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Revisiting Fe/S interplay in tomato: A split-root approach to study the systemic and local responses

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ABSTRACT

Based on our previous studies demonstrating an intriguing interplay between sulfur (S) and iron (Fe), a split-root experiment was performed to determine whether plant S status and/or S external concentration could modify plant capability to take up and accumulate Fe. This split-root system allowed the roots of each tomato plant to grow in two different compartments, both Fe-deficient, but one S-sufficient, and the other one S-free.

Although S was freely available to half root system and thus plant S status was preserved, S-deficient part of root apparatus exhibited a decrease of total S, thiols and protein content, an enhanced activity of both ATPsulfurylase and *O*-acetylserine(thiol)lyase, and a higher expression of *SlST1.1*, as occurring under S deficiency. The side of the root apparatus exposed to combined S and Fe deficiency, showed an over induction of the FeIII-reducing capacity (+40%) and of the expression levels of the gene codifying for this protein (*SlFRO1*), with respect to the Fe-deficient part of the root system. Interestingly, the regulation pattern of the *bHLH* transcription factor *SlFER*, controlling the expression of both *SlFRO1* and *SlIRT1* genes, was very close to that of *SlFRO1*. *SlIRT1* expression levels appeared unaffected by S supply, suggesting distinct regulatory processes targeting *SlFRO1* and *SlIRT1*.

1. Introduction

The lower Fe accumulation in leaves of S-deficient maize plants was the first clear indication of an intriguing relation between sulfur (S) and iron (Fe) acquisition in plants [1] and triggered a quite intense scientific production in the following years on this topic. It is interesting to note that the same response was recorded in other crops such as barley [2], durum wheat [3] and tomato [4], regardless of which mechanism is employed by plants to cope with Fe deficiency (*i.e.* Strategy I, or FeIII-reduction based mechanism, or Strategy II, or FeIII-chelation based mechanism) [5]. It should be highlighted that at the field scale multiple nutrient depletions are more common than single nutritional deficiencies and the response of plants to a combined deficiency is completely different when compared to the single one, at both physiological and molecular level [6,7]. The comprehension of the mechanisms underlying the responses to the combined deficiency are still mostly lacking, even if some theories have been postulated. In particular, it has been suggested that in grasses (Strategy II plants) the reduced Fe accumulation in plant tissues induced by S deficiency could be ascribed to a decrease in the production and release of phytosiderophores (PS) [2], whereas in tomato (a Strategy I plant) the effect was rather due to an impaired ethylene and nicotianamine (NA) production [4]. In addition, the involvement of sulfate in transcriptional regulation of cellular Fe homeostasis was described in both barley, in which *HvYS1* expression changed in response to S external supply [8], and tomato, in which S deficiency virtually abolished the expression of the NA synthase (*LeNAS*) gene and limited the expression of both *LeIRT1* and *LeFRO1* gene [4].

According to this theory, limited Fe availability results in a further sulfate demand that becomes the driving force which leading to an increased sulfate uptake and assimilation rate [6,9]. Interestingly, PS, ethylene and NA share a common precursor, namely S-adenosylmethionine (SAM), whose synthesis depends on the availability of methionine (Met) [10]. Furthermore, it has been shown in plants that Fe is normally linked with S when it is bound in Fe-S proteins suggesting that Fe-S clusters are the biggest sink for Fe within the plant [11]. However, higher S need to sustain the activation of Strategy I and II machinery cannot fully account for the observed link between the flow of these two essential nutrients in plant tissues, but also likely reflect a direct interference of Fe with the signal transduction pathway involved in S metabolism (and *vice versa*) and with the activation of different acquisition strategies.

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In this regard, evidences have been provided by using transcriptome [7] and metabolome-wide [6] approaches that Fe and S metabolism need to be coordinated to a certain extent, as *e.g.* maintenance of Fe-S-cluster formation and, on the other hand, being sulfur metabolism additionally involved in multiple processes of primary and secondary metabolism, they need also to be independently modulated. According to this model, an intensive overlap, as well as distinct responses by comparing the transcriptome and the metabolome of single and combined S and Fe starved plants has been observed [6,7,12]. Finally, only recently citrate has shown to represent a key role in the regulation of some Fe- and S-responsive genes, demonstrating the mitochondria involvement in the regulation of Fe and S interplay in plants [13].

