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Differential modulation of Target of Rapamycin activity under single and combined iron and sulfur deficiency in tomato plants

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SUMMARY

Over the past few decades, a close relationship between sulfur (S) and iron (Fe) in terms of functionality and nutrition was demonstrated in the tomato. However, very little is known about the regulatory mechanisms underlying S/Fe interactions. Recently, the potential role of citrate in plant adaptation to Fe deficiency and combined S and Fe deficiency has been described. It is known that an impaired organic acid metabolism may stimulate a retrograde signal, which has been proven to be linked to the Target of Rapamycin (TOR) signaling in yeast and animal cells. Recent reports provided evidence of TOR involvement in S nutrient sensing in plants. This suggestion prompted us to investigate whether TOR may play a role in the cross-talk of signaling pathway occurring during plant adaptation to combined nutrient deficiency of Fe and S. Our results revealed that Fe deficiency elicited an increase of TOR activity associated with enhanced accumulation of citrate. In contrast, S deficiency resulted in decreased TOR activity and citrate accumulation. Interestingly, citrate accumulated in shoots of plants exposed to combined S/Fe deficiency to values between those found in Fe- and S-deficient plants, again correlated with TOR activity level. Our results suggest that citrate might be involved in establishing a link between plant response to combined S/Fe deficiency and the TOR network.

Keywords: citrate, iron, nutrient interaction, sugars, sulfur, tomato, TOR.

INTRODUCTION

In the last few decades, a close relationship between sulfur (S) and iron (Fe) was established in tomato: S deficiency significantly impairs plant capability to cope with Fe deficiency (Zuchi et al., 2009), whereas Fe deficiency induces a significant increase in S uptake and assimilation rate (Paolacci et al., 2014).

Tomato follows Strategy I mechanism to improve Fe uptake under Fe deficiency, in which a proton pump P-type ATPase, acidifies the external medium to increase Fe^{3+} solubility, ferric reductase *FRO1* reduces Fe^{3+} into Fe^{2+}, which is then transported across the root epidermis cell membrane via the high-affinity Fe transporter *IRT1*. The *FRO1* and *IRT1* genes are upregulated by Fe deficiency, and it has been demonstrated that the Fe shortage induces the ethylene production of roots playing an important role in the modulation of the Fe^{3+}-reducing capacity (García et al., 2010; Lucena et al., 2006). Once inside the root, Fe is distributed within the plant, and nicotianamine (NA) is considered the main Fe chelator involved in both xylem and phloem Fe transport (Douchkov et al., 2002; Rudolph et al., 1985).

Based on the evidence that ethylene plays a role in the regulation mechanisms of Fe deficiency responses (García et al., 2010; Lucena et al., 2006), whereas NA is involved in Fe transport within the plant via xylem or phloem (Douchkov et al., 2002; Rudolph et al., 1985), the interaction between Fe and S was initially ascribed to methionine, which is well known as the common precursor of ethylene and NA (Hesse & Hoefgen, 2003).

Accordingly, Zuchi et al. (2009) revealed that S deficiency prevented ethylene production at the root of Fe-deficient tomato plants. Furthermore, the induction of Fe^{3+}-chelate reductase activity severely limited the expression of NA synthase (*LeNAS*), *LeFRO1* (ferric reductase oxidase 1), and *LeIRT1* (iron-regulated transporter 1) genes, suggesting that the lower Fe accumulation in tomato shoots could be attributed to at least partial lack of activation of Strategy I (ethylene) as well as impaired Fe translocation to the shoot (NA). Thus, it has been suggested that increased demand for reduced S under Fe deficiency could be functional to allow proper methionine availability to sustain ethylene and NA biosynthesis (Paolacci et al., 2014; Zuchi et al., 2009; Zuchi et al., 2015).

However, higher S needs to sustain the activation of the Strategy I mechanism cannot fully account for the observed link between these two essential nutrients in the plant, as demonstrated by using a metabolomic approach, which showed an intensive overlap but also distinct responses (Zuchi et al., 2015). On the other hand, the multi-faceted effects of combined S and Fe deficiency on plant metabolome cannot even be explained by the need to maintain Fe-S cluster synthesis (Balk & Pilon, 2011). In plants, Fe-S clusters are cofactors found in the plastids, mitochondria, cytosol, and nucleus, where they play an essential role in different physiological processes (Balk & Pilon, 2011) and for Fe-S cluster assembly it is essential to recruit a balanced amount of Fe and S.

Therefore, this latest link could only account for the reduced Fe accumulation under S deficiency as Fe and S need to be coordinated to maintain Fe-S-cluster formation, but not for increased S uptake and assimilation under Fe deficiency.

Many interacting factors and complex mechanisms must likely include a direct interference of Fe with the signal transduction pathway involved in S metabolism and *vice versa*.

Intriguingly, mitochondria represent a crucial cellular site for assembling Fe-S clusters (Balk & Pilon, 2011). Furthermore, mitochondria are the cell's powerhouse, providing energy and many compounds, such as carboxylic acids, necessary for the whole metabolism. Among these compounds, citric acid has been shown to play a role as a metabolite signal in several organisms (Finkemeier et al., 2013; Ostaszewska et al., 2014; Schwarzländer et al., 2012; Vigani & Briat, 2016). Recently, changes in intracellular citrate have been shown to correlate with some Fe- and S-responsive genes (Vigani et al., 2018). Moreover, positive feedback was found between root citric acid concentration and plant ability to cope with Fe starvation in terms of increased Fe(III)-chelate reductase activity and both *SlFRO1* (Ferric Reductase Oxidase 1) and *SlFER* (Fe deficiency-induced transcription factor) expression (Coppa et al., 2018). These data suggest a possible mechanism in which citrate may be involved in plant adaptation to the combined deficiency of Fe and S in tomato (Coppa et al., 2018; Vigani et al., 2018).

It is well known that an impaired organic acid metabolism (i.e., trichloroacetic acid [TCA] cycle activity) in cells may stimulate a retrograde signal (from mitochondria to the nucleus) and that this response is involved in the regulation of many different processes within the plant (Butow & Avadhani, 2004). Moreover, it has been proven that the retrograde response is linked to Target of Rapamycin (TOR) signaling in yeast and animal cells, but this link has not been resolved so far (Butow & Avadhani, 2004).

TOR is a large Ser/Thr kinase protein (Sugimoto, 2018), which consists of two different complexes, TORC1 and TORC2, in yeast and mammals, whereas only TORC1 complex, a strongly conserved sensor kinase, exists in plants (Albert & Hall, 2015; Fu et al., 2020).

