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

Differential modulation of Target of Rapamycin (TOR) 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 tomato. However, very little is known about the regulatory mechanisms underlying S/Fe interaction. 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.

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Significance statement of up to two sentences of no more than 75 words total

This is the first report on the Target of Rapamycin (TOR) response to nutrient starvation in the tomato crop. TOR activity has been modulated differently by single or combined Fe and S deficiency. In addition, evidence was presented suggesting that endogenous changes in citric acid content in plant tissues might act as an important modulator of TOR activity.

Introduction

In the last few decades, a close relationship between S and 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 up-regulated by Fe deficiency, and it has been demonstrated that Fe shortage induces the ethylene production of roots playing an important role in the modulation of the Fe^{3+}-reducing capacity (Lucena et al., 2006; Garcia et al., 2010). 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 (Rudolph et al., 1985; Douchkov et al., 2002).

Based on the evidence that ethylene plays a role in the regulation mechanisms of Fe deficiency responses (Lucena et al., 2006; Garcia et al., 2010), whereas NA is involved in Fe transport within the plant via xylem or phloem (Rudolph et al., 1985; Douchkov et al., 2002), the interaction between Fe and S was initially ascribed to methionine, which is well known as the common precursor of ethylene and NA (Hesse and 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 (Zuchi et al., 2009; Paolacci et al., 2014; 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 and 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 and Pilon, 2011) and for Fe–S cluster assembly it is essential to recruit balanced amount of iron and sulfur.

Therefore, this latest link could only account for the reduced Fe accumulation under S deficiency since 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 et al., 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 (Schwarzländer et al., 2012; Finkemeier et al., 2013; Ostaszewska et al., 2014; Vigani and 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 (Vigani et al., 2018; Coppa et al., 2018).

It is well known that an impaired organic acid metabolism (i.e. 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 and 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 and 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 and Hall, 2015; Fu et al., 2020).

Several metabolites like sugars, amino acids, hormones, and energy stimulate TOR activity to promote nutrient assimilation and protein production (Jamsheer et al., 2019; Fu et al., 2020; Liu and Sabatini, 2020). TOR activity induction results in the activation of mRNA translation and, in turn, cell growth and division (Shi et al., 2018; Jamsheer K et al., 2019; Ryabova et al., 2019; Fu et al., 2020). Furthermore, TOR represses catabolic processes such as autophagy or protein degradation (Xiong et al., 2013; Forzani et al., 2018; Fu et al., 2020). 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 (Wullschleger et al., 2006; Loewith and Hall, 2011; Yan et al., 2012; Dobrelen et al., 2016; Pu et al., 2017; Wu et al., 2019).

Some evidence indicates that TOR may play an integrative role in modulating sulfur nutrient sensing in plants (Wu et al., 2019; Fu et al., 2020). 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 signalling pathway occurring during plant adaptation to combined deficiency of Fe and S.

To disentangle this relation, we characterised 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 (Fig. 1 A, B), since 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) (Fig. 1 C, D and Suppl. Fig. 1). Tomato plants exposed to S deficiency (S condition) showed a strongly decreased TOR activity (-38%, concerning the control) at shoot level (Fig. 1A). In contrast, TOR activity in roots was similar to that of control plants (Fig. 1B). The withdrawal of Fe from nutrient solution (NS) significantly increased TOR activity in both shoots and roots (+ 89% and +68% compared to control, respectively) (Fig. 1). Finally, when plants were treated with combined deficiency (D), TOR activity showed values similar to those of control plants (Fig. 1A, B). However, significant differences were observed between D and S plants and between D and F plants. As reported in Fig.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 (Fig. 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 (Tab. 1). The greatest inhibition was observed for shoot growth and in response to the combined deficiency (-60% with respect to the control) (Tab. 1). F and S conditions resulted in 25 and 45% inhibition of shoot development respectively (Tab. 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 development (Tab. 1).

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.

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 sulfur 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 (Fig. 2A). On the other hand, there was no significant difference in root total sugar concentration among the four different treatments (Fig. 2B).

Then, endogenous concentrations of glucose in both shoot and root tissues were specifically quantified (Fig. 3A, B). Interestingly, only S starvation treatment significantly affected glucose

levels in both tissues. However, in shoots, glucose concentration increased by 20% (Fig. 3A), and the response was even more pronounced at the root level, where glucose concentration showed a 4-fold increase under S condition (Fig. 3B).

Also, the accumulation of not reducing sugar (in this case sucrose) was detected in plant tissues (Fig. 3C, D). Even in this case, only S starvation treatment significantly increased sucrose concentration in tomato shoots by 10% (Fig. 3C). At root level, the content of sucrose increased by 47 and 32% under S and D condition, respectively (Fig. 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 (Tab. 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 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 conditions), citric acid concentrations were significantly lower (-23% in both cases) than those in the shoots of control plants (Fig. 4A).

