Mitochondrial dysfunction under Fe and S deficiency: is citric acid involved in the regulation of nutrient-responsive genes?

Highlights

- Single and combined Fe and S deficiency strongly affect mitochondrial respiration activities.
- Single and combined Fe and S deficiency differentially affect Krebs-related organic acids accumulation.
- The citric acid levels correlated with the expression of some Fe and S deficiency induced genes.
- The Fe- and S-dependent variation in citric acid content might be involved in the regulation of some genes.

Abstract

Within the last years, extensive information has been accumulated on the reciprocal influence between S and Fe nutrition at both physiological and molecular level in several plant species, but the mechanisms regulating S and Fe sensing and signaling are not fully understood. Fe and S interact for the building of Fe–S clusters, and mitochondria is one of the cellular compartments where Fe–S cluster assembly takes place. Therefore, it would be expected that mitochondria might play a central role in the regulation of Fe and S interaction. The Fe deficiency-induced alteration in the synthesis of mitochondria-derived carboxylic acids, such as citric acid, and the evidence that such molecules have already been identified as important players of metabolite signaling in several organisms, further support this hypothesis.

Tomato plants were grown under single or combined Fe and S deficiency with the aim of verifying whether mitochondria activities played a role in Fe/S interaction. Both Fe and S deficiencies determined similar alteration of respiratory chain activity: a general decrease of Fe-S containing complexes as well as an increase of alternative NAD(P)H activities was observed in both Fe and S deficient-plants. However, the content of Krebs cycle-related organic acids in roots was substantially different in response to treatments, being the accumulation of citric acid always increased, while the others (*i.e.* succinic, malic, fumaric acids) always decreased. Interestingly, citric acid levels significantly correlated with the expression of some Fe and S deficiency induced genes.

Our results contribute to existing knowledge on the complexity of the S/Fe interaction, suggesting a model in which endogenous alteration of citric acid content in plant tissues might act as signal molecule for the regulation of some nuclear-encoded and nutrient-responsive genes and also provide a basis for further study of the mechanism underlying S and Fe sensing and signalling.

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Keywords

Citric acidIronMitochondria*Solanum lycopersicum*Sulfur

1. Introduction

Recent studies have demonstrated a close relationship between sulfur (S) and iron (Fe) nutrition in tomato (Strategy I) plants, at both physiological and molecular levels, although the mechanisms integrating the homeostasis of these two elements are still unknown (Paolacci et al., 2014; Zuchi et al., 2009, 2015). Yet, this aspect is of great importance not only in relation to the recent occurrence of S deficiency and the common limited Fe availability in soils, but also because in many areas S and Fe deficiency likely occur simultaneously.

The importance of S in improving plants ability to cope with Fe deficiency is well documented (Zuchi et al., 2009); S deprivation has been shown to cause a significant drop in leaf Fe concentration as a consequence of the influence of this macronutrient on the main components of the Strategy I mechanism (Zuchi et al., 2009, 2015). In particular, in plants challenged by severe S deficiency no induction of Fe(III)-chelate reductase activity (as well as of the expression of the corresponding gene, *LeFRO1*) and ethylene production was observed. Differently, the extent of the induction of Fe2+ uptake rate and of *LeIRT1* over-expression, well demonstrated in Fe deficiency, were more limited in a context of S-shortage (Zuchi et al., 2009).
Furthermore, the orchestration of the response machinery to Fe deficiency is assumed to be associated with an adjustment of the S uptake rate (Paolacci et al., 2014; Zuchi et al., 2015). The management of S homeostasis is achieved by the up-regulation of the sulfate transporter genes belonging to Groups 1, 2 and 4 (among which plasma membrane and tonoplast S transporters), as it commonly occurs under S-deficient conditions (Paolacci et al., 2014; Zuchi et al., 2015). In addition, the higher accumulation of total S and thiols measured in tomato Fe-starved plants indicates that Fe deficiency is also associated with an increased assimilation rate of sulfate and, subsequently, with a modulation of the normal distribution of S-containing compounds within the plant (Paolacci et al., 2014; Zuchi et al., 2009, 2015).
In Fe-deficient Strategy I plants, ethylene and nicotianamine (NA) synthesis is increased (Li and Li, 2004; Molassiotis et al., 2005; Zuchi et al., 2009) with relevant consumption of methionine, which represents the precursor of both compounds (Hesse and Hoefgen, 2003). It was thus suggested that the regulation of S uptake by roots and root-to-shoot translocation rate might be necessary to meet the increased demand for methionine and its derivatives in response to Fe starvation (Paolacci et al., 2014; Zuchi et al., 2009, 2015). On the other hand, limited availability of S could impinge on the Fe metabolism for its impact on the synthesis or assemblage of Fe–S clusters (Balk and Schaedler, 2014). Taken together, these indications point to the need for a strict regulation and coordination of the balance of these two essential nutrients in plant tissues; nevertheless, it remains unclear to what extent changes in S homeostasis in Fe deficient plants are simply the consequence of a higher S demand, in order to sustain Strategy I machinery, or the result of a direct Fe interference with the signal transduction pathway involved in S metabolism. Recently, Forieri et al. (2017) deeply characterized the cross-regulation in Fe/S homeostasis in *Arabidopsis* plants, showing the occurrence of specific nutrient-deficiency responses signalling pathway and highlighting the complexity of the Fe/S interplay.
It has been suggested that regulation of the Fe/S interactions might potentially occur by modulating the Fe-S cluster biogenesis and relative abundance in response to various nutritional stresses (Balk and Schaedler, 2014). The Fe-S cluster biogenesis is an intricate process involving at least three different assembly machineries located in chloroplast (SUF machinery), mitochondrion (ISC machinery) and cytosol (CIA machinery) compartments, leading to an even more complex regulation of Fe and S interplay (Balk and Schaedler, 2014). Nonetheless, mitochondria have been proposed to play a key role in the modulation of Fe and S homeostasis (Vigani and Briat, 2016).
Indeed, Fe and S are required in great amount as in mitochondria several Fe-S cluster containing proteins are present; particularly, one single respiratory chain unit requires at least 10 different Fe-S clusters corresponding to approximately 30 Fe atoms and 30 S atoms (Vigani and Briat, 2016). A strong alteration of all respiratory complexes have been observed in Fe-deficient plants (Vigani et al., 2009, 2015, 2016) and, more recently, a strong decrease in respiratory activity has been described in S-deficient *Arabidopsis* plants (Ostaszewska et al., 2014). In addition, both Fe and S deficiency severely affect mitochondrial morphology (Vigani

et al., 2015; Ostaszewska et al., 2014), impairing most likely also mitochondria function. Nevertheless, the plasticity of the respiratory chain probably enables plants to survive and grow, in spite of the nutritional stress imposed by either Fe or S deprivation (Vigani and Briat, 2016). Schwarzländer et al. (2012) suggested that impairments in the respiratory chains might affect the expression of nuclear genes, highlighting the role of mitochondria in regulating nuclear gene expression through the retrograde signalling pathway. Through a meta-analysis approach, potential genes targeted by the retrograde signalling pathway induced by mitochondrial dysfunctions have been identified (Schwarzländer et al., 2012). Interestingly, a similarity between such genes and those known to respond to both Fe and S has been observed (Vigani and Briat, 2016). Indeed, being mitochondria impaired by both Fe and S deficiencies, it is reasonable to consider that these organelles could be a source of retrograde signals. However, little evidence explaining the contribution of mitochondria in the regulation of Fe and S deficiency responses is available (Vigani et al., 2016; Vigani and Briat, 2016).

Therefore, the aim of this study was to verify mitochondrial functions in response to both single and combined Fe and S deficiency in order to provide insight into the regulation of Fe and S deficiency-induced responses.

