layers of Miracloth and centrifuged at 6000 *g* for 5 min at 4°C. Organelles were precipitated by centrifugation at 16 000 *g* for 10 min at 4°C. The resulting supernatant was centrifuged at 41 000 *g* for 1 h at 4°C in order to precipitate total cellular membranes, which were resuspended in 0.3 ml of a buffer containing 5 m*M* Tris–HCl pH 7.6, 2 m*M* DTT and 10% glycerol. The pellet from the previous centrifugation (organelles) was resuspended in a final volume of 400 ml of wash buffer containing 300 m*M* sucrose, 10 m*M* MOPS (pH 7.5) and 1 m*M* EGTA using fine hairbrush followed by recentrifugation at 16 000 *g* for 10 min and resuspension at a final volume of 2–3 ml of wash buffer. This fraction was loaded on top of a Percoll step gradient comprising 3 ml of 40% Percoll, 3 ml of 23% Percoll and 4 ml of 18% Percoll all in a solution containing 0.3 *M* sucrose and 2 m*M* MOPS–KOH, pH 7.2. The gradient was centrifuged at 12 000 *g* for 45 min, and the mitochondria were collected from the 23% and 40% Percoll interface into 200 ml of resuspension buffer containing 400 m*M* manitol, 10 m*M* Tricine–KOH pH 7.2 and 1 m*M* EGTA followed by centrifugation at 14 000 *g* for 15 min. The pellet was washed in 200 ml of resuspension buffer, resuspended in a total volume of 1.2 ml and transferred to 1.5 ml microfuge tube followed by centrifugation at 14 000 *g* for 15 min at 4°C. The final mitochondria pellet was resuspended in 100 ml of resuspension buffer.

RNA analysis

RNA was extracted from calli by means of the phenol–chloroform procedure as described before, with modifications (Sadka et al. 2000a). Approximately 1 g of frozen tissue was ground (using mortar and pestle) in liquid nitrogen and added to a tube containing 900 μl of grinding buffer containing 300 μl of phenol saturated with TLE (0.2 *M* Tris–HCl, pH 8.2, 0.1 *M* LiCl and 5 m*M* EDTA). Three hundred microliter of chloroform was then added, followed by incubation for 20 min at 50°C with occasional shaking. The mixture was then centrifuged at 10 000 *g* for 30 min at 4°C, and the upper phase was re-extracted with 500 μl of TLE-saturated phenol and 500 μl of chloroform and recentrifuged. The upper phase was extracted with 1 ml chloroform and centrifuged (10 000 *g*, 30 min, 4°C). LiCl was then added to the upper phase to a final concentration of 2 *M* (from 8 *M* stock solution), followed by an overnight incubation at 4°C and centrifugation at 10 000 *g* for 40 min at 4°C. The pellet was dissolved in 200 μl H_2O and precipitated by the addition of 0.1 volume of 3 *M* sodium acetate, pH 5.2, and 2.5 volumes of cold ethanol, followed by 30 min incubation at −80°C. Following washing with ethanol 70%, the pellet was dissolved in 30–50 μl of water, and the RNA content was determined spectroscopically. Northern blot analyses (Sambrook and Russell 2001)

were performed with [α^{32}P] 2′-deoxycytidine 5′-triphosphate radiolabeled probe. The membranes were scanned with a Fujifilm BAS-1500 Phosphoimager (Fugi Photo Film Co., Tokyo, Japan).

Results

Fe shortage leads to an increase in citric acid content in citrus fruit explants and callus

The question of whether there was a relationship between the content of citric acid and Fe shortage in citrus was investigated using fruit juice sacs calli and isolated juice vesicles. In order to avoid Fe-shortage-induced growth inhibition, the minimal and maximal Fe concentrations capable of supporting optimal cell growth were determined. The results showed that reducing the Fe concentration to 0.02 m*M*, one-fifth of the normal Fe concentration, did not affect calli growth, while 0.01 m*M* resulted in a clear growth inhibition, in particular after 35 days (Fig. 1). Similarly, increasing the Fe concentration to 0.2 m*M* resulted in significant growth retardation (not shown); therefore, the maximal Fe concentration used was 0.15 m*M*. The two higher Fe concentrations (0.1 and 0.15 m*M*) resulted in similar levels of citric acid of about 1.5 mg g^{-1} FW (Fig. 2A). A 2.5–fold increase in citric acid content was detected in calli grown for 3 weeks under low Fe (Fig. 2A). Other organic acids were also detected in the calli, albeit in much lower concentrations than that of citric acid (not shown). The effect of Fe shortage on acid level was studied in intact juice vesicles. Because citric acid is the major acid in the juice and changes in its levels directly affect the total acidity, we measured total acidity by titration (Sadka et al. 2000a). Within 1–2 days, a decrease in the total titratable acidity from 6 to 7% to about 3% was detected both in the vesicles incubated in the normal medium and in those incubated in Fe–free medium (Fig. 2B). After an additional week, the total acidity in vesicles incubated in normal medium fell to about 1%, while that of vesicles incubated in Fe–free medium fell to about 2%.