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

In the roots of plants exposed to single S deficiency (S), the concentration of citric acid was similar to that of control plants (Fig. 4B). However, combined deficiency treatment (D) caused a 55% increase in citrate concentration in roots relative to the control (Fig. 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 (Fig 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 (Fig 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 (Tab. 3).

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

Since the transcription of 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 to different levels in plants exposed to different nutritional treatments (Fig. 6). Iron 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 (Fig. 6). Interestingly, when plants were exposed to the combined deficiency, both 17S and 25S rRNA were upregulated at shoot level and 25S also at the root level (Fig. 6).

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 (Forieri *et al.*, 2013; Zuchi *et al.*, 2015; Zamboni *et al.*, 2017; Vigani *et al.*, 2018; Coppa *et al.*, 2018; Astolfi et al., 2021).

The relation has been partly attributed to methionine, being well known as the common precursor of ethylene and nicotianamine (NA) (Hesse and 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).

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

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

The inhibition of TOR activity observed in shoots of tomato plants exposed to single S deficiency (Fig. 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 et al., 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 ribosomal RNA 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 (Fig. 1B and 6); under Fe deficiency TOR induction occurred along with a lower rRNA amount of 17S and 25S, (Fig 1 A,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 (Fig. 3). On the other hand, plants exposed to single Fe deficiency (F) accumulated significantly more soluble sugars in their shoots (Fig. 2A), while showing significantly increased TOR activity (Fig. 1A).

Interestingly, combined deficiency (D) resulted in an intermediate pattern of TOR activity between F and S plants (lower and higher, respectively) (Fig. 1A), once again not associated with an increased sugars accumulation (Fig. 2). Indeed, no positive correlation was found between glucose and sucrose accumulation and TOR activity in both root and shoot (Tab. 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), it was possible to identify other reducing and not reducing sugars in both shoot and root tissues, but in neither case it was possible to detect a significant correlation with TOR activity (Fig. 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) the TOR kinase is activated above a given threshold of sugars; ii) the hexose-to-sucrose ratio alteration or a different subcellular localisation of sugars and the TOR complex prevent kinase activation and iii) an active TOR kinase is needed for the plant stress response. Yokawa and Baluška (2016) suggested that TOR could be activated by reactive oxygen species (ROS) as an escape strategy to avoid stress.

Since our results showed that S starvation resulted in an inhibition of TOR activity (Fig. 1) without a reduction in glucose concentration (Fig. 2), we could suggest that other mechanisms than glucose could be responsible for S-starvation-induced inhibition of TOR activity observed in 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 signalling 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 signalling 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 signalling pathway.

By re-analysing 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 (Fig. 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 might be due to the Fe-deficiency-induced synthesis of compounds such as nicotianamine, coumarins and putrescine (Klatte et al., 2009; Rajniak et al., 2018; Zhu et al., 2016). The evidence of tomato growth impairment under Fe deficiency (Tab. 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 signalling 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 and 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 and 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 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 TOR activation's downstream effect with respiration impairment only under Fe deficiency. Conversely, the amino acids profile did not support their possible upstream role in TOR signalling under single or combined Fe and S deficiency, indicating that the variation in amino acids level observed likely represents an effect of TOR activity modulation occurring under Fe and S deficiency.

Besides amino acids, disruption of TOR signalling 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 respiration rate (Ramanathan and Schreiber, 2009; Caldana et al., 2013).

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 (Vigani et al., 2018; Coppa et al., 2018). As reported in the literature, Fe deficiency commonly increases citrate production (Marschner, 1995). Consistently with this pattern, the roots of tomato plants deprived of Fe exhibited increased levels of citrate accumulation (Fig. 4B), which, however, resulted unchanged in shoots compared to control plants (Fig. 4A).

Interestingly, it has been recently demonstrated that increased citric acid levels correlate with the expression of some Fe deficiency-induced genes, such as *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 (Tab. 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 both in root and shoot of tomato plants, while S deficiency limits TOR activity in shoot; ii) the Fe deficiency-induced TOR activity is dependent on the S availability of plants as also reflected by amino acids profiles; iii) the 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 by 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 discrepancy might be linked to the different threshold levels of S deficiency perceived by plants under the experimental conditions imposed 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 and 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 (NS), containing 1.2 mM sulfate and 40 μM FeIII-EDTA) (Zuchi et al., 2009). After one week, plants were transferred to different NS in order 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} s^{-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 d 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) 2x Laemmli Buffer supplemented with 3% phosphatase inhibitor cocktail 2 (Sigma). Proteins were denatured for 7 min at 95 °C. The immunological detection of S6K-p proteins was separated on 10% SDS-PAGE, while the immunological detection of S6K1/2 proteins was separated on 15% SDS-PAGE 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 four °C: anti-S6K1-p (abcam, phospho T449 ab 207399, 1:2500), anti-S6K1/2 (Agrisera, Ribosomal S6 kinase 1/2 AS12 1855, 1:3000). The primary antibodies were detected using the HRP-conjugated secondary antibody (1:20000). The bands intensity was quantified by Image Quant LAS4000 version 1.21 and normalised by the loading control. Full scans of blots are shown in Supplementary Fig. 1.