In addition, an uneven nutrient distribution in soil is further complicating the understanding of what is happening at the soil/root interface. In this respect, all these studies have been carried out by exposing the whole root system to a single (Fe or S) or combined (Fe and S) nutritional stress, conditions that do not completely represent the heterogeneous availability of nutrients often present at the rhizosphere level. This aspect is of particular relevance also in relation to the extent, and the relative efficacy, of the root responses to nutritional deficiency. The aim of this study was to assess whether the S-nutritional state of the plant (systemic effect) and/or the S availability in a restricted part of the root system (local effect) could modify the plant capability to take up Fe and, then, to allocate it at the shoot level.

Therefore, a split-root hydroponic system was developed to allow the root system of each single tomato plant to grow in two different and independent compartments, both Fe-free, but half of the root apparatus was in a S-starved condition and the other one received S (1.2 mM sulfate). After one-week treatment, the root capability to cope with Fe deficiency was evaluated by analysing the FeIII-reducing activity and the expression of the *SlFRO1*, *SlIRT1* and *SlFER* genes. Changes in plant sulfate uptake and assimilation rate were investigated by analysing the expression of a high affinity sulfate transporter gene, *SlST1.1*, and the activity of ATPsulfurylase (ATPS) and *O*-acetylserine(thiol)lyase (OASTL), two S pathway-related enzymes.

2. Materials and methods

2.1. Plant growth conditions

Tomato (*Solanum lycopersicum* L., cv. Marmande) seeds were germinated in the dark at room temperature for five days and then seedlings were grown hydroponically in plastic pots containing 2.2 L of complete nutrient solution (NS) [14], being exposed to 1.2 mM sulfate and 40 μM FeIII-EDTA [4]. After two weeks, seedlings having root system of sufficient length and approximately equal size were chosen for planting in split-root (SR) containers filled with NS (Fig. 1). Each container consisted of two equal compartments, where each half of the tomato root system was allowed to grow separately being exposed to two different treatments: sole Fe deficiency (F, 1.2 mM sulfate and 0 μM FeIII-EDTA) and combined Fe and S deficiency (D, 0 mM sulfate and 0 μM FeIII-EDTA). The plants were grown an additional week in order to induce nutrient deficiency responses and then shoot and root samples were harvested (21 days after sowing). As control conditions, whole (W) tomato plants were also grown hydroponically being exposed to sole Fe deficiency (F condition, WF) and combined Fe and S deficiency (D condition, WD).

Nutrient solution was continuously aerated and renewed every 3 days. 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.

2.2. Determination of ferric chelate reductase activity

Root capability to reduce FeIII-EDTA was measured *in vivo* by using the bathophenanthrolinedisulfonate (BPDS) reagent [4]. Briefly, roots were carefully washed with deionized water, transferred to an assay solution containing 0.5 mM $CaSO_4$, 0.25 mM FeIII- EDTA, 0.6 mM BPDS, 10 mM Mes at pH 5.5 adjusted by 1 M KOH, and incubated in the dark at 25 °C for 20 min, continuously aerated. The absorbance of the assay solution was recorded at 535 nm and the amount of reduced FeIII was calculated by the concentration of the FeII-$BPDS_3$ complex using an extinction coefficient of 22.1 $mM^{-1}\ cm^{-1}$.

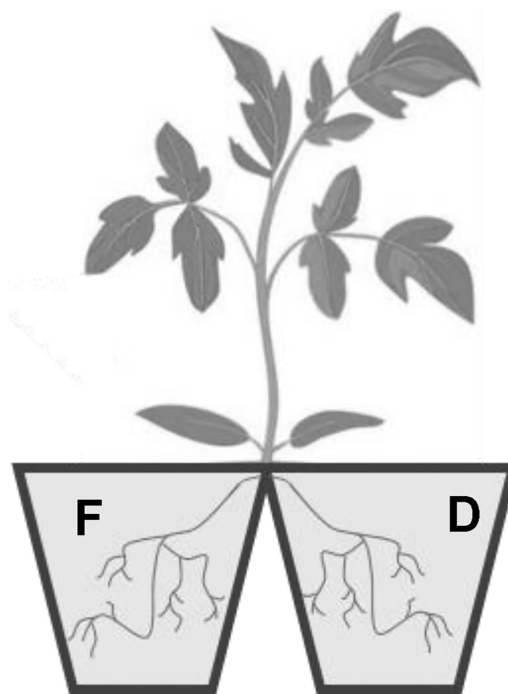

Fig. 1. Design of the split-root system. The terms used to describe the various parts of split-root plants are indicated in the text. F indicates condition where the Fe-deficient compartment (1.2 mM sulfate and 0 μM FeIII-EDTA), D condition of the dual deficient compartment (0 mM sulfate and 0 μM FeIII-EDTA).