2. Materials and methods

2.1. Plant material

Tomato (*Solanum lycopersicum* L. cv. Gimar) plants were grown as described by Zuchi et al. (2015). Briefly, seeds were germinated in distilled water and, after germination, seedlings were grown for seven days in a full nutrient solution, containing 1.2 mM sulfate and 40 μM FeIII-EDTA. Half of the plants were then transferred for a further week to an S-free nutrient solution. Thereafter, half of the plants deriving from the two S growth conditions (+S and −S) were transferred to a Fe-free nutrient solution for three days. The resulting four different nutrient conditions were named as C (control condition, with sufficient availability of both S and Fe), F (Fe deficiency condition), S (S deficiency condition) and D (dual deficiency condition, with neither S nor Fe).

Nutrient solution was continuously aerated and changed every 2 days. Plants were grown in a growth chamber under 200 μmol photons m−2 s−1 PPF and 14h/10h day/night regime (27/20 °C day/night temperature cycling; 80% relative humidity). Plants were harvested 17 days after sowing.

2.2. ICP-OES analysis

Shoot tissues were oven-dried at 80 °C until constant weight was reached and thereafter digested with concentrated ultrapure HNO3 (65% v/v, Carlo Erba, Milano, Italy), using a Single Reaction Chamber (SRC) microwave digestion system (UltraWAVE, Milestone, Shelton, CT, USA). The elements concentration was subsequently analyzed by ICP-OES (Spectro Arcos, Spectro, Germany). Elements quantifications were carried out using certified multi-element standards (CPI International, https://cpiinternational.com). Tomato leaves (SRM 1573a) and spinach leaves (SRM 1547) have been used as external certified reference material.

2.3. Bioinformatic analyses

Plasma membrane (PM) H+-ATPases were identified in tomato genome on the basis of amino acid sequence similarity with orthologous genes from *Nicotiana* plumbaginifolia Viv., *Oryza sativa* L., *Arabidopsis* thaliana (L.) Heynh. (Arango et al., 2003), *Vitis vinifera* (Pii et al., 2014), *Fragaria vesca* (Valentinuzzi et al., 2015) and *Zea mays* L. (Pii et al., 2016a). The putative tomato PM H+-ATPases were identified through BLASTP (Altschul et al., 1997) research using the Phytozome version 12 database (https://phytozome.jgi.doe.gov/pz/portal.html). The similarity research was carried out by using each known protein as query and selecting the putative tomato proteins on the basis of the highest sequence homology value (≥80%). The

selected tomato proteins, as well as the protein sequences mentioned above, were used for a phylogenetic analysis, by aligning the sequences through ClustalW ver. 2.1 algorithm. Phylogenetic tree was built using the Phylogenetic Interference Package program (PHYLIP; University of Washington) and visualized by the FigTree ver. 1.4.2 software.

2.4. Extraction of total RNA, cDNA preparation and expression analyses

Total RNA was extracted from shoots and roots of control and treated (F, S and D) tomato plants as described by Zuchi et al. (2015). The resulting RNA was treated with RNase-free DNase I (Promega) according to the manufacturer's protocol. Following digestion, nucleotides were removed from RNA using a G50 Sepharose buffer exchange column (Amersham). RNA concentration and quality were checked using a NanoDrop ND-1000 spectrophotometer (Labtech, East Sussex, UK). The quality of RNA samples was further assessed by electrophoresis on 1% formaldehyde agarose gels. First-strand cDNA was synthesized from 1 µg of RNA by the M-MLV (H-) Reverse Transcriptase (Invitrogen) and the resulting cDNA was diluted 1/5 for quantitative real-time RT–PCR (qRT–PCR) analyses.

The expression of genes Solyv06g068660.2 (*SlCI1*), Solyc01g060070.2.1 (*SlOEP16-1*), Solyc04g040180.2 (*SlSAM-MT*), Solyc06g050980.2 (*SlFER1*) and Solyc07g017780.2.1 (*SlAHA2*) were analyzed by qRT–PCR. Briefly, gene-specific primers for both target and housekeeping genes have been designed (Supplemental Table 1). Real-time RT–PCR experiments were carried out in biological triplicates and the reaction was performed by using the SsoFast EvaGreen Supermix (Bio-Rad) as previously described (Pii et al., 2016b). The amplification efficiency was calculated from raw data using LinRegPCR software (Ramakers et al., 2003). The expression of genes were normalized using two housekeeping transcripts, namely Tomato *LeEF-1* mRNA for elongation factor 1 alpha (X14449.1) and Tomato *Ubi3* gene for ubiquitin (X58253.1), previously described (Zamboni et al., 2012). The relative expression ratio value was calculated for treated samples relative to the corresponding untreated sample at the same time-point according to the Pfaffl equation (Pfaffl, 2001). Standard error values were calculated according to Pfaffl et al. (2002).

2.5. Mitochondrial purification

Mitochondria were purified from tomato roots according to Vigani et al. (2009), with few modifications. Fresh root tissues were homogenized with a mortar and pestle in 0.4 M mannitol, 25 mM MOPS pH 7.8, 1 mM EGTA, 8 mM cysteine and 0.1% (w/v) bovine serum albumin (BSA). The filtered homogenized plant material was centrifuged 5 min at 4000 × g. The supernatant was further centrifuged for 15 min at 12,000 × g to pellet mitochondria. The crude mitochondrial pellet was then resuspended in Resuspension Buffer (RB: 0.4 M mannitol, 10 mM Tricine pH 7.2, 1 mM EGTA) and lightly homogenized with a potter; mitochondria were further purified on a 40, 28, and 13.5% (v/v) Percoll (Pharmacia) step gradient obtained in RB buffer. The fraction at the 28/40% interface was collected and washed by differential centrifugation in RB buffer.

2.6. Enzyme activity

Mitochondrial NADH dehydrogenase activities (Rotenone sensitive and Rotenone insensitive) were measured on intact mitochondria according to Rasmusson and Agius (2001), with a small modification in the buffer composition (0.4 M mannitol, 10 mM MOPS pH 7.2, 2.5 mM MgCl2, 0.1 mM EGTA, 0.4 mM carbonylcyanidep-(trifluoromethoxy)phenylhydrazone (FCCP)) as reported in Vigani and Zocchi (2010).

The activity of Complex IV (EC 1.9.3.1) was assayed by monitoring the oxidation of reduced cytochrome *c* at 550 nm at 38 °C according to Vigani et al. (2009).

The activity of *O*-acetylserine(thiol)lyase (OASTL; EC 4.2.99.8) activity was determined in root crude extracts by detecting cysteine production according to the method described by Celletti et al. (2016).

2.7. Plasma membrane-associated enzymes

Plasma membrane (PM) enriched vesicles were isolated from roots of tomato plants by following the small-scale procedure described by Astolfi et al. (2005) with minor changes. Briefly, fresh root tissue was homogenized in a pre-cooled mortar with 2.5–fold volumes of a freshly prepared medium containing 250 mM sucrose, 2 mM MgSO4, 25 mM BTP, 10 mM glyerol-1-phosphate, 2 mM EGTA, 2 mM EDTA, 10% (v/v) glycerol, 0.5% (w/v) BSA, 6% (w/v) choline-iodide, 1 mM PMSF, 2 mM ATP, 2 mM DTT, 1% (w/v) PVPP, titrated to pH 7.6 with MES. The homogenized tissue was filtered through four layers of cheesecloth and then centrifuged in a microcentrifuge at 13000 g for 3 min; the supernatant was recovered and centrifuged at 13000 g for 25 min to obtain a microsomal membrane pellet. The microsomes were gently resuspended in 0.4 ml of homogenization medium and loaded onto a discontinuous sucrose gradient made by layering 700 µl of sucrose solution (30%) onto 300 µl of sucrose solution (40%) cushion and centrifuged at 13000 g for 1 h. Vesicles banding at the 30–40% interface were collected, quickly frozen in N2 and stored at −80 °C until used. Vesicles obtained with the above-described procedure were enriched in PM vesicles as tested for each membrane preparation in experiments run in the presence of diagnostic inhibitors. The K+-stimulated ATPase activity was more than 70% inhibited by 100 µM vanadate, whereas the activity, measured at pH 8.0, was almost unaffected (about 10–15%) by 1 mM NaN3 or 100 mM KNO3. Inhibition by Na-molibdate was less than 15%.