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 (cat. n. 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 (cat. n. A51119500C, Thermo Fisher Scientific, Oslo, Norway). 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 (cat. n. 1708840, Bio-Rad, Hercules, CA, USA) 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 (cat. n. 1861096, Bio-Rad, Hercules, CA, USA) at 25 °C for 5 min, 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 (cat. n. 1725270, Biorad, Hercules, CA, USA) and 0.5 µM of each primer. qRT-PCR analysis was performed by using a CFX96 Real-Time System (cat. n. 1845097, Biorad, Hercules, CA, USA) and data analysis was done using LinRegPCR software.

The expression levels of 17S and 25S ribosomal RNA (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 Supplementary Table 1.

A melting curve analysis of all PCR 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 homogenised 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

12000 rpm, 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 (ca. 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 oxidised 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 O2 electrode (Hansatech) at 25°C (according to Vigani et al 2009). Calibration was made from the difference in signal between aerated water and Na-dithionite saturated water. The difference in between the initial O_2 consumption rate and the residual signal after the addition of respiration inhibitors KCN (2mM) and salycilichydroxamic acid (SHAM, 2mM) defined the respiration (R) 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′-dithio-bis-2-nitrobenzoic acid (DTNB); thionitrobenzoic acid (TNB) 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 DTNB, 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 dichloroindophenol (DCIP) concentration at A_{600} at 30°C. Purified mitochondria (50 µg protein) in 50 mm phosphate buffer, pH 7.0, 0.1 mM DICP, 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 $p< 0.05$.

Acknowledgments

All authors gratefully acknowledge the scientific advice and discussion provided by Markus Wirtz.

Short legends for Supporting Information

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*).

Name gene	Accession number	Forward primer 5'→ 3'	Reverse primer 5'→ 3'	Amplicon length (bp)	Reference
Sl_EFα1 (house keeping)	X14449.1	GGAACTTGAGAAGGAGCCTAAG	CAACACCAACAGCAACAGTCT	158 bp	Løvdal and Lillo (2009)
Sl_17S	NR_137327.1	GCTGCTGCGATGATTCATGA	ATTGTCACTACCTCCCCGTG	239 bp	-
Sl_25S	NR_137326.1	GATTCCCCTAGTAACGGCGA	ACAACCCGACTCGTAGACAG	200 pb	-

Fig. 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.

Fig. S2

Changes in amino acids production in shoot and root of tomato plants grown under control (C), Fe deficiency (F), S deficiency (S) and double deficiency (D) condition (mod. from Zuchi et al., 2015).

Fig. S3

Changes in reducing and non-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 (mod. from Zuchi et al., 2015).

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Table 1

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) condition. 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 ($p < 0.01$).

	Treatment			
	C	**F**	**S**	**D**
				
Shoot FW (g plant^{-1})	2,577 ± 0.20 a	1,954 ± 0.17 c	1,439 ± 0.19 bc	1,120 ± 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

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) condition. 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 ($p < 0.05$).