Several metabolites such as sugars, amino acids, hormones, and energy stimulate TOR activity to promote nutrient assimilation and protein production (Fu et al., 2020; Jamsheer et al., 2019; Liu & Sabatini, 2020). TOR activity induction results in the activation of mRNA translation and, in turn, cell growth and division (Fu et al., 2020; Jamsheer et al., 2019; Ryabova et al., 2019; Shi et al., 2018). Furthermore, TOR represses catabolic processes such as autophagy or protein degradation (Forzani et al., 2018; Fu et al., 2020; Xiong et al., 2013). Recent evidence gathered in plants supports their role as "regulatory hub" in a wide range of processes such as cell metabolism, biogenesis, organ growth, and development transitions in response to nutrient availability, energy, hormone, and environmental signals (Dobrenel et al., 2016; Loewith & Hall, 2011; Pu, Luo, et al., 2017; Pu, Soto-Burgos, et al., 2017; Wu et al., 2019; Wullschleger et al., 2006; Yan et al., 2012).

Some evidence indicates that TOR may play an integrative role in modulating S nutrient sensing in plants (Fu et al., 2020; Wu et al., 2019). In particular, in *Arabidopsis*, S deficiency results in reduced TOR activity in leaves, monitored by the phosphorylation of its target, S6K protein (Dong et al., 2017).

This intriguing suggestion prompted us to investigate whether TOR may play a role in the cross-talk signaling pathway occurring during plant adaptation to combined deficiency of Fe and S.

To disentangle this relation, we characterized changes in TOR activity in roots and shoots of tomato plants grown under single and combined deficiency of S and Fe. Results revealed that the TOR activity correlated with a citric acid content rather than glucose content, suggesting that citrate might be involved in establishing a link between plant response to combined S/Fe deficiency and the TOR network.

RESULTS

Differential modulation of TOR activity under single or combined Fe and S deficiency

TOR activity was determined by the ratio p-S6K/S6K in shoots and roots of tomato plants (Figure 1a,b), as S6K is an established target and readout for TOR activity. Antibodies also detected it against p-S6K and S6K (apparent sizes: 52 kDa) (Dong et al., 2017) (Figure 1c,d and Figure S1). Tomato plants exposed to S deficiency (S condition) showed a strongly decreased TOR activity (−38%, concerning the control) at shoot level (Figure 1a). In contrast, TOR activity in roots was similar to that of control plants (C condition) (Figure 1b). The withdrawal of Fe from nutrient solution (F condition) significantly increased TOR activity in both shoots and roots (+89% and +68% compared with control, respectively) (Figure 1). Finally, when plants were treated with double deficiency (D condition), TOR activity showed values similar to those of control plants (Figure 1a,b). However, significant differences were observed between D and S plants and between D and F plants. As reported in Figure 1, D plants had about 50% and 20% lower TOR activity in their shoots and roots than F plants. On the other hand, D plants had about 50% and 60% higher TOR activity in their roots and leaves than S plants (Figure 1a,b).

It has been previously reported that an increase in TOR activity levels is accompanied by shoot and root growth in Arabidopsis (Deprost et al., 2007). All nutrient shortage treatments resulted in inhibition of both shoot and root development (Table 1). The greatest inhibition was observed for shoot growth and in response to the combined deficiency (−60% with respect to the control C) (Table 1). F and S conditions resulted in 25% and 45% inhibition of shoot development respectively (Table 1). The growth rate of the root apparatus was also reduced by starvation treatments, even if to a lesser extent than that of the shoot. Specifically, F, S, and D conditions resulted in 25%, 20%, and 30% reduction in root biomass (Table 1).

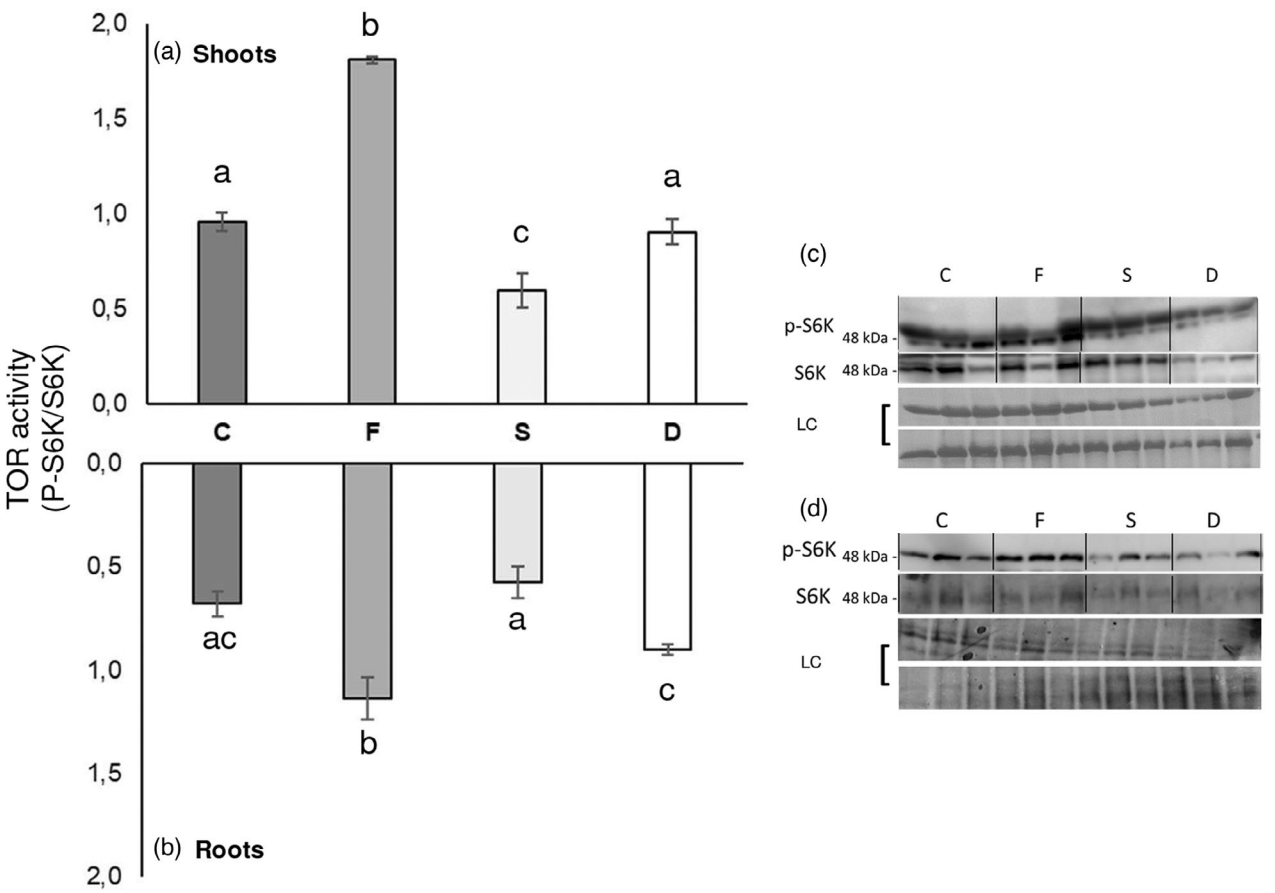

Figure 1. Effect of single and double Fe and S deficiency on TOR activity. Target of Rapamycin (TOR) activity was calculated by the ratio of p-S6K/S6K (a and b, respectively for shoots and roots) and was determined by antibodies against p-S6K, S6K (apparent sizes: 52 kDa) (c and d, respectively for shoots and roots) in tomato plants grown under control (C), Fe deficiency (F), S deficiency (S), and double deficiency (D) conditions. Data are means ± SD of three independent replications run in triplicate. The statistical significance was tested by means of ANOVA with Tukey post-hoc test. Different letters indicate statistically different values ($P < 0.01$).