ATP-hydrolyzing activity was measured by determining the release of inorganic phosphate according to Astolfi et al. (2005).

Proton accumulation into inside-out pm vesicles was measured as absorbance decrease of acridine orange spectrophotometrically at 492 nm, as decribed in Astolfi et al. (2005).

2.8. Statistical analyses

Statistical analyses were carried out using either Student's t-test or ANOVA using GraphPad Prism version 6.00 for Mac OS X, GraphPad Software, San Diego California USA. Multivariate analyses were carried out by using PAST 3.14 software for Mac OSX. The validity of the PCA models were assessed by the cross-validation approach previously described (Pii et al., 2015).

3. Results

3.1. Tomato plants differentially respond to single or combined Fe and S deficiencies

The shoot tissues of plants grown in control (C), Fe deficiency (F), S deficiency (S) and combined Fe and S deficiencies (D) conditions were analysed by ICP-OES in order to assess the nutritional status of tomato plants (Table 1). As expected, F plants showed a reduced Fe concentration as compared to C plants ($p < 0.001$); in agreement with previous evidence, F plants also showed an increased concentration of other divalent cations, as for instance copper (Cu; $p < 0.05$), manganese (Mn; $p < 0.05$) and zinc (Zn; $p < 0.0001$). On the other hand, S deficiency in plants caused a reduction in Fe ($p < 0.05$), Cu ($p < 0.05$) and Zn ($p < 0.05$) content (Table 1). Interestingly, the dual deficiency (D) resulted as a distinct nutritional status, characterized by the strong accumulation of several mineral elements, such as boron (B), barium (Ba), calcium (Ca), potassium (K), lithium (Li), magnesium (Mg), manganese (Mn), sodium (Na) and Zn, as compared to the other conditions considered in the experiment (C, F and S) (Table 1 and Fig. S1). In line with these observations, the principal component analysis (PCA) model obtained with the ionomic profile, explaining about 94.5% of the total variance, showed the separation of the samples into at least two distinct clusters long the first component (PC1): the first cluster encompassed C, F and S plants, whilst the second only D plants, highlighting that the dual deficiency represents a different physiological condition from both single S and Fe deficiency (Fig. S1).

Table 1. Mineral elements concentration in leaf of tomato plants subjected to the different nutritional regimes. The statistical significance was tested by means of ANOVA with Tukey post-test. Different letters indicates statistically different values.

	C			D			F			S			
	Mean	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		
mg gDW−1													
Al	0.137	0.013	a	0.188	0.005	b	0.113	0.015	a	0.128	0.025	a	p = 0.0041
B	0.760	0.197	a, b	1.094	0.060	b	0.751	0.070	a	0.742	0.140	a	p = 0.0269
Ca	1.995	0.381	a	4.600	0.496	b	2.860	0.216	a	2.395	0.350	a	p = 0.0001
Cu	0.021	0.002	a, b	0.025	0.003	b	0.023	0.002	a, b	0.018	0.001	a	p = 0.0258
Fe	0.064	0.002	b	0.041	0.002	a	0.039	0.006	a	0.054	0.005	b	p = 0.0002
K	22.107	3.187	a	30.475	2.970	b	25.292	0.277	a, b	22.564	2.190	a	p = 0.0106
Mg	0.892	0.162	a	2.719	0.305	b	1.395	0.308	b	1.094	0.136	a	p < 0.0001
Mn	0.041	0.013	a	0.121	0.027	b	0.068	0.006	a	0.045	0.011	b	p = 0.0011
Na	2.856	0.373	a	4.037	0.151	b	2.881	0.660	a	2.858	0.268	a	p = 0.0179
Sr	0.026	0.026	a	0.035	0.035	a	0.034	0.034	a	0.027	0.027	a	p = 0.9846
Zn	0.056	0.007	a	0.189	0.043	b	0.203	0.003	b	0.046	0.007	a	p < 0.0001
µg gDW−1													
Ba	2.551	0.565	a	5.573	0.511	b	2.221	1.251	a	3.560	0.513	a	p = 0.0030
Cd	0.166	0.025	a	0.410	0.164	a	0.209	0.152	a	0.212	0.067	a	p = 0.1259
Co	0.015	0.007	a	0.020	0.000	a	0.021	0.010	a	0.015	0.014	a	p = 0.7939
Cr	0.217	0.018	a	0.211	0.045	a	0.196	0.017	a	0.226	0.027	a	p = 0.6517
Li	3.931	0.931	a	5.934	0.305	b	3.577	0.537	a	3.181	0.350	a	p = 0.0019
Ni	0.885	0.456	a	0.323	0.051	a	0.513	0.080	a	0.528	0.049	a	p = 0.0946

It has been previously demonstrated that D treatment differs from both single deficiencies at physiological, molecular and metabolomic level (Zuchi et al., 2015). Results obtained by testing the activities of H+-ATPase and the OASTL enzyme (Fe- and S-responsive enzyme, respectively) in tomato roots are clearly in agreement with this observation (Fig. 1).

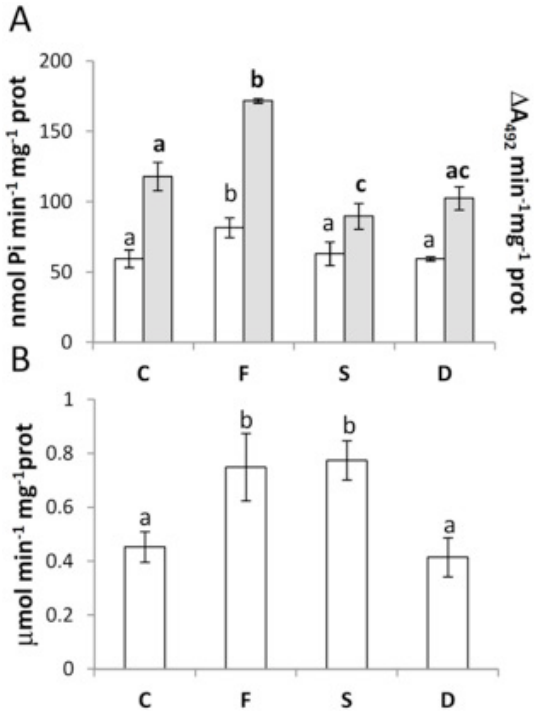

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Fig. 1. Changes in H+ATPase activity (**a**) (white bars indicate ATP hydrolysis activity and grey bars proton transport activity) and OASTL activity (**b**) in roots of tomato plants subjected to different treatments (C = control, F = Fe deficiency, S = S deficiency, D = dual deficiency). Data are means ± SD of four independent replications run in triplicate. Significant differences between samples are indicated by different letters ($P < 0.05$).