	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

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

	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

Figure legends

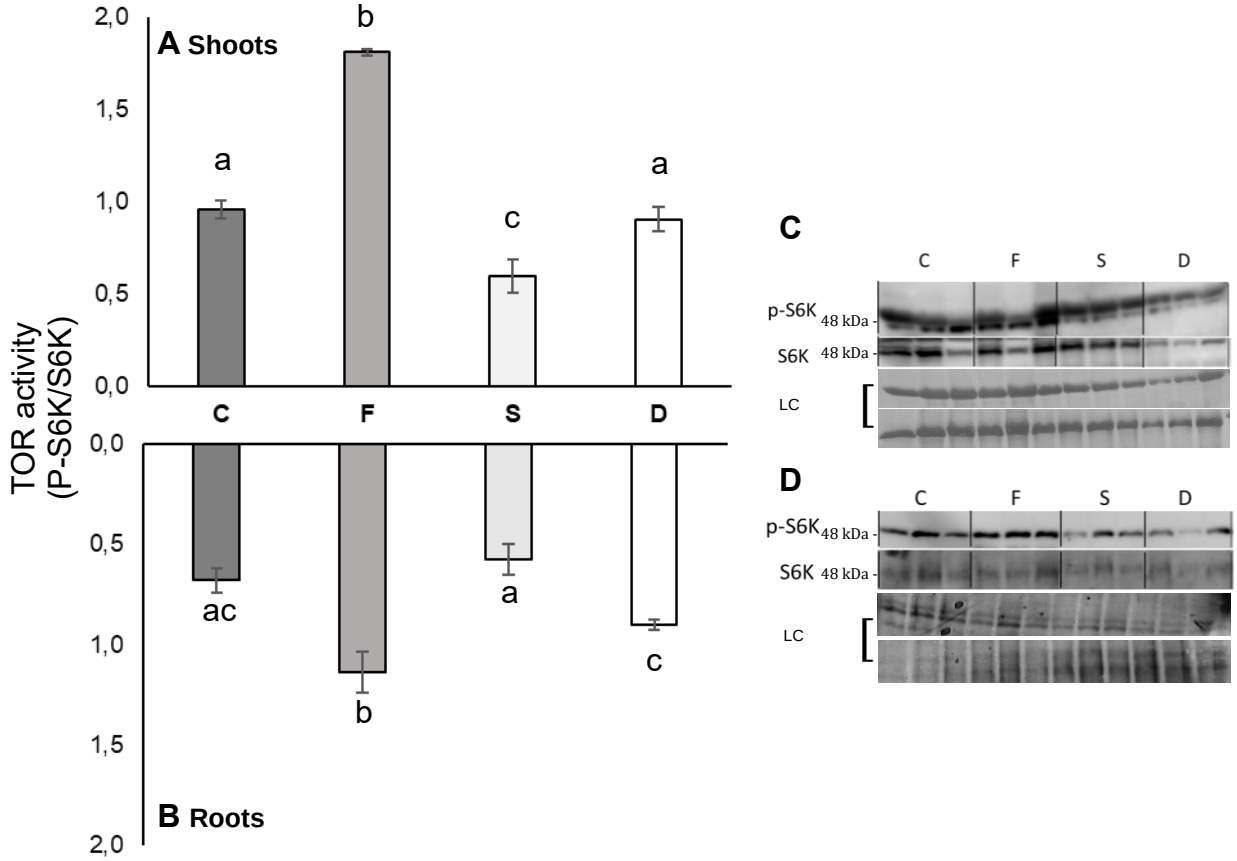


Fig. 1

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) condition. 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 ($p < 0.01$).

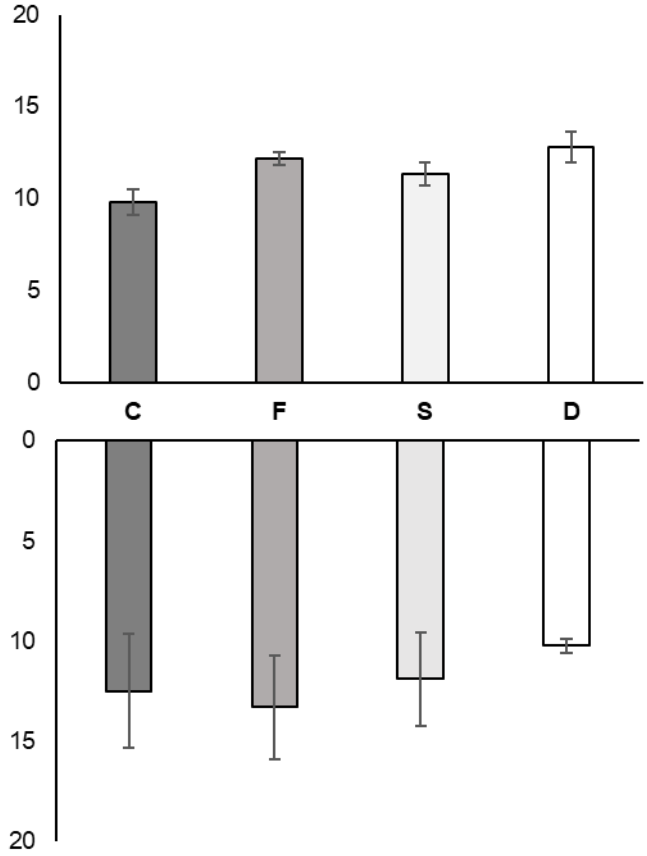


Fig. 2

Total soluble sugars concentration in shoot (A) and root (B) of tomato plants grown under control (C), Fe deficiency (F), S deficiency (S) and double deficiency (D) condition. Treatments and statistics as in Fig. 1.