Table 1 Nutrient shortage treatments and plant growth. Shoot and root fresh weight (FW) (expressed as g plant^{-1}) of tomato plants grown under control (C), Fe deficiency (F), S deficiency (S), and double deficiency (D) conditions

	Treatment			
	C	F	S	D
Shoot FW (g plant^{-1})	2577 ± 0.20 a	1954 ± 0.17 c	1439 ± 0.19 bc	1120 ± 0.09 b
Root FW (g plant^{-1})	0.558 ± 0.05 a	0.408 ± 0.06 b	0.452 ± 0.04 ab	0.376 ± 0.06 b

Data are means ± SD of three independent replications run in triplicate. The statistical significance was tested by means of ANOVA with the Tukey post-hoc test. Different letters indicate statistically different values ($P < 0.01$).

Single and combined Fe and S deficiency affect the concentration of total soluble sugars in the shoot and glucose in the roots of tomato plants

The regulation of plant TOR by glucose was previously reported (Xiong et al., 2013), and later research supported the role of the glucose-TOR signaling under S deprivation (Dong et al., 2017).

Our results showed that the total soluble sugar (including reducing and not reducing sugars) concentration increased in shoots of plants grown under treatments (by about 24%, 15%, and 30% in F, S, and D plants, respectively) but to a different extent with respect to the control plants (Figure 2a). On the other hand, there was no significant difference in root total sugar concentration among the four different treatments (Figure 2b).

Then, endogenous concentrations of glucose in both shoot and root tissues were specifically quantified (Figure 3a,b). Interestingly, only S starvation treatment significantly affected glucose levels in both tissues. However, in shoots, glucose concentration increased by 20% (Figure 3a), and the response was even more pronounced at the root level, where glucose concentration showed a four-fold increase under the S condition (Figure 3b).

In addition, the accumulation of not reducing sugar (in this case sucrose) was detected in plant tissues (Figure 3c,d). Even in this case, only the S starvation treatment significantly increased sucrose concentration in tomato shoots by 10% (Figure 3c). At root level, the content of sucrose increased by 47% and 32% under S and D conditions, respectively (Figure 3d).

These results indicate that neither total soluble sugars nor glucose or sucrose are significantly correlated with TOR activity in both shoots and roots of the tomato plants (Table 3).

Single or combined Fe and S deficiency differentially affect citric acid concentration in tomato plants

The modulation of TOR activity affects (or depends) on mitochondrial respiration (O'Leary et al., 2020). We, therefore, investigate the impact of single and combined Fe and S deficiency on mitochondrial respiration, in root and leaf

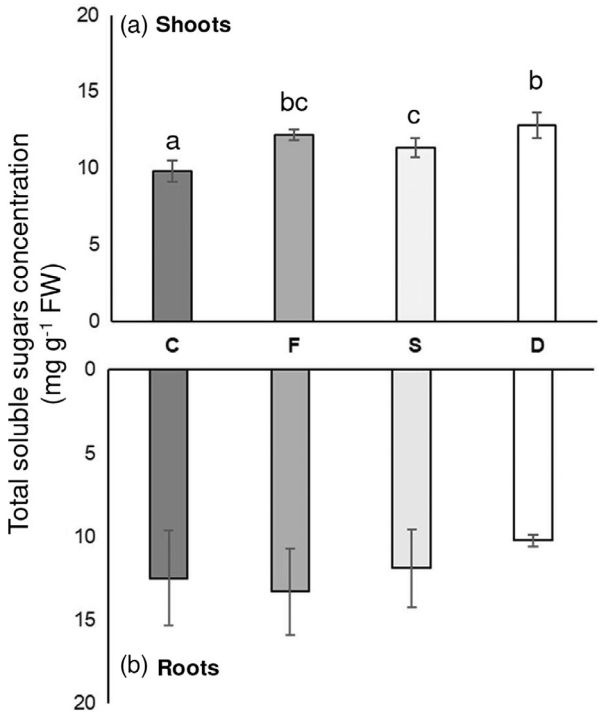

Figure 2. Effect of nutrient shortage treatments on total soluble sugars. Total soluble sugars concentration in (a) shoots and (b) roots of tomato plants grown under control (C), Fe deficiency (F), S deficiency (S), and double deficiency (D) conditions. Treatments and statistics are as in Figure 1.

tissues of tomato plants. Respiration (defined here as the oxygen consumption rate sensitive to KCN + SHAM treatment) strongly decreased in F, S, and D plants compared with C plants. Notably, in D plants, respiration displayed lower values with respect to C and F at the leaf level, while in roots single or combined Fe and S deficiency displayed a comparable impact on mitochondrial respiration. TCA-related metabolites are other possible metabolic targets of TOR activity related to mitochondria. Among these, citric acids represent one of the mitochondrial-derived metabolites playing a crucial role in mineral nutrient deficiencies.

In our experiment, citric acid concentration in tomato shoots was not affected by Fe deficiency; whereas when the external supply of sulfate was limited (both S and D