ATP hydrolysis (µmol Pi min−1 mg−1 prot) and H+ transport (ΔA492 min−1 mg−1 prot) activity were studied in plasma membrane-enriched vesicles isolated from roots of tomato plants. Iron withdrawal from the nutrient solution substantially stimulated both ATPase and Mg:ATP-dependent proton transport activity (37% and 45% increase, respectively) (Fig. 1A). A different trend was observed when plants are deprived of S: ATPase activity was not affected whereas the initial rate of H+ accumulation was significantly inhibited (24% decrease) (Fig. 1A). Finally, the exposure of tomato seedlings to the combined deficiency of S and Fe (D condition) did not significantly affect both activities (Fig. 1A).

As expected, the removal of S from the nutrient solution significantly stimulated OASTL activity (+70% with respect to the control) in roots of tomato seedlings (Fig. 1B). Interestingly, an increase of 65% in OASTL activity was observed also in roots from plants exposed to complete Fe deficiency (Fig. 1B). However, the imposition of the dual deficiency of Fe and S resulted in OASTL activity values similar to those of control plants (Fig. 1B).

3.2. Fe and S deficiencies similarly affect respiratory chain activity

In order to investigate the comparative effect of Fe and S deficiencies we focused on mitochondria, as they represent an important cell compartment where Fe and S interplay take place. Firstly, the respiratory activity was tested by determining the O2 consumption rate *in vivo* at root tips levels of plant grown under control (C), Fe deficiency (F), S deficiency (S) and combined Fe and S deficiencies (D). All the nutritional stress conditions affect initial O2 consumption rate (IR) suggesting that the low Fe and S availability limit the O2 consumption of tomato roots (Fig. 2A). After the addition of both KCN (inhibitor of complex IV activity) and SHAM (inhibitor of alternative oxidase activity), the O2 consumption rate strongly decreased in tomato roots under all treatments imposed, indicating that the observed variation of root O2 consumption was attributable to the respiration activity (Fig. 2A).

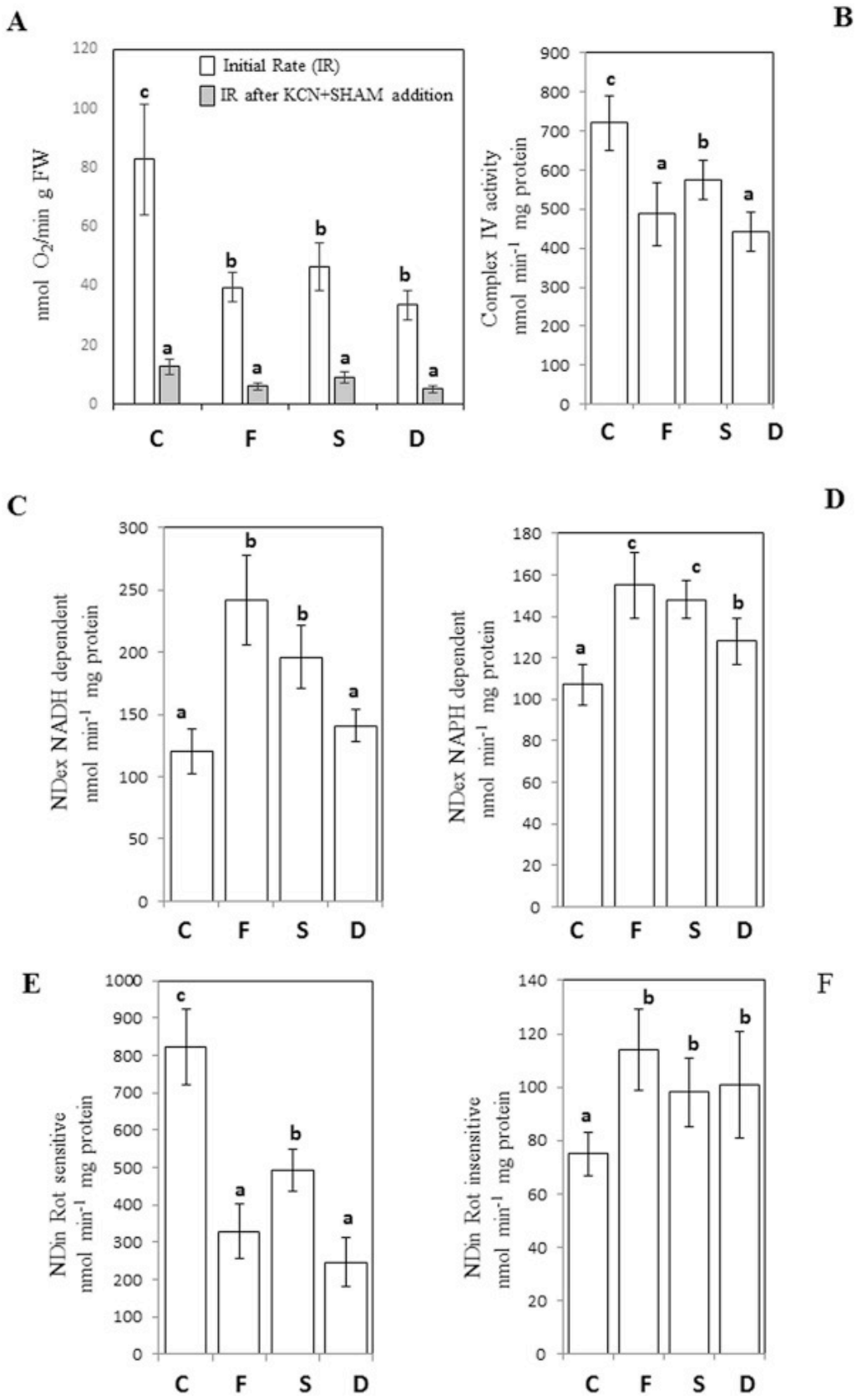

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Fig. 2. Changes in respiratory chain activity in tomato plants subjected to different treatments (C = control, F = Fe deficiency, S = S deficiency, D = dual deficiency). **a**) *In vivo* analysis of O2 consumption rate in tomato root tips. **b**) Complex IV activity on purified mitochondria. **c**)

External dehydrogenase activity NADH dependent, **d**) External dehydrogenase activity NADPH dependent; **e**) Internal NADH dehydrogenase rotenone-sensitive (complex I) and **f**) rotenone insensitive (alternative NADH dehydrogenase) on purified mitochondria from tomato roots. The values reported are means ± SD of three independent replications). Significant differences between samples are indicated by different letters ($P < 0.05$).

To gain a further insight on the *in vivo* observation, specific enzymatic activities of the respiratory chain were determined using a purified mitochondrial fraction obtained from roots of plants exposed to C, F, S and D treatments. In particular, the activity of complex IV, the external NADH-dependent dehydrogenase (NDex NADH dependent), the external NADPH-dependent dehydrogenase (NDex NADPH dependent), the NADH dehydrogenase activity rotenone-sensitive [complex I-mediated activity, NDin Rot sentive)] and the NADH dehydrogenase activity mediated by the type II alternative NAD(P)H dehydrogenases [internal NADH dehydrogenase rotenone-insensitive (NDin Rot insentive)] were determined (Fig. 2B–F).

Under F, S and D treatments, the activities of both NDin Rot sensitive (Complex I) and complex IV were significantly decreased (Fig. 2B, E), whereas a significant increase of alternative NADH dehydrogenase activity was observed with respect to C plants (Fig. 2C, D, F). In particular, NDex NADH dependent activity was significantly induced under F and S treatment (Fig. 2C), while NADPH-dependent activity was significantly induced in all stressed plants (Fig. 2D, F). However, as expected, both Fe and S deficiency similarly impaired the respiratory chain activity.