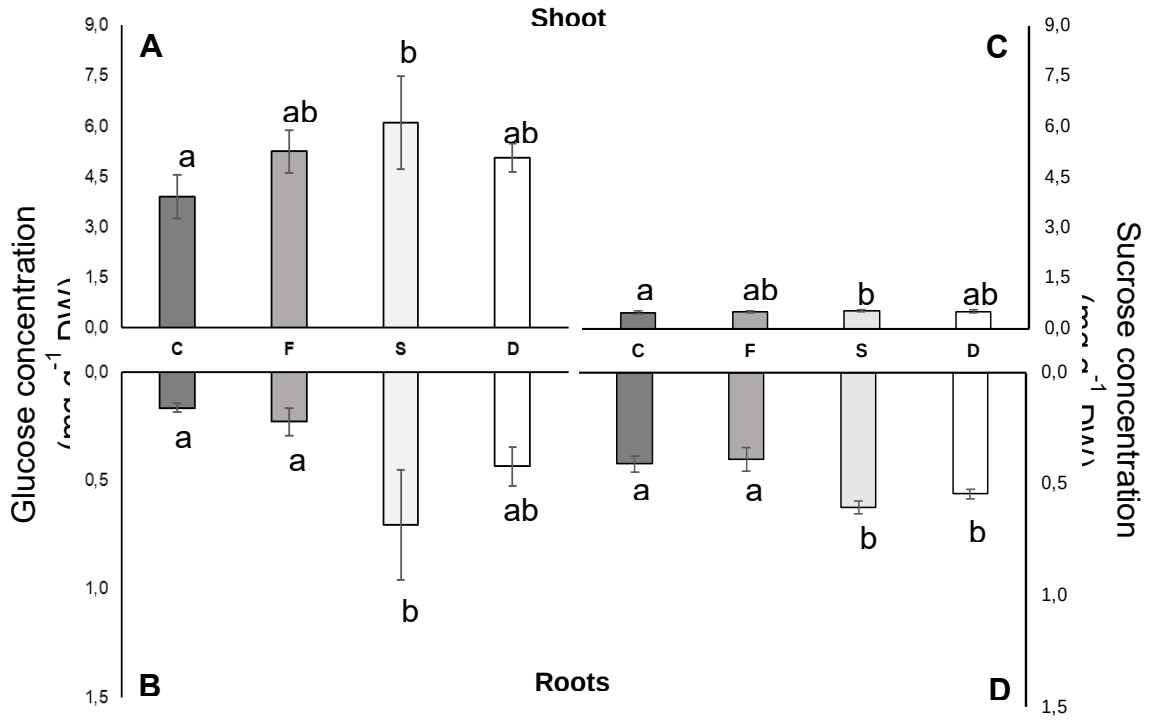


Fig. 3

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) condition. Treatments and statistics as in Fig. 1.

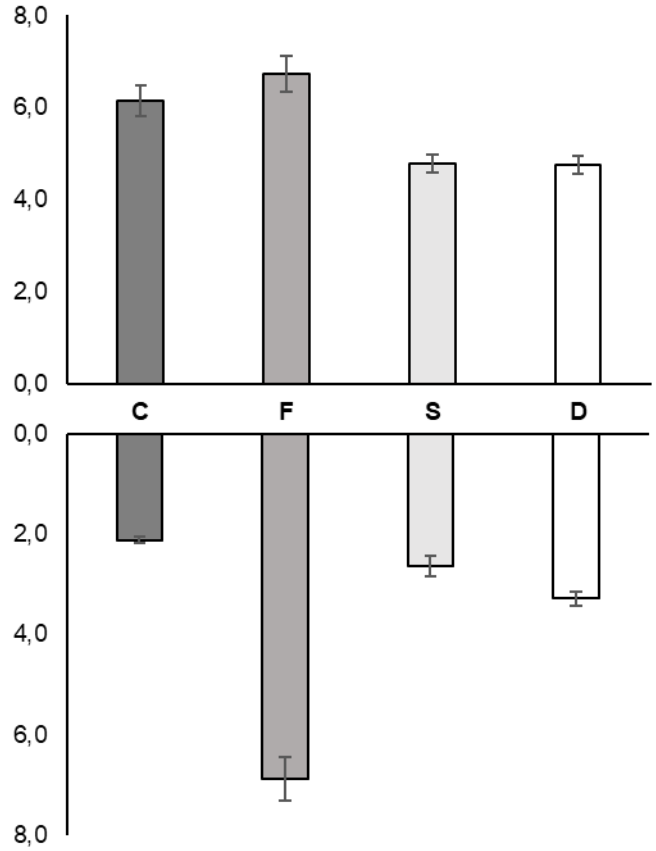


Fig. 4

Citric acid concentration in shoot (A) and root (B) of tomato plants grown under control (C), Fe deficiency (F), S deficiency (S) and double deficiency (D) condition. Treatments and statistics as in Fig. 1.