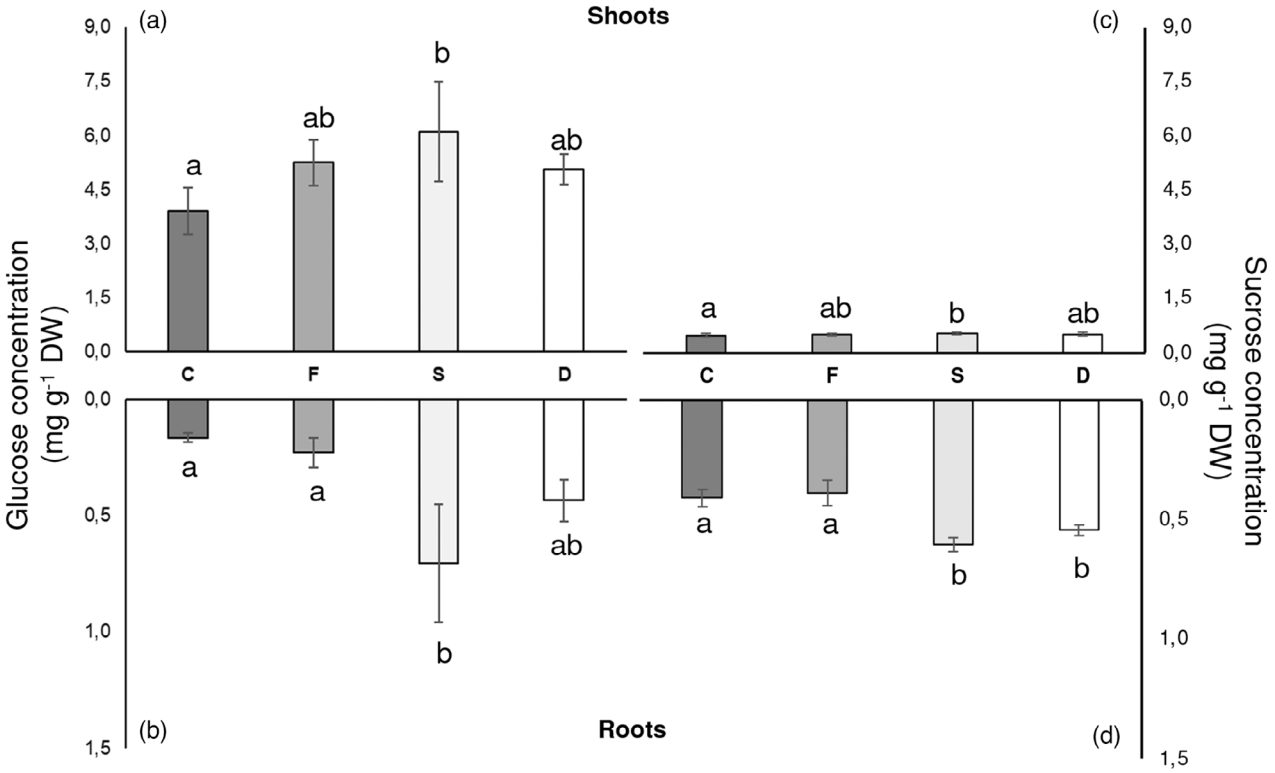

Figure 3. Effect of single and combined Fe and S deficiency on glucose and sucrose concentration measured in shoot (a and c, respectively) and root (b and d, respectively) of tomato plants grown under control (C), Fe deficiency (F), S deficiency (S), and double deficiency (D) conditions. Treatments and statistics as in Figure 1.

conditions), citric acid concentrations were significantly lower (−23% in both cases) than those in the shoots of control plants (Figure 4a).

The citric acid concentration in roots closely followed the TOR activity pattern (Figure 4b). The highest citric acid concentration values were found in plants exposed to Fe deficiency (F) roots and were more than three-fold higher than those found in control roots (Figure 4b).

In the roots of plants exposed to single S deficiency (S), the concentration of citric acid was similar to that of control plants (Figure 4b). However, combined deficiency treatment (D) caused a 55% increase in citrate concentration in roots relative to the control (Figure 4b).

We further evaluated the activity of some specific TCA cycle-related enzymes from an enriched mitochondrial fraction of root tissues to understand their contribution to the citric acid synthesis rate. Citric acid accumulation in roots was related to the changing activity of TCA cycle-related enzymes (Figure 5). Under Fe deficiency, the significant induction of citrate synthase and the slowing down of succinate dehydrogenase and aconitase resulted in citrate accumulation in root cells. Under S deficiency, only aconitase activity significantly decreased, while the combined Fe and S deficiency displayed a sightly induction of citrate synthase and a decrease of aconitase and succinate dehydrogenase activities (Figure 5).

These data raise the question of the possible impact of different citric acid concentrations on TOR activity, and actually, a positive correlation was observed between the two parameters in both shoots and roots of tomato plants (Table 3).

Expression of ribosomal protein genes under single or combined Fe and S deficiency

As the transcription of ribosomal RNAs (rRNAs) and translation in plants are under the positive control of TOR (Kim et al., 2014) we further determined the level of rRNA transcription in the root and shoot of tomato plants.

Messenger RNAs (mRNAs) encoding for ribosome proteins and proteins associated with ribosome biogenesis (17S and 25S) accumulated at different levels in plants exposed to different nutritional treatments (Figure 6). Fe deficiency resulted in significantly lower amounts of 17S and 25S rRNA in both shoot and root tissues. In contrast, S deficiency did not induce significant changes in the expression of both ribosomal protein genes (Figure 6). Interestingly, when plants were exposed to the combined deficiency, both 17S and 25S rRNA were upregulated at shoot level and 25S at the root level (Figure 6).

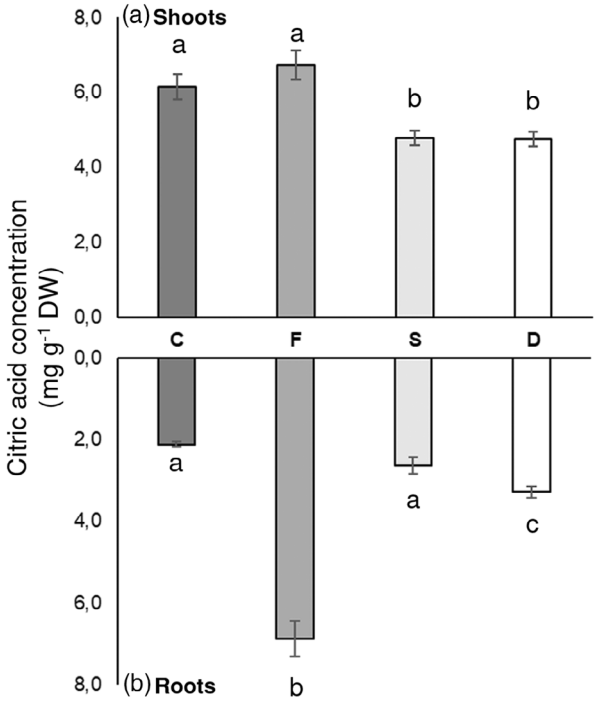

Figure 4. Single or combined Fe and S deficiency differentially affect citric acid concentration in tomato plants (a) shoot and (b) root of tomato plants grown under control (C), Fe deficiency (F), S deficiency (S), and double deficiency (D) conditions. Treatments and statistics as in Figure 1.

DISCUSSION

Modulation of TOR activity is not dependent on sugars under Fe and S deficiency in tomato plants

A close relationship between S and Fe has been demonstrated in plants (Astolfi et al., 2021; Coppa et al., 2018; Forieri et al., 2013; Vigani et al., 2018; Zamboni et al., 2017; Zuchi et al., 2015).

The relation has been partly attributed to methionine, being well known as the common precursor of ethylene and NA (Hesse & Hoefgen, 2003), and partly to Fe-S cluster synthesis. However, neither fully accounts for the observed link between the flow of these two essential nutrients in the plant but it seems associated with many complex mechanisms that could likely lead to the direct interference of Fe with the signal transduction pathway involved in S metabolism and *vice versa* (Zuchi et al., 2015).

A role for TOR in the adaptation response to S deficiency in Arabidopsis (Dong et al., 2017; Fu et al., 2020; Wu et al., 2019) has been recently demonstrated.

Thus, putting all these into context, this study is focused on the potential role TOR plays in the cross-talk of signaling pathways occurring during plant adaptation to the combined deficiency of Fe and S.