3.3. Fe and S deficiencies differentially affect Krebs cycle-related activities

Fig. 3A reports the relative changes of selected organic acids (*i.e.* citric, succinic, malic and fumaric acids) belonging to the Krebs cycle; the data have been re-elaborated from a previously obtained dataset (see Fig. 3 legend). In particular, the data measured in both roots and shoots of plants grown under F, S and D were referred to the C treatments, in order calculate the variation magnitude in the relative organic acids concentration (Fig. 3A).

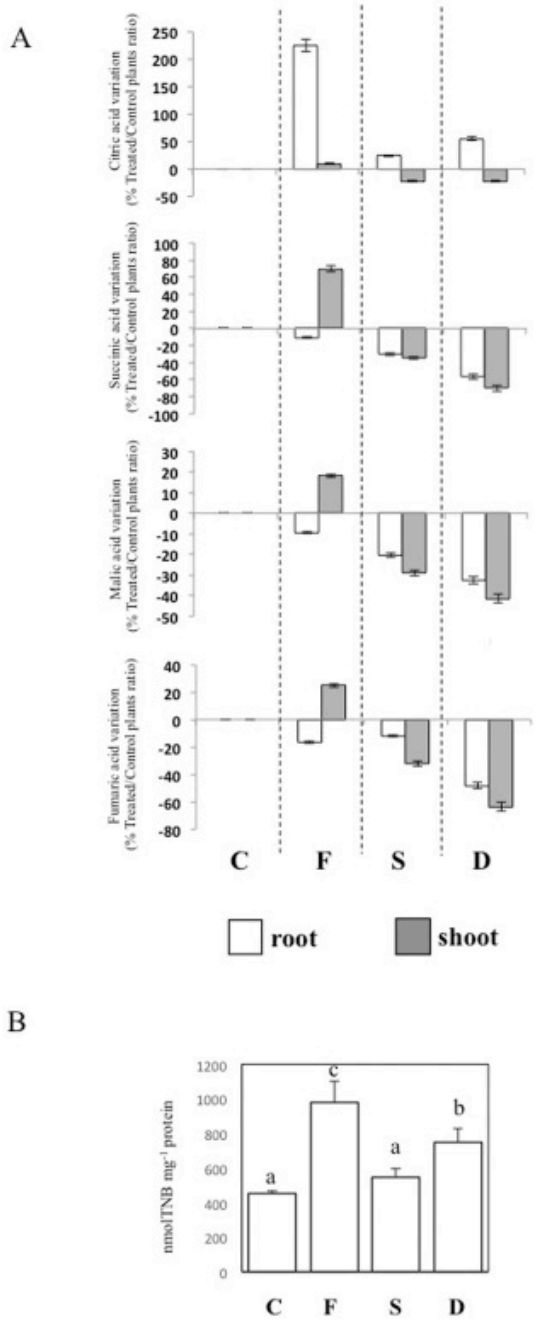

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Fig. 3. Changes in Krebs cycle-related pathways in tomato plants subjected to different treatments (C = control, F = Fe deficiency, S = S deficiency, D = dual deficiency). **a**) TCA-related carboxylic acids content in tomato roots and shoots (data reported in percent changes respect to control were re-elaborated from previous experiments where the original datasets were available (Zuchi et al., 2015)]. **b**) Citrate synthase activity determined on purified mitochondria from tomato roots.

The most significant change in the relative concentration was observed for citric acid in root tissue. Indeed, citric acid concentration increased by about 250% in root of Fe-deficient plants respect to the control (Fig. 3A). The relative citrate concentration was also increased in both S and D roots as compared to C, even though to a lower extent with respect to F roots (20% and 60%, respectively) (Fig. 3A). Accordingly, the citrate synthase activity determined on mitochondrial purified fraction from roots significantly increased only in F- and D-treated plants (Fig. 3B). Furthermore, roots of F-treated plants showed a decrease of succinic, malic

and fumaric acid content by about −11%, −9% and −13% respectively when compared with the C plants. In shoot of F-treated plants, succinic, malic and fumaric acid increased by about 70%, 19% and 25% respectively when compared with the C plants. Plants from S condition showed lower levels of succinic, malic and fumaric acid in both roots (−31%, −20%, −6%) and shoots (−38%, −29%, −32%). Similarly to S plants, also D plants showed a decreased content of succinic, malic and fumaric acid in both roots (−57%, −33%, −44%) and shoot (−69%, −41%, −64%) when compared with C plants. Interestingly, citric acid concentration correlated with the concentration of succinic acid, fumaric acid and malic acid concentration only at the shoot levels (Table 2), suggesting that in the root the level of citrate might be modulated regardless of the level of other organic acids.

Table 2. Pearson's correlation coefficients between the variation of the citrate concentration and the concentration of the succinate, fumarate and malate (*, $p < 0.05$; **, $p < 0.01$).

	Succinate		**Fumarate**		**Malate**	
	Root	**Leaves**	**Root**	**Leaves**	**Root**	**Leaves**
Citrate	0.2848	0.9545*	0,1242	0.9522*	0.6046	0.9776*

3.4. Expression of putative citrate-induced genes in tomato plants

To gain a deeper insight in the possible role of citrate in the mitochondria-mediated response to single and combined nutrient(s) deficiency, we have investigated the transcriptional modulation of citrate-regulated genes. A recent study suggested that in *Arabidopsis* plants a set of genes, namely *citrate-induced1* (*CI1, At1g73120*), *ferritin* 1 (*FER1, At5g01600*), *outer-envelope proteins16-1* (*OEP16-1, At2g28900*) and SAM-dependent *methyltransferase* (*SAM-MT, At2g41380*), are responsive to exogenously-applied citrate (Finkemeier et al., 2013). On these bases, the orthologous genes of *CI1*, *FER1*, *SAM-MT* and *OEP16-1* were identified in tomato genome by means of amino acid sequence similarity (Table 3). Additionally, we extend such analysis also to plasma membrane H+-ATPase (PM H+-ATPase) *AHA2* gene that was shown to be induced citrate in *Arabidopsis* plants (Finkemeier et al., 2013); tomato genome was found to contain 11 transcripts putatively encoding PM H+-ATPase (*Solyc03g117150.2.1, Solyc07g017780.2.1, Solyc00g257110.2.1, Solyc06g071100.2.1, Solyc12g010360.1.1, Solyc08g078200.1.1, Solyc02g088060.1.1, Solyc10g079300.1.1, Solyc04g016260.2.1, Solyc01g096190.2.1, Solyc09g082870.1.1*), which were aligned to generate a phylogenetic tree (Fig. S2). The PM H+-ATPase transcript encoded in the tomato genome, most closely related to AHA2 from *A. thaliana,* was *Solyc07g017780.2.1*, displaying 87% of identity and 96% of similarity in the amino acid sequence (Fig. S2).

Table 3. Nomenclature and accession number of the genes analysed in this study.

Gene ID	**Accession A. *thaliana***	**Accession *Solanum lycopersicon***
Citrate Induced 1	At1g73120	Solyc06g068660.2
Outer Envelope Protein 16-1	At2g28900	Solyc01g060070.2.1
SAM-MT	At2g41380	Solyc04g040180.2
Ferritin	At5g01600	Solyc06g050980.2
AHA2	At4g30190	Solyc07g017780.2.1

The expression analyses of *Solyc06g068660.2* (orthologous to *CI1* and hereafter referred to as *SlCI1*), *Solyc01g060070.2.1* (orthologous to *OEP16-1* and hereafter referred to as *SlOEP16-1*), *Solyc04g040180.2* (orthologous to *SAM-MT*, and hereafter referred to as *SlSAM-MT*), *Solyc06g050980.2* (orthologous to *FER1*, and hereafter referred to as *SlFER1*) and *Solyc07g017780.2.1* (orthologous to *AHA2*, and hereafter referred to as *SlAHA2*) in the different tissues and in the different growth conditions were performed by qRT-PCR (Fig. 4).