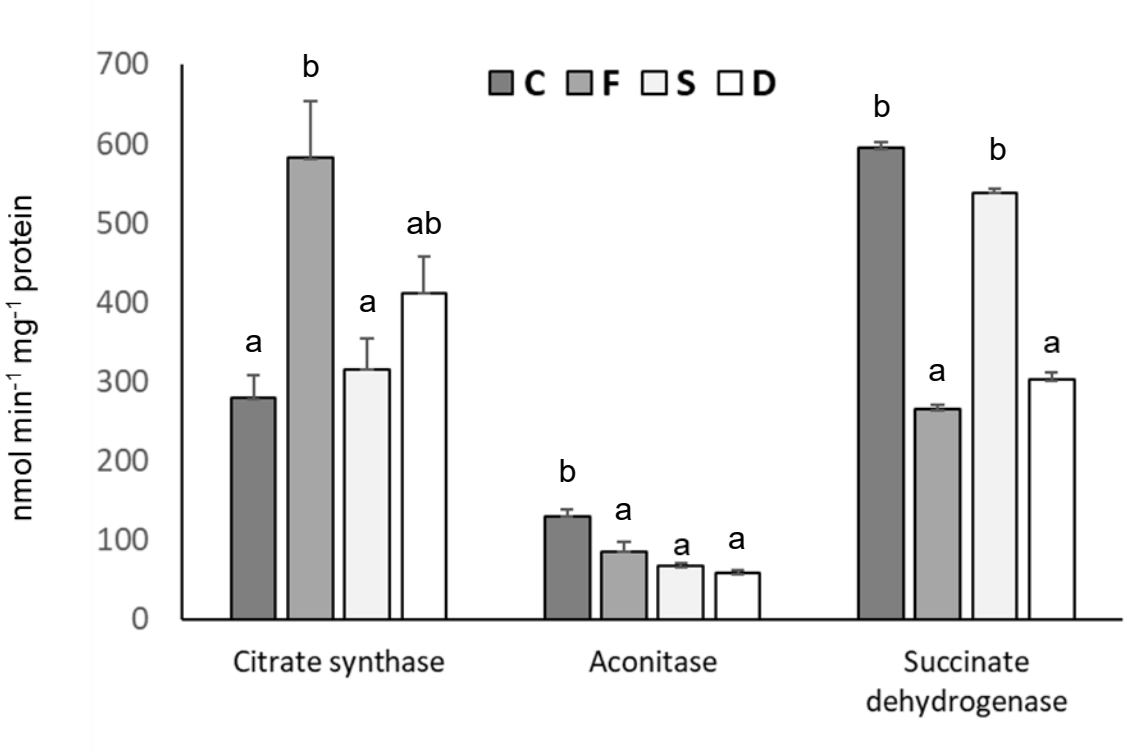


Fig. 5

Fig. 5

TCA cycle-related enzymatic activities in tomato roots. Citrate synthase, aconitase and succinate dehydrogenase activities in mitochondrial enriched fraction from plants grown under control (C), Fe deficiency (F), S deficiency (S) and double deficiency (D) condition.

Activities are reported as formation of TNB (citrate synthase), formation of NADPH (aconitase) and consumption of DCIP (see materials and methods section). Data are means ± SD of three independent replications. The statistical significance was tested by means of ANOVA with Tukey post-hoc test. Different letters indicate statistically different values ($p < 0.05$).