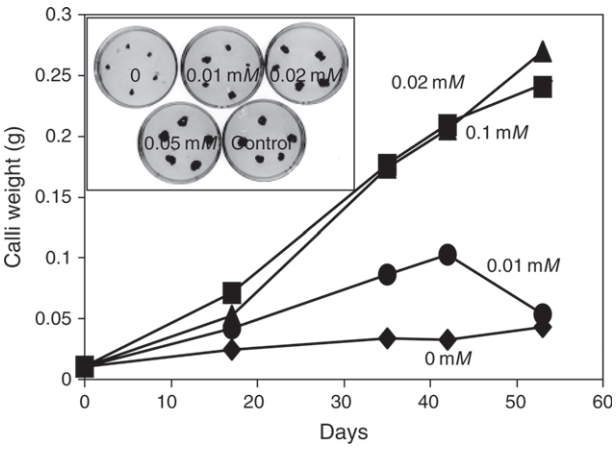

Fig. 1. Calli growth under elevated Fe concentrations. Pieces of lemon calli of about 0.01 g each were incubated in solid media containing different Fe concentrations as indicated. Growth was estimated by FW.

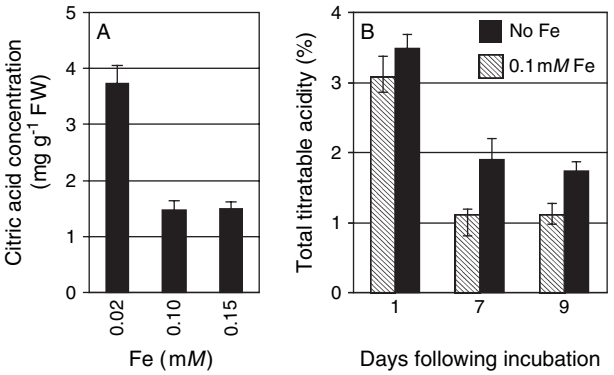

Fig. 2. Citric acid is induced upon Fe shortage lemon calli were grown in a medium containing 0.02 or 0.1 m*M* (control) Fe, and their citric acid content was measured after 3 weeks (A). Lemon juice vesicles were isolated and washed a few times with a medium containing 0.01 m*M* Fe (control) or Fe-free medium, followed by their incubation in the same medium. At the indicated times, their total titratable acidity was measured (B). Values are the mean ± SE (n = 10).

Fe shortage reduces cytosolic aconitase activity, but not protein or mRNA levels

In order to investigate whether the increase in citrate was because of a lower rate of its catabolism by aconitase, the effect of Fe shortage on aconitase expression and activity were next investigated. Calli were grown in reduced Fe levels and RNA was extracted after 6 and 8 weeks, and aconitase transcripts were detected using a radiolabeled probe. The analysis showed that the treatments did not result in any significant reduction in the aconitase mRNA levels after 3 and 6 weeks of incubation (Fig. 3A). Aconitase activity was assayed using isozyme gel electrophoresis, which enabled detecting the effect of treatments on the activity of each of the isozymes separately. The activity staining showed two isozymes in calli grown in 0.1 m*M* Fe, slow and fast migrating, displaying different intensity; the intensity of the slow-migrating isozyme was higher than that of the fast-migrating one. While no significant change in activity was detected in the fast-migrating isozyme under lower Fe concentrations, the slow-migrating isozyme was barely detectable in 0.02 and 0.03 m*M* Fe. The antibodies cross-reacted with a protein band that comigrated with