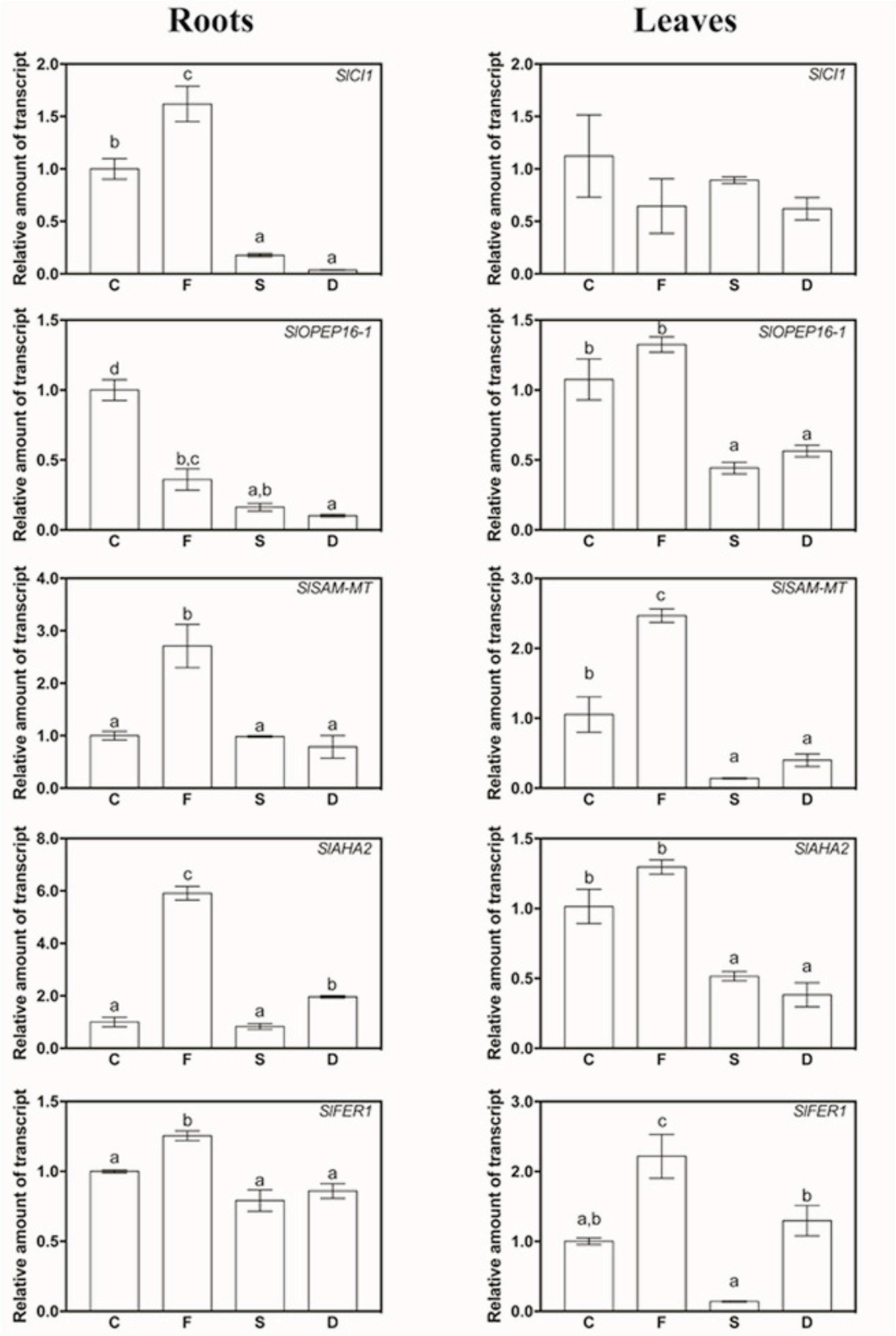

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Fig. 4. Relative expression rate of citrate-induced genes in tomato root and leaves. The expression levels of *SlCI1*, *SlOEP16-1*, *SlSAM-MT*, *SlFER1*, *SlAHA2* were assessed by quantitative reverse transcription– PCR. The data were normalised to two internal controls,

Tomato *LeEF-1* mRNA for elongation factor 1 alpha and Tomato *ubi3* gene for ubiquitin. The relative expression ratios were calculated using C plants as a calibrator sample. The values reported are means ± SE (n = at least 3). The statistical significance was tested by means of ANOVA with Tukey post-test. Different letters indicates statistically different values ($P < 0.001$).

At root level, the expression of *SlCI1, SlSAM-MT, SlFER1* and *SlAHA2* genes was higher under Fe deficiency, even though *SlFER1* mRNA levels displayed only a slight increase respect to control plants (Fig. 4). Both *SlSAM-MT* and *SlFER1* did not display any significant variation in S and D conditions as compared to C, whilst *SlAHA2* was upregulated in D plants (Fig. 5). The expression levels of *SlCI1* were strongly downregulated by about 73 and 96% in S and D plants, respectively. Differently from the other genes analysed, the expression of *SlOEP16-1* showed a decreasing trend, being reduced by about 64, 84 and 90% in the F, S and D treatments, respectively, as compared to control plants (Fig. 4). To assess the possible role of citric acid in inducing the expression of these genes in tomato plants, a correlation analysis between the relative amount of transcript (Fig. 4) and the concentration of citrate in the root tissue (Fig. 3A) in the different growth conditions was carried out. As shown in Table 4, the relative expression of *SlCI1, SlSAM-MT, SlFER1* and *SlAHA2* showed a positive and statistically significant correlation with the variation in the citrate concentration in roots, showing, at least in the case of *SlAHA2,* a dose response correlation (Figs. 3A and 4). In the root tissue, the modulation of *SlOEP16-1* appeared to be independent from the intracellular concentration of citrate (Table 4).