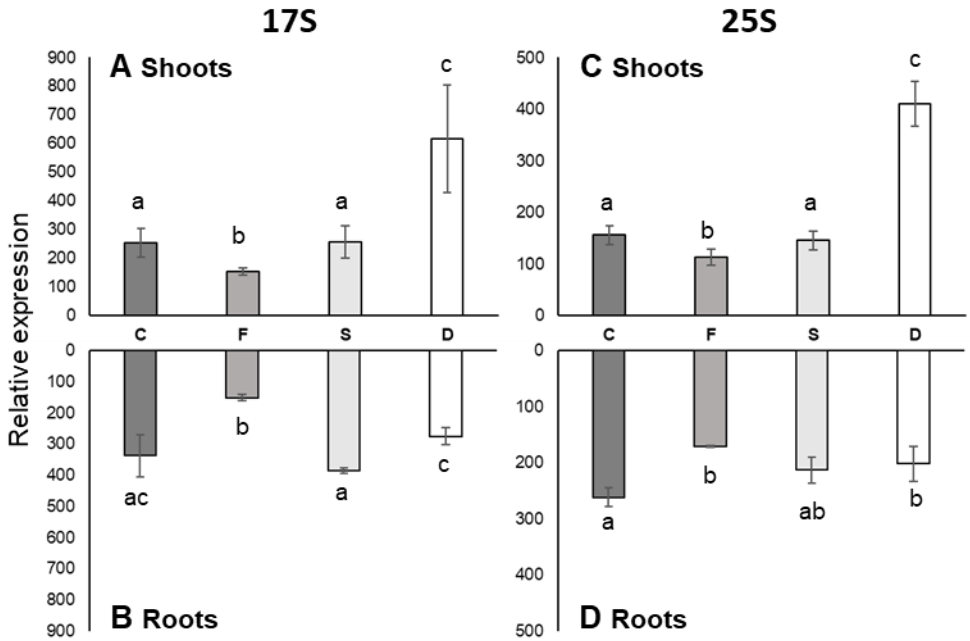
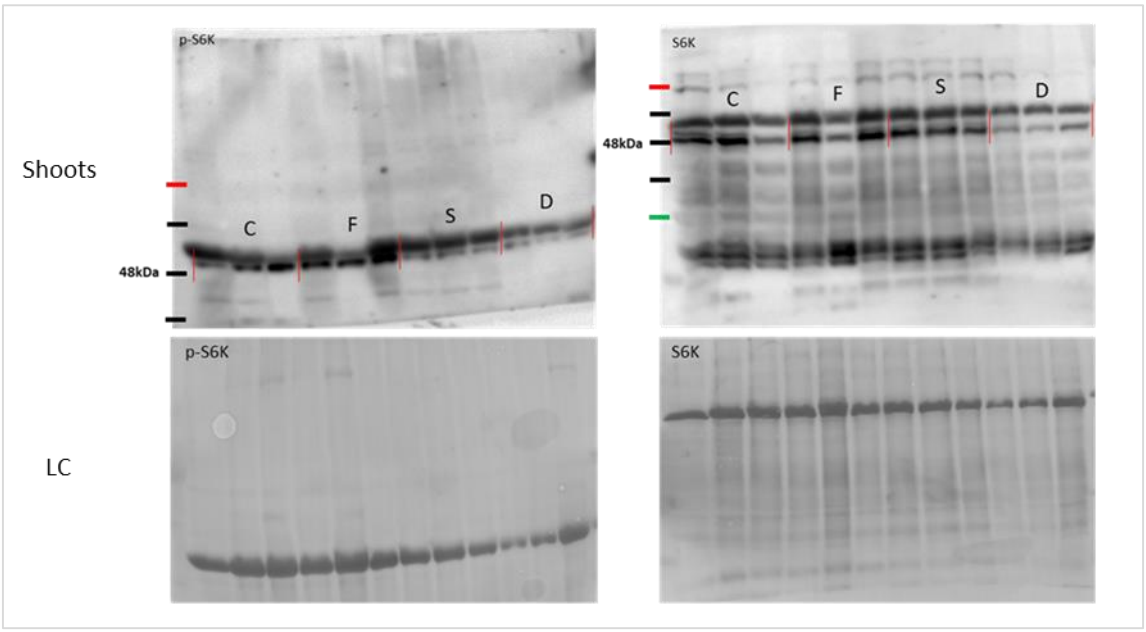
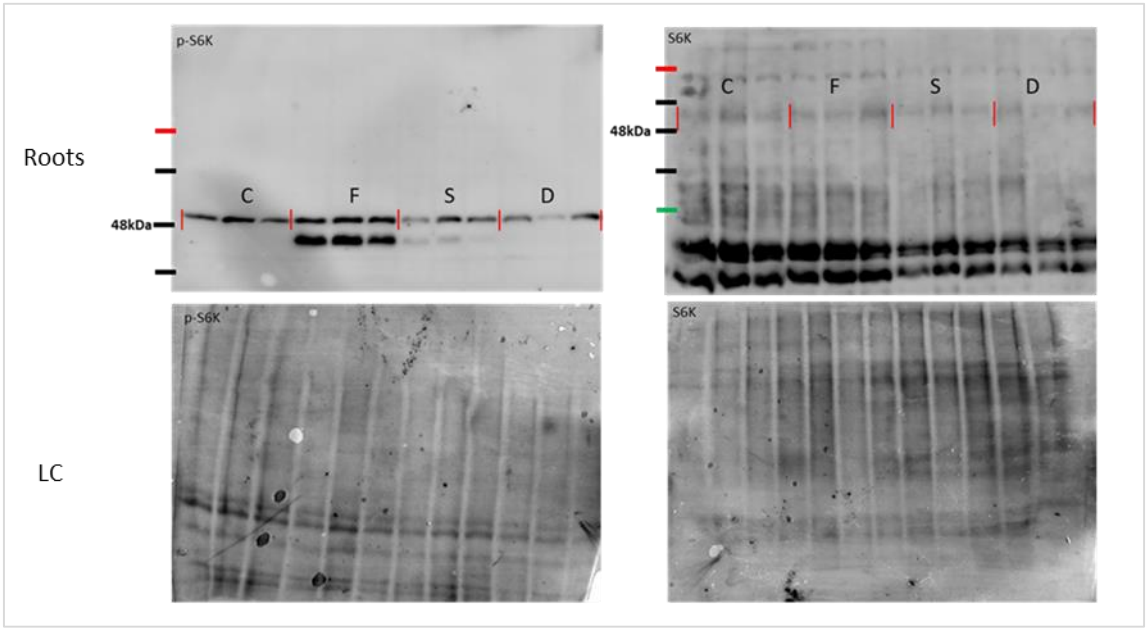


Fig 6

17S (A and B, respectively for shoots and roots) and 25S (C and D, respectively for shoots and roots) transcript levels were analysed by qPCR in tomato plants grown under control (C), Fe deficiency (F), S deficiency (S) and double deficiency (D) condition. Relative expression data was normalised against the expression of the reference gene *SlEFα1*. Statistics as in Fig. 1.