The inhibition of TOR activity observed in the shoots of tomato plants exposed to single S deficiency (Figure 1) is consistent with that reported in the literature. For instance, Dong et al. (2017) demonstrated that TOR activity strongly decreased in *Arabidopsis* shoots, without phosphorylation of S6K protein, under S starvation conditions. This response has been ascribed to autophagy in shoots induced by decreased TOR activity, resulting in the remobilization of the internal resources under the S deficiency condition (Dong et al., 2017). This could be considered a general response of plants to S stress and probably a protective response against it. However, this hypothesis needs further experimental substantiation in tomato.

Furthermore, some authors have proposed that TOR stimulates the expression of rRNA (Schepetilnikov

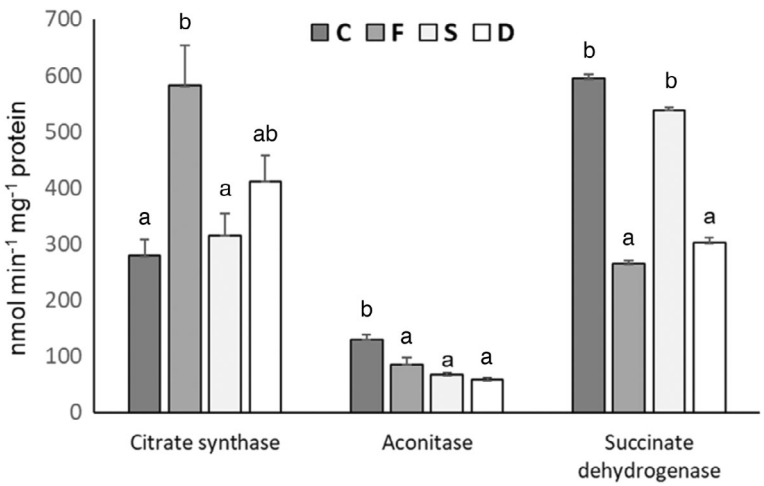

Figure 5. Trichloroacetic acid cycle-related enzymatic activities in tomato roots. Citrate synthase, aconitase, and succinate dehydrogenase activities in the mitochondrial enriched fraction from plants grown under control (C), Fe deficiency (F), S deficiency (S), and combined deficiency (D) conditions. Activities are reported as formation of thionitrobenzoic acid (citrate synthase), formation of NADPH (aconitase), and consumption of dichloroindophenol (see Experimental procedures section). Data are means ± SD of three independent replications. Statistical significance was tested by means of ANOVA with the Tukey post-hoc test. Different letters indicate statistically different values ($P < 0.05$).

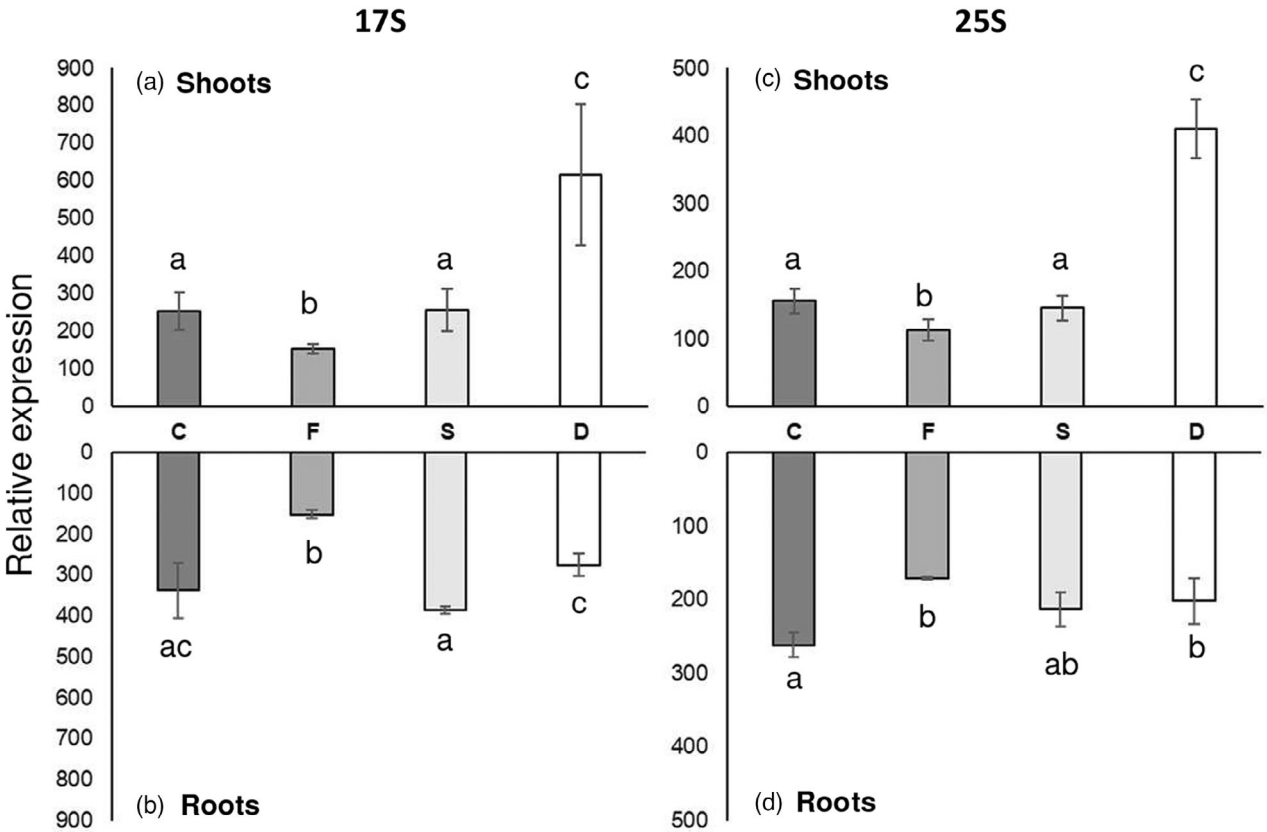

Figure 6. Expression of ribosomal protein genes under single or combined Fe and S deficiency 17S (a and b, respectively for shoots and roots) and 25S (c and d, respectively for shoots and roots) transcript levels were analyzed by quantitative polymerase chain reaction in tomato plants grown under control (C), Fe deficiency (F), S deficiency (S), and double deficiency (D) conditions. Relative expression data were normalized against the expression of the reference gene *SlEFα1*. Statistics as in Figure 1.

& Ryabova, 2018). Accordingly, Dong et al. (2017) demonstrated that TOR inhibition, mediated by S deficiency, decreased rRNA expression (18S and 25S rRNA) in Arabidopsis. However, our results did not highlight the link between TOR activity modulation and rRNA expression in tomato plants grown under single and combined Fe and S conditions. Although a decrease of 25S was observed in S-deficient tomato root associated with a slight decrease of TOR (Figures 1b and 6); under Fe deficiency TOR induction occurred along with a lower rRNA amount of 17S and 25S (Figures 1a,b and 6). Accordingly, impairment of root ribosomal proteome in Fe-deficient *Arabidopsis* plants has been previously observed (Wang et al., 2013), supporting the conclusion that low Fe availability widely affects the cell's translational machinery. In addition, most reports on plant TOR are mainly limited to the model plant Arabidopsis, with the consequent difficulty in applying knowledge from Arabidopsis to crops.