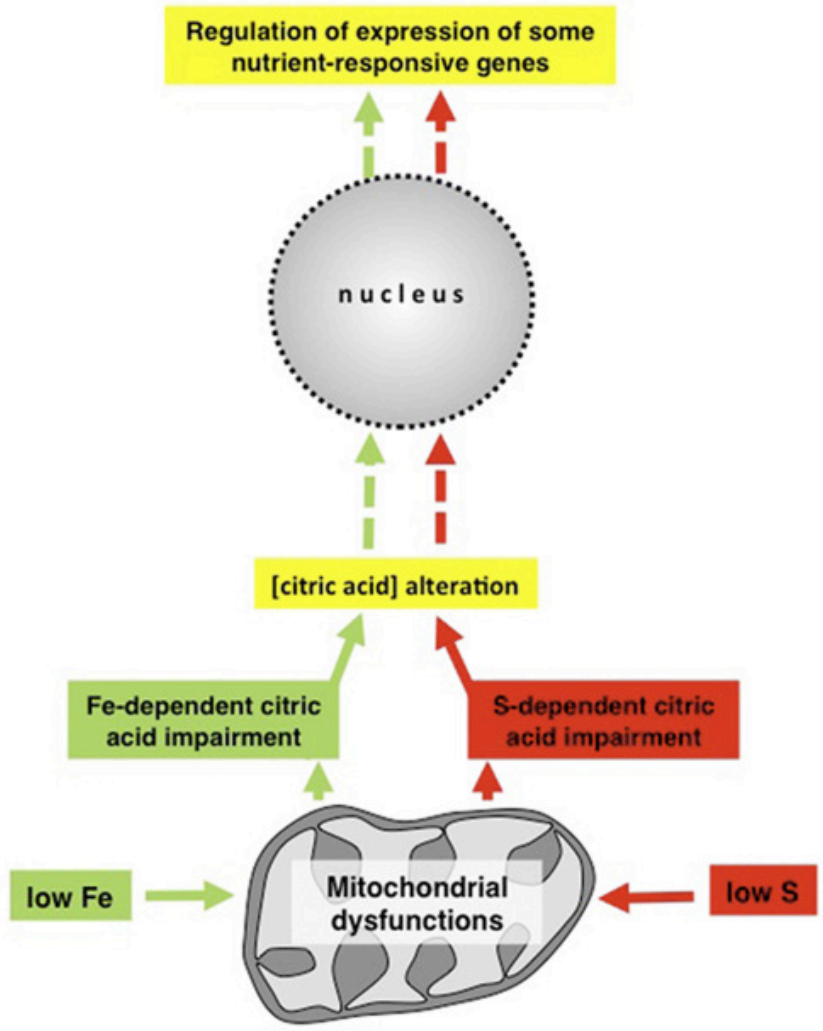

Fig. 5. Proposed model of the potential role of citrate in the regulation of some genes under Fe and S deficiencies in tomato plants. Here we present again the model, previously proposed by Vigani and Briat (2016), providing evidences about the role of citric acid as a signal molecule for a still unknown mitochondria retrograde signalling pathway. Iron (Fe) and Sulfur (S) deficiencies similarly affect mitochondrial functions, but they differently affect citric acid contents (reported as [citric acid]). Such variations might be perceived in the nucleus and thereby might be involved in the regulation of some nutrient-responsive genes (*SlCI1*, *SlOEP16-1*, *SlSAM-MT*, *SlFER1*, *SlAHA2)*. Dotted lines indicate hypothetical retrograde pathways involving citric acid as signal molecule.

Table 4. Pearson's correlation coefficients between the variation of the citrate, succinate, fumarate and malate concentration and the relative amount of transcript of target genes (*, $p < 0.05$; **, $p < 0.01$).

Relative amount of transcripts	**Citric acid**		**Succininc acid**		**Fumaric acid**		**Malic acid**	
	Root	**Leaves**	**Root**	**Leaves**	**Root**	**Leaves**	**Root**	**Leaves**
SlAHA2	0.9978**	0.9768*	0.2848	0.9939**	0.1232	0.9364*	0.6046	0.9449*
SlCI1	0.9018*	0.2389	0.4591	0.1661	0.2439	0.4749	0.6945	0.4310
SlFER1	0.9707*	0.6472	−0.0001	0.5946	0.1594	0.3916	0.3540	0.4723
SlOPEP16-1	0.1046	0.9800*	0.9469*	0.9364*	0.8022	0.8768	0.7193	0.9161*
SlSAM-MT	0.9656*	0.8737	0.2190	0.9023*	0.1293	0.7349	0.5919	0.7662

To further investigate the role of the above-mentioned genes in regulating plant responses to either Fe, S or the combined deficiency (D), their transcriptional regulation was determined also in the shoot tissue of tomato plants (Fig. 4). The expression of *SlCI1* did not show any significant difference among the treatment (Fig. 4), whilst *SlAHA2* and *SlOEP16-1* were significantly downregulated in both S and D conditions as compared to C plants (Fig. 4). On the other hand, the transcript levels of *SlSAM-MT* and *SlFER1* were significantly upregulated in Fe deficiency conditions (F) and reduced in both S and D plants as compared to control ones (Fig. 4). The assessment of the correlation between relative amount of transcript (Fig. 4) and the concentration of citrate in the shoot tissue (Fig. 3A) showed that only the expression of *SlAHA2* and *SlOEP16-1* might be putatively regulated by the increased concentration of citrate (Table 4). However, the expression of *SlAHA2, SlOPEP16-1* and *SlSAM-MT* genes might depend on the concentration of the other organic acids in shoot tissues: *SlAHA2* expression depend on succinate, fumarate and malate contents; *SlOPEP16-1* expression depend on succinate and malate while *SlSAM-MT* expression depend on succinate concentration.

Additionally a correlation analysis was carried out among the citrate contents and some specific genes involved in the uptake and translocation of Fe (*SlFER, SlFRO, SlIRT1*) and S (*SlST1.1, SlST1.2; SlST2.1, SlST2.2, SlST4,1*) (Table S2). At the root level, the expression of such genes did not correlate with citrate contents, whilst, in the shoot, only the S transporters negatively correlated with the citrate contents (Table S2).

4. Discussion

In most agricultural soils it is unlikely that plants are exposed to a single nutritional disorder, whereas multiple and combined nutritional stresses are more likely to occur. Notwithstanding, so far most studies have mainly characterized the effects of single nutrient deficiencies on plants.

The combined S/Fe deficiency has been recently studied and it was shown that responses of plants subjected to a double-deficiency are different from those activated when subjected to the shortage of a single element at the time, at both molecular and metabolic level (Zuchi et al., 2015). For these reasons, research focusing on multiple nutrient deficiency conditions,

likely occurring under field scale, is urgently required in order to get more detailed information on how plants respond to this type of nutritional stress. In fact, these pieces of information are pivotal to set up agronomic practices ensuring an equilibrate growth of the crop and its productivity, in particular when marginal lands are considered.
The data reported in Fig. 1, showing changes in H+-ATPase and OASTL activity of tomato plants in response to single and combined S and Fe deficiencies, are coherent with previous statements. The acidification of rhizosphere through the secretion of protons (H+) operated by the plasma membrane (PM) H+-ATPase is an essential mechanism of Strategy I to cope with Fe-deficiency (Kabir et al., 2012; Colangelo and Guerinot, 2004); consistently, our data showed that the maximum enzyme activity was found in roots of plants exposed to the sole Fe deficiency. Nonetheless, when the plants were exposed to dual (concurrent Fe and S) deficiency, the increase of activity observed before was completely lost. The OASTL activity, a hallmark of S deficiency response, was even more surprising: compared with control condition (C), both single deficiencies promoted the enzyme activity, but the combined deficiency (D) suppressed it. These evidences clearly confirm that plants responses to combined nutritional shortages are unique and cannot be deduced from single stress experiments.
Along with the data already reported in literature, such results highlight the complexity of the interplay occurring between Fe and S homeostasis in plants. Unravelling the interaction between Fe and S in plant is of high priority since both nutrients are essential cofactors for several crucial processes for plant life. Indeed, protein complexes catalysing photosynthesis and respiration require an elevate number of Fe-S clusters. Despite the progress obtained so far, the mechanisms regulating S and Fe sensing and signalling are not fully understood. Considering the strong request of Fe-S cluster proteins, few reports suggested that chloroplast and mitochondria might be involved in such mechanisms (Vigani et al., 2013; Vigani and Briat, 2016). Particularly, it has been hypothesized that the impairment of mitochondrial function observed under Fe deficiency in cucumber plant and under S deficiency in *Arabidopsis* might be source of signals responsible of regulation of Fe- and S-responsive genes.
In this context, we aimed at comparing the effects of Fe and S deficiency on the mitochondrial functionality in tomato plants. Interestingly, we observed that these nutrient deficiencies similarly affect the functionality of the respiratory chain, by decreasing the activities of both complexes I and IV (and more in general the mitochondrial O2 consumption rate) and by inducing that of the alternative NADH dehydrogenases. However, low Fe availability seems to have a stronger impact on the mitochondrial respiration rather than S deficiency (Fig. 2). Overall, such results are in agreement with the observation obtained in Fe-deficient cucumber mitochondria and in S-deficient *Arabidopsis* mitochondria (Vigani et al., 2009; Vigani and Zocchi, 2010; Ostaszewska et al., 2014).
It has been hypothesized that the mitochondrial dysfunction might be source of retrograde signals in cells (Schwarzländer et al., 2012) and it was found that both Fe and S deficiency strongly affect mitochondrial function. Despite being the observed alterations of the respiratory chain activity similar in F and S plants (Fig. 2), those affecting the Krebs cycle-related activity (*i.e.* relative concentration of organic acids) were different among the treatments (Fig. 3). Interestingly, citric acid was the only carboxylic acid accumulated in the root tissues of treated plants as compared to control ones and showed the highest concentrations in the Fe-deficient roots. Accordingly, the citrate synthase activity determined on the mitochondrial fraction purified from roots strongly increased under single Fe deficiency and under combined deficiency (D). Therefore, it is reasonable to suggest that signals addressed to specifically regulate Fe and S-responsive genes might come from an