Supplementary Fig1

Aminoacids	F	S	D
Alanine	0,78	2,17	3,65
Valine	0,87	3,68	10,79
Isoleucine	0,91	3,85	12,48
Glycine	1,42	21,43	19,65
Proline	0,65	0,97	1,09
Serine	0,87	3,42	5,78
Threonine	1,00	2,90	6,26
Alanine, beta-	0,67	2,40	3,46
Serine, O-acetyl-	0,84	17,40	75,76
Proline, 4-hydroxy	1,15	5,04	14,32
Aspartic acid	0,56	0,56	0,49
Methionine	0,75	0,44	0,64
Glutamic acid	0,79	0,89	0,69
Phenylalanine	0,78	4,77	10,69
Asparagine	0,43	3,55	14,65
Glutamine	0,52	6,43	8,65
Arginine	0,61	8,22	25,11
Lysine	1,04	11,44	44,36
Glutamine	0,52	6,43	8,65
Arginine	0,61	8,22	25,11
Lysine	1,04	11,44	44,36
Ornithine	0,73	11,16	39,28
Tryptophan	0,61	4,11	12,59
Tyrosine	0,66	4,08	19,17

Aminoacids	F	S	D
Alanine, DL- (2TMS)	1,17	2,57	1,72
Valine, DL- (2TMS)	0,79	2,23	3,62
Isoleucine, L- (2TMS)	0,54	1,75	3,42
Proline, L- (2TMS)	0,80	1,22	1,69
Serine, DL- (3TMS)	0,89	1,68	1,32
Threonine, DL- (3TMS)	0,85	1,88	3,38
Alanine, beta- (3TMS)	0,82	2,11	1,45
Serine, O-acetyl-, DL- (2TMS)	0,95	17,98	15,64
GABA	1,09	1,50	1,45
Aspartic acid, L- (3TMS)	0,79	1,49	1,03
Methionine, DL- (2TMS)	0,52	0,52	0,52
Ornithine, DL- (4TMS)	1,02	3,90	7,60
Asparagine, DL- (3TMS)	0,58	6,43	22,79
Arginine	0,55	6,90	15,39
Lysine, L- (4TMS)	0,63	2,29	6,02
Glutamine, DL- (3TMS)	0,81	5,90	10,61
Tyrosine, DL- (3TMS)	0,53	2,00	2,49
Tryptophan, L- (2TMS)	0,45	1,06	2,10

Sugars	F	S	D
Ribose	1,01	1,39	1,50
Rhamnose	0,89	0,97	1,16
Fructose	1,05	1,15	1,02
Glucose	1,00	1,16	0,96
Inositol, myo-	1,01	1,41	1,52
Glucose-6-phosphate	0,83	0,83	0,54
Sucrose	1,07	1,11	1,07
Maltose	1,16	1,58	0,96

Sugars	F	S	D
Glycerol-3-phosphate, DL- (4TM	0,96	2,07	1,89
Glucose-6-phosphate (1MEOX) (	0,85	1,22	0,79
Fructose, D- (1MEOX) (5TMS)	1,23	5,02	3,89
Sorbitol, D- (6TMS)	0,91	1,14	1,46
Glucose, D- (1MEOX) (5TMS)	1,40	4,30	2,65
Saccharic acid (6TMS)	0,48	0,46	0,23
Inositol, myo- (6TMS)	1,35	2,15	5,01
Sucrose, D- (8TMS)	0,95	1,47	1,33

Supplementary Fig. 2

Linear correlations between TOR activity and the concentrations of reducing sugars (glucose-6-phosphate, fructose, maltose, ribose, rhamnose, sorbitol, glycerol-3-phosphate) and non-reducing sugars (galactinol) in the shoots and roots of tomato plants grown with different availability of iron (Fe) and sulfur (S): C, control; F, Fe deficiency; S, S deficiency; D, double deficiency of Fe and S (n.d.=not detected).

	TOR activity	
	Shoots	*Roots*
Glucose-6-phosphate	0,095	-0,821
Fructose	-0,337	-0,470
Maltose	-0,342	n.d.
Ribose	-0,643	n.d.
Rhamnose	-0,532	n.d.
Sorbitol	n.d.	-0,144
Glycerol-3-phosphate	n.d.	-0,478
Galactinol	n.d.	0,150

Supplementary Fig 3