2.3. Extraction of total RNA and qRT-PCR analysis

Total RNA extraction from F and D roots was performed by using Spectrum Plant Total RNA Kit (Sigma-Aldrich Co. LLC) according to the manufacturer's instructions.

One mg of DNase-treated RNA was reverse-transcribed by ImProm-II Reverse Transcription System kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

The expression of genes involved in Fe homeostasis (*SlFER, SlFRO1, SlIRT1)* and coding for a high affinity sulfate transporter (ST) of group 1 (*SlST1.1)* were analyzed by qRT–PCR as described in Zuchi et al. [6].

Nevertheless, the identity of each amplicon was determined by sequencing. The amplification efficiency was calculated from raw data using LinRegPCR software [15]. The expression of genes was normalized using two housekeeping transcripts, namely Tomato *LeEF-1* mRNA for elongation factor 1 alpha (X14449.1) and Tomato *Ubi3* gene for ubiquitin (X58253.1), previously described [13]. The relative expression ratio value was calculated for treated samples relative to the corresponding untreated sample at the same time-point according to the Pfaffl equation [16]. Standard error values were calculated according to Pfaffl et al. [17].

2.4. Total sulfur and non-protein thiols extraction and determination

For the determination of total S concentration, 1 g of each shoot and root sample was first oven-dried at 105 °C and then ashed in a muffle furnace at 600 °C. After complete mineralization, the ashes were

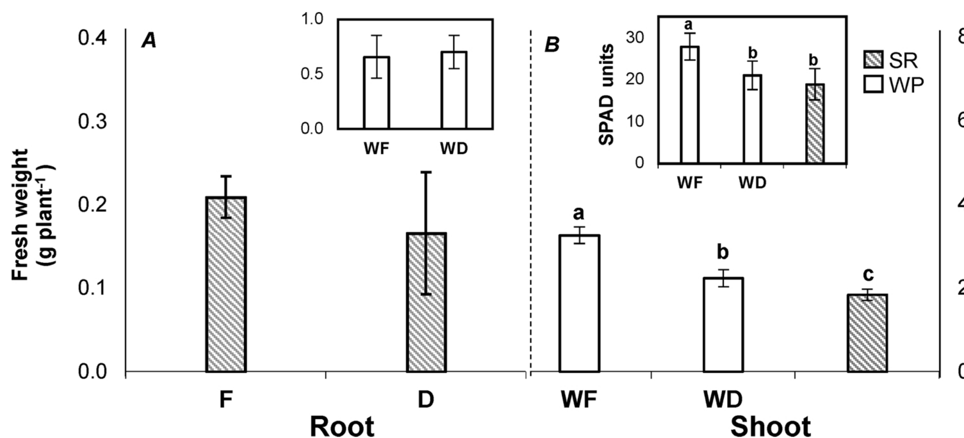

Fig. 2. Fresh weight of roots (A) and shoots (B) from split-root (SR) (lined bars) or not split-root (whole, W) (white bars) tomato plants grown under sole Fe deficiency (F and WF, 1.2 mM sulfate and 0 μM FeIII-EDTA) and under combined S and Fe deficiency (D and WD, 0 mM sulfate and 0 μM FeIII-EDTA). *Insert in A*: Fresh weight of roots from not split-root plants under Fe deficiency (WF) or dual deficiency (WD). *Insert in B*: Chlorophyll content, measured by SPAD meter, in leaf of split- and not split-root tomato plants. Data are means ± SD of four independent replications run in triplicate. Significant differences between samples are indicated by different letters ($P < 0.01$) ($n = 4$). Stars indicate significant differences between F and D (or WF and WD) samples of both split and not split-root tomato plants (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ and **** $P < 0.001$).

dissolved in 10 mL of 3 N HCl and filtered through Whatman No. 42 papers. In contact with $BaCl_2$, a $BaSO_4$ precipitate was formed, which was determined according to the turbidimetric method described by Bardsley and Lancaster [18].