Furthermore, the reduction in TOR activity due to S starvation has been associated with the reduction of the glucose concentration in *Arabidopsis* (Dong et al., 2017), based on the so-called glucose-TOR axis first described by Xiong et al. (2013).

However, our results showed not only increased glucose (reducing sugar) concentration but also increased sucrose (not reducing sugar) accumulation both in the roots and shoot tissues of S-deficient tomato plants (Figure 3). On the other hand, plants exposed to single Fe deficiency (F) accumulated significantly more soluble sugars in their shoots (Figure 2a), while showing a significantly increased TOR activity (Figure 1a).

Interestingly, combined deficiency (D) resulted in an intermediate pattern of TOR activity between F and S plants (lower and higher, respectively) (Figure 1a), once again not associated with increased sugars accumulation (Figure 2). Indeed, no positive correlation was found between glucose and sucrose accumulation and TOR activity in both roots and shoots (Table 3).

Furthermore, through data processing from an untargeted metabolomic analysis of tomato plants grown under single and combined Fe and S deficiency (Zuchi et al., 2015) (Figure S2), it was possible to identify other reducing and not reducing sugars in both shoot and root tissues,

Table 2 Mitochondrial respiration (R) in root and shoot tissue of tomato plant grown under control (C), Fe deficiency (F), S deficiency (S), and double deficiency (D) conditions

	C	F	S	D
Root	70.21 ± 12.36 a	29.56 ± 6.65 b	35.72 ± 8.11 b	31.37 ± 4.66 b
Shoot	58.19 ± 9.61 a	44.52 ± 4.33 b	42.33 ± 3.87bc	36.54 ± 3.21 c

R is determined by the oxygen consumption rate (nmol O_2 mg FW min^{-1}) sensitive to KCN and SHAM treatments. The statistical significance was tested by means of ANOVA with Tukey post-hoc test. Different letters indicate statistically different values ($P < 0.05$).

but in neither case it was possible to detect a significant correlation with TOR activity (Figure S3).

However, sugar accumulation has been observed under several abiotic stresses (Signorelli et al., 2019). Recently, Rodriguez et al. (2019) provided possible explanations that might link sugar content and TOR activity: (i) TOR kinase is activated above a given threshold of sugars; (ii) hexose-to-sucrose ratio alteration or a different subcellular localization of sugars and TOR complex prevent kinase activation; and (iii) active TOR kinase is needed for the plant stress response. Yokawa and Baluška (2016) suggested that TOR could be activated by reactive oxygen species as an escape strategy to avoid stress.

As our results showed that S starvation resulted in the inhibition of TOR activity (Figure 1) without a reduction in glucose concentration (Figure 2), we could suggest that other factors than glucose could be responsible for S-starvation-induced inhibition of TOR activity observed in our tomato plants. However, under Fe deficiency and combined Fe and S deficiency, the TOR activity modulation could not be linked to the variation in glucose concentration, indicating that a Glu-TOR signaling pathway might be directly activated under single and combined Fe and S deficiency in tomato plants.

Amino acids and citric acid levels are linked to TOR activity modulation under Fe and S deficiency

It has been stated that TOR perceives S limitation via a decrease in sulfite reduction to sulfide in the plastids through a decline in glucose and sucrose concentrations. Although the mechanistic link between sulfite/sulfide and sugar metabolism remains uncovered, it has been suggested that plastidic sulfide or sulfite levels may be the signal connecting S assimilation and growth through sugar-regulated changes in TOR kinase activity. In tomato plants, sulfide content is strongly affected by both Fe and S deficiency, with a substantial decrease under low S availability (S and D treatments) (Zuchi et al., 2015), indicating that the differential modulation of TOR activity under F and S treatments might be linked to other processes.

The TOR kinase pathway is a master regulator of eukaryotic carbon metabolism, where its activation generally promotes anabolic metabolism and inhibits catabolic metabolism (Dobrenel et al., 2016). A further aspect affecting TOR activity is linked to the N metabolism, and amino acids have been considered one of the primary signals activating the TOR pathway in other eukaryotes (Jewell et al., 2013). O'Leary et al. (2020) recently demonstrated that amino acid levels impact plants' metabolic activities via the TOR kinase signaling pathway. Furthermore, they provided evidence that TOR activity is influenced by amino acid levels leading to a downstream effect on the use of respiratory substrates. Notably, O'Leary and coworkers suggested that the accumulation of amino acids and TCA cycle organic acids might occur after plants' impairment of the TOR signaling pathway.

By re-analyzing metabolic data obtained from tomato plants grown under single and combined Fe and S deficiency (Zuchi et al., 2015), a general decrease of amino acids can be observed in both shoots and roots of Fe-deficient plants, while S deficiency, either in single or in combination with Fe deficiency, led to a general accumulation of amino acids both in leaves and roots tissues (Figure S2).

Therefore, the differential TOR activity in tomato plants might be linked to the different impacts of Fe and S deficiency in amino acid metabolism, although no clear relations can be provided at this stage. It has been suggested that under Fe deficiency, primary metabolites could be allocated to synthesizing compounds required to maintain Fe homeostasis and support Fe acquisition (Chutia et al., 2021). Therefore, the decreased levels of amino acids

Table 3 Linear correlations between TOR activity and the concentrations of citrate, total soluble sugars, glucose and sucrose in the shoots and roots of tomato plants grown with different availability of iron (Fe) and sulfur (S)

	TOR activity	
	Shoots	Roots
Citrate	0.840**	0.905***
Soluble sugars	0.266	0.234
Glucose	−0.509	−0.506
Sucrose	−0.180	−0.567

C, control; F, Fe deficiency; D, double deficiency of Fe and S; S, S deficiency.
** and *** indicate statistical significance at $P < 0.01$ and $P < 0.001$.

might be due to the Fe-deficiency-induced synthesis of compounds such as NA, coumarins, and putrescine (Klatte et al., 2009; Rajniak et al., 2018; Zhu et al., 2016). The evidence of tomato growth impairment under Fe deficiency (Table 1) suggests that the increased TOR activity would activate specific anabolic pathways aiming to improve Fe uptake and translocation. Accordingly, the amino acids accumulation observed in S-deficient tomato plants mirrors, at least in the shoot, the observed decrease of TOR activity. Accordingly, it has been observed that disruption of TOR signaling leads to the accumulation of a high levels of amino acids in plants (Caldana et al., 2013). Such accumulation might be due to an increase of protein breakdown by nutrient recycling processes (e.g., senescence or autophagy) or *de novo* synthesis of amino acids or to a decrease of amino acid incorporation into newly synthesized proteins.