impaired Krebs cycle rather than an impaired respiratory chain. Indeed, carboxylic acids have already been identified as important players of cellular signalling in several organisms, as they can be exported from or imported into mitochondria or other cellular compartments (McCammon et al., 2003; Wellen et al., 2009; Yang et al., 2012; Meyer et al., 2010).
Recently, it has been demonstrated that the exogenous application of relatively high concentrations of organic acids (1 mM), and in particular of citric acid, can impact the expression of some nuclear-encoded genes in *Arabidopsis*, as for instance *CI1, SAM-MT, FER1, OEP 16-1* and *AHA2* (Finkemeier et al., 2013). Such pieces of evidence suggest that, in *Arabidopsis,* the abundances of citric acid could be perceived and translated into a signal by still unknown pathway(s). Interestingly, the genes *CI1, AHA2, SAM-MT, FER1* are strictly linked to the Fe homeostasis responses (Li and Lan, 2017). In particular, *CI1* has been considered to be robustly regulated by FIT (FER_LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR) (Mai et al., 2016). Indeed, the mRNA abundance of *CI1* showed a 0.67 and a 2.64 fold increase in the FIT-overexpressing line HA-FIT when compared with wild-type plants, under Fe sufficiency and Fe deficiency conditions, respectively (Mai et al., 2016). Moreover, the expression of *CI1* showed a 3.14 fold increase in Fe-deficient wild-type plants when compared control ones (Mai et al., 2016). These pieces of evidence further indicate that, along with FIT, other factors related to the Fe deficiency might affect the regulation of *CI1* gene.
In this work, the orthologous genes of *CI1, SAM-MT, FER1, OEP 16-1* and *AHA2* were identified in tomato genome and their expression was quantified in both roots and shoots of C, F-, S- and D-treated plants. At the root level, the expression of *SlCI1, SlSAM-MT,* and *SlAHA2* genes increased in F plants (Fig. 4), which also strongly accumulated citric acid (Fig. 3). Although FER1 gene is typically down regulated under Fe deficiency, its orthologous gene *SlFER1,* considered in this work, showed a slight increase expression in F. The Fe content of F-treated tomato plants decreased by about 40% respect to C treatment, indicating that, likely, the degree of Fe deficiency imposed did not repress the expression of FER1 at this stage. On the other hand, the *SlFER1* orthologous gene in *A. thaliana, At5g01600*, showed a consistent expression induction upon exogenous application of citrate (Finkemeier et al., 2013). On the whole, the expression of these genes showed a positive correlation with the increase of citric acid concentration (Table 4). In *Arabidopsis OEP16-1* gene was considered as a citrate-specific down-regulated marker gene (Finkemeier et al., 2013). Accordingly, in tomato roots *SlOEP16-1* was down-regulated in those conditions (F, S and D) that induced a significant accumulation of citric acid in comparison to control plants. Overall, these results might suggest that the expression of *SlCI1, SlSAM-MT, SlFER1* and *SlAHA2* could be linked to the physiological fluctuation of citric acid concentration in the cell. To provide more insights on the possible role of citric acid as regulator of gene expression, we determined the expression of the selected genes in tomato shoots. Only the expression of *SlAHA2* and *SlOEP16-1* genes showed a correlation with the level of citric acid concentration, suggesting that most likely the regulation of genes expression in leaf might be even more complex. Additionally, the lack of correlation between citric acid content and *SlFER, SlFRO, SlIRT1, SlST1.1, SlST1.2; SlST2.2, SlST2.2, SlST2,1* genes (Table S2) in roots suggests that citric acid might not act as signal molecule for the regulation of genes involved in the uptake and translocation of Fe and S in tomato plants.
Importantly, while in *Arabidopsis* plants the role of citric acid as signal molecule has been demonstrated by exogenous application (Finkemeier et al., 2013), here we suggest that an endogenous variation in citric acid concentration might be involved in the regulation of expression of target nuclear genes.
In mammal cells, the citric acid exported from mitochondria was already shown to mediate histone acetylation to specifically regulate the expression of glycolysis-related genes in liver

cells (Wellen et al., 2009) and a specific role for citric acid in metabolite signalling was shown for Fe di-citrate (Gomez et al., 2010). Hence, citric acid might indeed have specific roles in signalling that differ from other carboxylic acids, especially due to its high affinity for divalent cations such as Fe or even calcium (for review, see Schwarzländer and Finkemeier, 2013). Consistently, Zamboni et al. (2016) reported that the supplementation of Fe-deficient tomato plants with Fe-citrate specifically affects the expression of genes belonging to protein synthesis, oxidative stress and cell wall-related metabolisms in the roots; the same functional categories were also specifically regulated by mitochondrial dysfunctions (Schwarzländer et al., 2012). Indeed, when Fe is supplied as Fe-citrate, such organic acid might be taken up by roots (Bell et al., 2003), suggesting that exogenous citric acid supply could act as elicitor for the expression of target genes belonging to the mitochondrial retrograde signalling pathway in tomato plants. In addition, previous studies also highlighted the possible involvement of gibberellin (GA) in the citrate-mediated modulation of genes expression, most likely regulating the abundance of members of the DELLA proteins family (Finkemeier et al., 2013; Zamboni et al., 2016). Despite these suggestions, specific evidence about a possible citrate-mediated signalling pathways as well as mechanism by which the abundance of key metabolites such as citric acid is perceived and signalled within the cell is still lacking.
On the basis of our and others' observations, we hereby propose a model in which the alteration of carboxylic acid content observed under nutrient deficiency might impact the regulation of nutrient-responsive genes (Fig. 5), with citric acid possibly playing a role as signal molecule originating from mitochondria.

5. Conclusions

Our results contribute to existing knowledge on the complexity of the S/Fe interaction showing alterations of mitochondrial activities in tomato plants as a result of both single and combined Fe and S deficiency. Such impaired metabolic status of mitochondria might produce specific and non-specific signals. Therefore, the specific regulation of Fe- and S-responsive genes might result from integrated signalling pathways, involving both specific and non-specific mitochondria-derived molecules. The most convincing hypothesis emerging from our findings suggests a model in which the alteration of endogenous citric acid content in plant tissues induced by changing Fe and S supply might be part of such integrated signalling pathways, for the regulation of some nuclear-encoded genes. Although a specific citric-acid mediated signalling pathway has not been characterized yet, this work provides a basis for further study of the mechanism underlying S and Fe sensing and signalling.

Authors' contributions

GV conceived the idea; GV, SA and YP planned the experimental approach: SA and SiC performed the biochemical characterization of tomato plants. GV and MM performed biochemical characterization of mitochondrial fraction from tomato root. YP performed gene expression and statistical analyses. GV, SA, YP, TM and StC discussed the data. GV and SA wrote the manuscript with the contributions of YP, TM, StC to the various drafts.

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