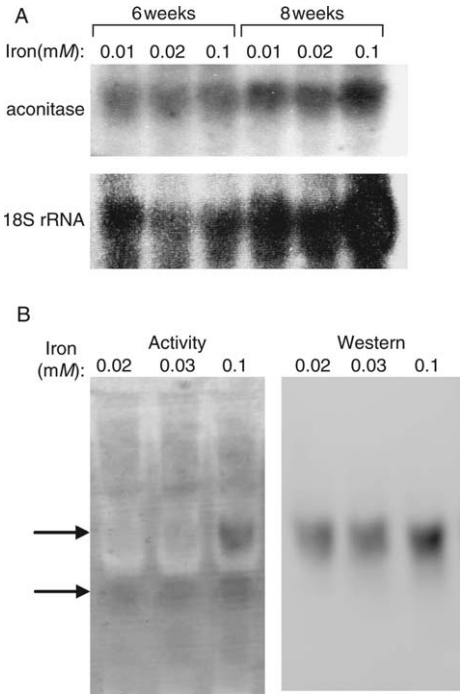

Fig. 3. Changes in the mRNA, protein and aconitase isozyme activity under elevated Fe concentrations. Calli were grown for the indicated times in various Fe concentrations, and the mRNA level of aconitase was analyzed using Northern blot analysis (A). Total protein extracts of calli grown for 6 weeks under the indicated Fe concentrations were separated in duplicates by non-denaturing PAGE. One half of the gel was stained for aconitase activity (B, left panel), and the other half was blotted and analyzed by Western blot analysis using anti-aconitase antibodies (B, right panel). The arrows indicate the position of the aconitase isozymes. Gels are representative of three independent experiments.

this isozyme, while no cross-reaction to the fast-migrating isozyme was detected (Fig. 3B). Some reduction at the protein level of the slow-migrating isozyme was seen between 0.1 m*M* and the lower Fe concentrations. However, this reduction was minor while compared with the change in the isozyme intensity.

To assess the cellular localization of the aconitase isozymes, calli were fractionated into soluble, membrane and mitochondrial fractions, and the proteins of each fraction were resolved by non-denaturing gels. One half of the gel was stained for aconitase activity (Fig. 4, left panel), while the other half was blotted and analyzed with antibodies (Fig. 4, right panel). The slow-migrating isozyme was solely localized into the soluble fraction, while the other isozyme was present mostly in the mitochondria fraction, although a faint band was also evident in the soluble fraction. No aconitase activity was detected in the membrane-associated fraction. The Western blot analysis confirmed that the only isozyme detected by the antibodies was localized

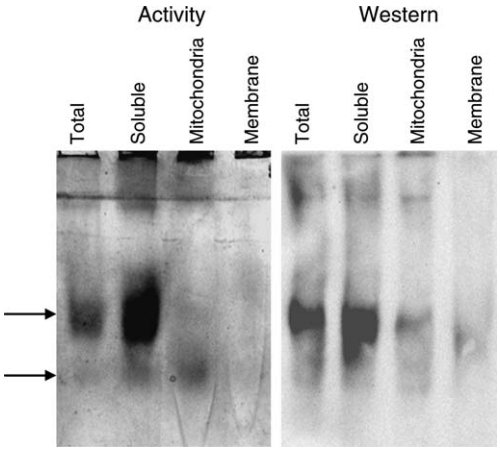

Fig. 4. The cytosolic form of aconitase is recognized by the antibodies. Calli were fractionated into soluble, membrane and organelle fractions, and mitochondria were purified from the organelle fraction. Samples of equal volumes (equivalent to 10 μg protein in the total extract) were separated in duplicates by non-denaturing PAGE. One half of the gel was stained for aconitase activity (left panel), and the other half was blotted and analyzed by Western blot analysis using anti-aconitase antibodies (right panel). The arrows indicate the position of the aconitase isozymes. Gels are representative of three independent experiments.

into the soluble fraction. Very faint signals were also detected in the membranes and mitochondria, resulting most likely from contamination.