Notably, under combined (D) deficiency, TOR activity decreased with respect to the Fe deficiency condition resulting in an increased level of amino acids. Such observation highlights that S availability is crucial for Fe-deficiency-induced modulation of TOR activity and metabolism in tomato plants (Zuchi et al., 2015). In animals and yeast, the level of amino acids seems to act upstream of TOR activity (González & Hall, 2017). In plants, although evidence that amino acids levels influence TOR activity has been reported (O'Leary et al., 2020), the components of amino acid sensing upstream of TOR and described in animals and fungi (Wolfson & Sabatini, 2017) are lacking. Therefore, whether the level of amino acid modulates TOR activity or whether it is an effect of TOR modulation under Fe and S deficiency requires further investigation. However, it has been demonstrated that a high level of amino acid leads to TOR activation with a subsequent downregulation of mitochondrial respiratory pathways (O'Leary et al., 2020). Our results indicate that single and combined Fe and S deficiency similarly impair respiration in tomato plants, linking the downstream effect of TOR activation with respiration impairment only under Fe deficiency. Conversely, the amino acid profile did not support their possible upstream role in TOR signaling under single or combined Fe and S deficiency, indicating that the variation in amino acid levels observed likely represents an effect of TOR activity modulation occurring under Fe and S deficiency.

Besides amino acids, disruption of TOR signaling also affects the TCA cycle organic acid level in plants. However, the inhibition of TOR leads to opposite responses in plant and animal cells: organic acid levels increase and decrease in plant and animal cells, respectively, in line with changes in the respiration rate (Caldana et al., 2013; Ramanathan & Schreiber, 2009).

Among others, citric acid plays a central role in plant responses to nutrient starvation. Recently, citric acid could be involved in adapting to the combined deficiency of S and Fe in tomato (Coppa et al., 2018; Vigani et al., 2018). As reported in the literature, Fe deficiency commonly increases citrate production (Marschner, 2012). Consistently with this pattern, the roots of tomato plants deprived of Fe exhibited increased levels of citrate accumulation (Figure 4b), which, however, was unchanged in shoots compared with control plants (Figure 4a).

Interestingly, it has been recently demonstrated that increased citric acid levels correlate with the expression of some Fe deficiency-induced genes, such as the *SlFRO1* gene (Coppa et al., 2018). Nevertheless, a significant positive correlation between the increase of citric acid concentration and TOR activity was observed in both shoots ($R^2 = 0.840$) and roots ($R^2 = 0.905$) of tomato plants (Table 3), indicating that TOR activity modulation and citric acid reflect the effect of Fe/S interplay tomato plants. A link between TOR and citric acid has been previously reported (Caldana et al., 2013). However, the authors provided evidence that TOR disruption leads to the accumulation of TCA-related organic acid (including citric acid) in Arabidopsis. Such an effect has been explained as a consequence of decreased anabolic activities and increased catabolic activities. By contrast, this work provides evidence that citric acid levels and TOR activity are similarly modulated in tomato plants when Fe and/or S nutrition is impaired.

In conclusion, this work suggests that (i) Fe deficiency induces TOR activity in both roots and shoots of tomato plants, while S deficiency limits TOR activity in shoots; (ii) Fe deficiency-induced TOR activity is dependent on the S availability of plants, as also reflected by amino acids profiles; and (iii) accumulation of citrate might be linked to the modulation of TOR activity under Fe deficiency, supporting the hypothesis of TOR involvement in the activation of Fe deficiency response in tomato. This evidence is even more interesting given that citrate levels are also correlated with the expression of some S deficiency-induced genes in tomato plants (Coppa et al., 2018) and underlines the importance of examining whether S deficiency response could be mediated by the inhibition of TOR activity resulting in decreased citrate accumulation.

Our data seem to rule out the prediction about the importance of glucose as a TOR inducer, at least in tomato, confirming that distinct differences occur between the Arabidopsis model system and tomato (Zuchi et al., 2015). Such a discrepancy might be linked to the different threshold levels of S deficiency perceived by plants due to the imposed experimental conditions or a different mechanism operating in tomato with respect to Arabidopsis. Furthermore, considering that the regulatory properties of the plant TOR pathway are likely different between tissues and developmental stages (Xiong & Sheen, 2014), it will also be important to spend efforts by performing specific studies addressing the role of TOR in shoot and root under nutrient starvation.

The reported evidence represents a further step towards unraveling the mechanisms underlying Fe/S interaction, but some mysterious responses have been revealed. Thus, new research based on the current knowledge would help answer unsolved questions.

EXPERIMENTAL PROCEDURES

Plant growth conditions

Tomato (*Solanum lycopersicum* L., cv. Marmande) plants were grown hydroponically in plastic pots containing 2.2 L of complete nutrient solution, containing 1.2 mM sulfate and 40 μM FeIII-EDTA) (Zuchi et al., 2009). After 1 week, plants were transferred to different nutrient solutions to obtain four different nutritional conditions defined as control (C; 1.2 mM sulfate and 40 μM FeIII-EDTA), Fe-deficient (F; 1.2 mM sulfate and 0 μM FeIII-EDTA), S-deficient (S; 0 mM sulfate and 40 μM FeIII-EDTA) and dual deficient (D; 0 mM sulfate and 0 μM FeIII-EDTA). Plants were grown in a growth chamber under 200 μmol photons m^{-2} sec^{-1} photosynthetic photon flux, with 16/8 h photoperiod (27°C/20°C day/night temperature cycling and 80% relative humidity. Plants were harvested 17 days after sowing.

Immunological assay

Immunological detection of S6K-p and S6K1/2 was performed as described by Dong et al. (2017), with minor modifications. Total soluble proteins were extracted from 50 μg (FW) of root and shoot tissues by adding a ratio of 1:5 (w/v) 2× Laemmli Buffer supplemented with 3% phosphatase inhibitor cocktail 2 (Sigma, St. Louis, MO, USA). Proteins were denatured for 7 min at 95°C. The immunological detection of S6K-p proteins was separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, while the immunological detection of S6K1/2 proteins was separated on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis supplemented with 8 M urea, and then for both, S6K-p and S6K1/2 detection, proteins were transferred on PVDF membrane. The blocking solution was supplemented with Twin 0.1% only for S6K1/2 detection. Membranes were incubated with the primary antibodies overnight at 4°C: anti-S6K1-p (Abcam, Cambridge, UK; phospho T449 antibody 207 399, 1:2500), anti-S6K1/2 (Agrisera, Vannas, Sweden; Ribosomal S6 kinase 1/2 AS12 1855, 1:3000). The primary antibodies were detected using the horseradish peroxidase-conjugated secondary antibody (1:20 000). The bands intensity was quantified by Image Quant LAS4000 version 1.21 and normalized by the loading control. Full scans of blots are shown in Figure S1.

Total RNA extraction and expression analyses

RNA was extracted from 100 mg (FW) of shoots and roots of tomato plants by using the Aurum™ Total RNA Mini Kit (catalog no. 7326820; Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions.

The optical density (OD) of 2 μl of extracted RNA was measured at 230, 260, and 280 nm with Thermo Scientific™ Multiskan SkyHigh Microplate Spectrophotometer (catalog no. A51119500C; Thermo Fisher Scientific, Waltham, MA, USA). The RNA concentration was quantified from OD at 260 nm, and the OD ratio evaluated the purity at 260/280 nm for protein contamination and the OD ratio at 260/230 nm for polysaccharide, phenol, and other contaminations. In addition, RNA integrity was checked on 2% agarose gel by electrophoresis.