The regulation of aconitase during citrus fruit development

The previous results suggested that Fe shortage did not affect the mRNA level of the cytosolic aconitase, slightly reduced its protein level and remarkably reduced its activity. Therefore, it was suggested that similar to animal cells, in citrus fruit Fe regulated the cytosolic aconitase at the post-transcriptional level. We have shown previously that the aconitase mRNA levels were constant during lemon fruit development, while the activity of

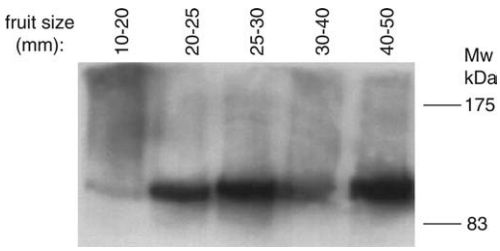

Fig. 5. The protein level of aconitase during lemon fruit development. Total proteins of fruits of different developmental stages, as indicated by their diameters, were analyzed by Western blot analysis using anti-aconitase antibodies. Blots are representative of four independent experiments.

the cytosolic enzyme was induced during the acid decline stage (Sadka et al. 2000b). Therefore, the level of regulation of aconitase throughout lemon fruit development was examined using antibodies in protein extracts following protein separation by denaturing gel electrophoresis (Fig. 5). A single band of approximately 95 kDa, the expected size of aconitase from the deduced amino acid sequence, was detected. In fruit of 10–20 mm in diameter, the protein was barely detectable, and it was greatly induced in 20–25 mm diameter fruits. Further induction was detected in 25–30 mm fruits, with some reduction in larger fruits of 30–40 mm diameter with a second induction peak in 40–50 mm diameter fruits. The constant levels of aconitase mRNA (Sadka et al. 2000b) together with the increase in the protein level detected between fruits of 10–20 mm diameter and 20–25 mm diameter indicate that aconitase was regulated at the post-transcriptional level early in fruit development.

Discussion

A survey performed in grapefruit trees grown in calcareous soils, displaying a typical, Fe deficiency, chlorosis phenotype, showed that the fruit juice Fe concentration was inversely related to the total acidity (Fig. 6). Although the linear relationships between juice Fe and total acidity were not strong, the results provide support to the notion that Fe shortage induces juice acidity. Because of the difficulties associated with the study of the relationships between Fe homeostasis and citric acid content in the field, as a result of the large variation in Fe contents among individual fruits (Fig. 6), we chose to work on isolated juice vesicles and calli. We have previously shown that juice cell calli might provide an ideal system to study organic acid metabolism for several reasons (Sadka et al. 2000a). Among these, similar to the fruit, citrate is the major organic acid accumulated in the calli;

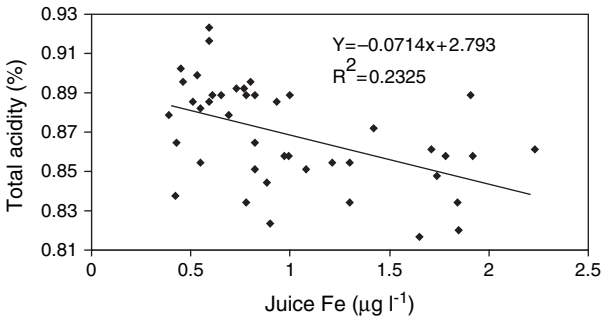

Fig. 6. The relationships between total acidity and Fe level in the juice. The total titratable acidity and the Fe level were measured in the juice of individual fruits (n = 50) of grapefruit grown in calcareous soil, showing typical Fe-chlorosis phenotype.

treatments that altered acid level in the fruit, such as arsenic compounds, acted similarly in the calli, having also similar effects on the expression of several tested genes. Our results supported the hypothesis that Fe-shortage-induced acid accumulation in citrus (Fig. 2B). It has been shown previously that juice vesicles can be maintained for months and even proliferate in situ by budding (Altman et al. 1982, Gulsen et al. 1981, Unger and Feng 1978). However, shortly following their isolation, the acid was released from the vesicles, resulting in the acidification of the media (not shown). Because the acid release stopped after a week, while it is reasonable to assume that citrate metabolism continued, the effect of Fe shortage became noticeable, i.e. acid levels were higher in vesicles incubated in Fe-free medium as compared with control vesicles, similar to what was shown in the calli. These results were in line with previous findings that showed the increase in the organic acid concentration, and citrate in particular, upon Fe starvation appeared not only in leaves and roots but also in the xylem sap and apoplastic fluids and were associated with a reduction in the xylem sap pH (Abadìa et al. 2002, Rombola et al. 2002). Similarly, fruits of Fe-deficient trees also exhibited higher values of organic acids than Fe-sufficient trees, leading to an increase in total acidity of the fruit pulp (Alvarez-Fernandez et al. 2003).

Abadìa et al. (2002) discussed possible reasons for the increase in organic acid concentrations upon Fe shortage. These included, (1) reduced aconitase activity, (2) an increase in phosphoenolpyruvate carboxylase (PEPC) activity, resulting in net carbon fluxes toward organic acid production and (3) increased CO_2 fixation from bicarbonate accompanied with increased PEPC activity in the root. We have suggested previously that Fe shortage reduced the activity of the cytosolic aconitase in citrus fruit, in particular, during the acid-decline induction of cytosolic aconitase, leading to a reduction of citrate turnover and concomitant increase in fruit juice acidity (Sadka et al. 2000b). Similar to animals and yeast, the plant aconitase could play a link between Fe shortage and organic acid accumulation, not necessarily through its RNA binding activity, but because of possible role(s) of Fe on the enzyme activity. Nevertheless, it should be noted that the available data in the literature regarding aconitase activity under Fe shortage is sometimes in contradiction. The aconitase activity from enzymes extracted from Fe-deficient mustard leaves was 50% of that extracted from Fe-sufficient leaves (Bacon et al. 1961), although the purified enzyme was not affected by Fe (Palmer 1963). Roots of Fe-deficient plants that showed increased citrate concentration displayed also reduced aconitase activity (McCluskey et al. 2004). In other cases, extracted aconitase activity decreased, increased or

remained unchanged under Fe deficiency (Reviewed in Abadìa et al. 2002). Noteworthy, in all these studies, aconitase activity was assayed spectroscopically in total protein extracts. Therefore, the specific activities were mean values of all the cellular forms and differential response of specific form(s) to Fe shortage could not be detected. Our finding showing that only one aconitase isozyme was sensitive to Fe shortage might contribute to explain the discrepancies found in the literature, as distinct enzyme forms might respond differentially, even within the same tissue. Although less likely, the possibility that the activity of some aconitase forms do not require Fe cannot be excluded at this stage. An alternative possibility is that the efficiency of Fe release from the active site cleft under limiting Fe varies between different isozymes.

The fractionation experiments along with the activity staining assays showed that the cytosolic aconitase activity was reduced under Fe shortage, while the other isozyme, most likely the mitochondrial aconitase, remained unaffected by Fe availability. Mitochondrial and cytosolic isoforms of aconitase were detected in all plant species analyzed so far, while oil-seed plants contain most likely also a glyoxisomal enzyme (Cots and Widmer 1999, De Bellis et al. 1993, 1995, Hayashi et al. 1995). Assuming that the activity-staining assays reflect the ratio between the two forms, our results would suggest that the cytosolic form of aconitase was more abundant than the mitochondrial form, as shown in pumpkin cotyledons (De Bellis et al. 1994). In animal cells, the mitochondrial and cytosolic aconitases display divergent sequences, while in plants different aconitase clones within the same plant species share high sequence similarity, thus complicating a sequence-based identification of the cytosolic and the mitochondrial genes. Citrus expressed sequence tags (ESTs) databases (http://harvest.ucr.edu/) contain over 225 000 ESTs, with more than 45 000 unigenes, among them several aconitase unigenes. Although some of these unigenes show a high level of similarity, others display some differences in the region used for the production of antibodies. This dissimilarity could explain the lack of cross-reactivity between the mitochondrial aconitase and the antibodies, as demonstrated also in other cases (De Bellis et al. 1994, Hayashi et al. 1995).

In summary, our data demonstrate the higher susceptibility of cytosolic aconitase and the lower susceptibility of mitochondrial enzyme to Fe shortage in citrus fruits. We suggest that the reduced activity of the cytosolic aconitase results in a reduced rate of citrate catabolism. This might explain the increase in pulp acidity of citrus fruit detected in trees grown under Fe-limiting conditions. In many citrus varieties, high acid content in mature fruits reduces quality or delays harvest. A practical question, arising from the above results, is whether fruit acidity can be further reduced following an Fe treatment during the citrate decline stage. Preliminary results in our group (Zur, L. Shlizerman, A. Sadka, The Volcani Center, Bet Dagan, in preparation) have shown that the application of Fe treatments during fruit maturation is capable of reducing the acid content of the fruit juice. Whether the enhanced rate of acid decline is because of Fe-treatment-induced cytosolic aconitase activity in the fruit is presently under investigation.

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