The reverse transcription was performed using the iScript gDNA clear cDNA synthesis kit (catalog no. 1708840; Bio-Rad) according to the manufacturer's instructions. All reaction volumes were 20 μl, and contained 1 μg of RNA. The mix was first processed on T100 Thermal Cycler (catalog no. 1861096; Bio-Rad) at 25°C for 5 min and 75°C for 5 min to remove any genomic DNA contaminations. The iScript Reverse Transcription Supermix was added, and the reverse transcription reaction was performed at 25°C for 5 min, 46°C for 20 min, 95°C for 1 min.

According to the manufacturer's instructions, cDNA was amplified from 50 ng of total RNA in a 20-μl reaction mixture containing 1× SsoAdvanced Universal SYBR green Supermix (catalog no. 1725270; Bio-Rad) and 0.5 μM of each primer. Quantitative real-time polymerase chain reaction analysis was performed by using a CFX96 Real-Time System (catalog no. 1845097; Bio-Rad) and data analysis was done using LINREGPCR software.

The expression levels of 17S and 25S rRNA genes, relative to *Elongation Factor 1 alpha* (*EFα1*), were determined using a modification of the Pfaffl method (Pfaffl, 2001) and expressed in arbitrary units. Primer sequences are shown in Table S1.

A melting curve analysis of all polymerase chain reaction amplicons was performed to assess that only fluorescence from specific target sequences was considered in the analysis and to exclude the possibility that any artifact, including those possibly related to the formation of primer dimers, can affect the obtained results.

Determination of soluble sugars and glucose

The total soluble sugars content of roots and shoots was determined after hot water extraction according to Stephan and Rudolph (1984), with minor modifications. First, plant tissue was homogenized to a fine powder with liquid nitrogen. Distilled water (100°C) was added to aliquots of the powdered tissue (500 ml mg^{-1} FW) and homogenates were incubated for 10 min at 80°C. Insoluble material was removed by 10 min centrifugation in a microliter centrifuge at 5 000 ***g***, and the pellet was then re-extracted with 500 ml boiling water as described above. After a second centrifugation step, the supernatant was used for spectrophotometric determination of reducing sugars and sucrose, according to Blakeney and Mutton (1980).

The concentration of glucose and sucrose in root and shoot tissues (approximately 0.1 g FW) of tomato plants was determined by the Glucose Colorimetric/Fluorometric Assay Kit (MAK263; Sigma-Aldrich) and Sucrose Colorimetric/Fluorometric Assay Kit (MAK267; Sigma-Aldrich), respectively. Glucose was oxidized to generate a colorimetric (570 nm) product proportional to the amount of glucose in plant tissues. Sucrose was converted to glucose by invertase and the generated glucose was determined as described above (λ = 570 nm).

The citric acid concentration in root and shoot samples of tomato plants was determined by Citrate Assay Kit (MAK057; Sigma-Aldrich). Citrate concentration is determined by a coupled enzyme assay, which results in a colorimetric (570 nm) product proportional to the citrate present in plant tissues.

Mitochondrial respiration in tomato tissues

Leaf disc (0.5–1 cm diameter) and apical root segments (1–2 cm in length) were excised under water at room temperature from plants. Oxygen consumption rates were measured under dark condition in an aqueous phase with a Clark-type oxygen electrode (Hansatech) at 25°C (according to Vigani et al., 2009). Calibration was made from the difference in signal between aerated water

and sodium dithionite saturated water. The difference in between the initial O_2 consumption rate and the residual signal after the addition of respiration inhibitors KCN (2 mM) and salicylhydroxamic acid (SHAM, 2 mM) defined the respiration parameter indicated in Table 2.

Determination of root mitochondrial activities

The mitochondrial enriched fraction was collected by tomato root, according to Vigani et al. (2018). In addition, citrate synthase and succinate dehydrogenase activity were performed according to Vigani et al. (2009), while aconitase activity was performed according to Lee et al. (2008). Methods for enzymatic activity determination are reported below.

Citrate synthase activity was assayed by reducing acetyl-CoA to CoA with 5-5′-dithiobis-2-nitrobenzoic acid; thionitrobenzoic acid formation was monitored at A_{412}. The reaction was carried out by adding purified mitochondria (50 μg protein) in 75 mM Tris–HCl, pH 8.0, 0.1 mM 5-5′-dithiobis-2-nitrobenzoic acid, 0.1% Triton X-100, 0.4 mM acetyl-CoA. After a preincubation at 30°C for 2 min, the reaction was started by the addition of oxalacetate to 0.5 mm final concentration. Aconitase activity was assayed by monitoring the formation of NADPH in A_{340} in the following mixture: 80 mM HEPES-NaOH (pH 7.5), 0.5 mM NADP, 0.5 mM $MnCl_2$, 2 units of NADP-isocitrate dehydrogenase, 0.05% (v/v) Triton X-100. The reaction was initiated by the addition of 8 mM of *cis*-aconitate. Succinate dehydrogenase activity was assayed by monitoring the decrease in the dichloroindophenol concentration at A_{600} at 30°C. Purified mitochondria (50 μg protein) in 50 mm phosphate buffer, pH 7.0, 0.1 mM dichloroindophenol, and 1.5 mM NaCN were preincubated at 30°C for 2 min. The reaction was followed by the addition of 16 μM succinate and 50 μM CoQ1 final concentration. Absorbance was registered for 2 min after an incubation time of 30 s.

Each reported value represents the mean ± SD of data from four independent experiments on three biological replicates per experiment. Statistical data analyses were carried out by ANOVA tests with the GraphPad InStat Program (version 3.06). Significant differences were established by post-hoc comparisons (HSD test of Tukey) at $P < 0.01$ or $P < 0.05$.

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DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Full scans of blots used to calculate TOR activity for shoots and roots of tomato plants grown under control (C), Fe deficiency (F), S deficiency (S), and double deficiency (D) condition (apparent sizes of p-S6K, S6K: 52 kDa) with the respective loading control below.

Figure S2. Changes in amino acids production and in reducing and not reducing sugars accumulation in shoot and root of tomato plants grown under control (C), Fe deficiency (F), S deficiency (S), and double deficiency (D) condition (modified from Zuchi et al., 2015).

Figure S3. Linear correlations between TOR activity and the concentrations of reducing sugars (glucose-6-phosphate, fructose, maltose, ribose, rhamnose, sorbitol, glycerol-3-phosphate, myo-inositol) and non-reducing sugars (galactinol) in the shoots and roots of tomato plants grown under control (C), Fe deficiency (F), S deficiency (S), and double deficiency (D) condition (n.d. = not detected).

Table S1. Primer pairs used to evaluate the expression levels of 17S and 25S ribosomal RNA (rRNA) genes, relative to *Elongation Factor 1 alpha* (*EFα1*).

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