Thiols extract was prepared from shoot and root tissues of plants grown as described above. Briefly, one g of frozen plant tissue was homogenized in a solution containing 80 mM trichloroacetic acid, 1 mM ethylenediaminetetraacetic acid, 0.15% (w/v) ascorbic acid and 10% (w/v) polyvinylpyrrolidone (PVP), in a ratio of 1:5 (w/v). After a centrifugation step of 30 min at 4000 g and 4 °C, the supernatants were collected, and the concentrations of DTNB-reactive compounds were detected by VIS absorption at 415 nm.

2.5. Enzyme extraction and assay

Shoot and root tissues (1 g fresh weight) were powdered in a pre-chilled mortar with liquid N_2 and extraction steps were performed according to the method described by Celletti et al. [18]. Briefly, extraction buffer contained 50 mM HEPES-KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), and 1% (w/v) polyvinylpyrrolidone (PVP) and was added in a ratio of 1:7 and 1:3 (w/v) for shoots and roots, respectively. The activity of ATP sulphurylase (ATPS; EC 2.7.7.4) and *O*-acetylserine(thiol) lyase (OASTL; EC 4.2.99.8) activity was determined by the bioluminescence technique and by detecting cysteine production, respectively, as described by Celletti et al. [19]. Protein concentration in the extracts of plant tissues was determined according to the dye-binding method of Bradford [20] using BSA as standard.

2.6. Citric acid extraction

Shoot and root tissues were powdered in a mortar with liquid N_2 and extracted with 10 mM H_2SO_4 using a ratio of 1:10 (w/v). The determination of the concentration of citric acid was performed by HPLC using a cation exchange column Aminex 87-H column (300 × 7.8 mm, 9 mm, Bio-Rad) using an isocratic elution with 10 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL min^{-1}. Citric acid was detected at 210 nm with a Waters 2998 photodiode array detector (Waters Spa, Italy). Standard acid was prepared as individual stock solutions and then prepared to give diluted reference standards. Citric acid was identified by comparing retention times of the unknown to the pure compound. Standards were purchased from Sigma–Aldrich (St. Louis, MO, United States).

2.7. Other measurements and statistics

The measurements of chlorophyll content per unit area was determined in the youngest fully expanded leaves by the SPAD porFaig apparatus (Minolta Co., Osaka, Japan) and presented as SPAD units.

Each reported value represents the mean ± SD of measurements carried out in triplicate and obtained from four independent experiments. All the data were statistically analysed using ANOVA and data from each treatment was then compared with the control treatment using Tukey's post hoc test at $P < 0.01$ by using the GraphPad InStat Program (version 3.06).

3. Results

3.1. Plant growth

Stimulation of root growth, and following reduction of shoot:root ratio, is one of the earliest and distinct symptoms of plant S deficiency [3,19]. When the performance of hydroponically grown split-root plants was evaluated, no difference in root growth was observed between the two portions of the split root system, one part subjected only to Fe deficiency (F) and the other grown under dual deficiency of Fe and S (D) condition (Fig. 1A). Interestingly, there was no difference in the root system development also considering not-split (whole, W) plants between roots of plants exposed only to the Fe deficiency (WF) or to the combined Fe and S shortages (WD) (Fig. 2A, insert). On the other hand, shoots of tomato seedlings exhibited obvious changes in phenotype, showing different total fresh biomass and chlorophyll levels depending on the nutrient regime and the growing system. In fact, the shoots of split-root plants displayed a reduced growth as compared to both shoots from not-split plants (WF and WD) (Fig. 2B) and leaf chlorosis when compared to shoots from Fe-deficient not-split plants (WF) (insert in Fig. 2B). The highest shoot fresh matter production was observed when tomato seedlings were cultivated without Fe and with S (WF condition). The reduction of biomass accumulation was greater in shoots from split-root plants than in those from whole plants grown in D condition (WD). Accordingly, the latter were reduced by 30%, whereas the former by 44% when compared to Fe-deficient shoots (WD) (Fig. 2B). The same result occurred with the chlorophyll level, which was highest in WF seedlings, but in this case no significant difference in chlorophyll content was observed between shoots from split-root plants and those from whole plants grown in D condition (WD) (insert in Fig. 2B).

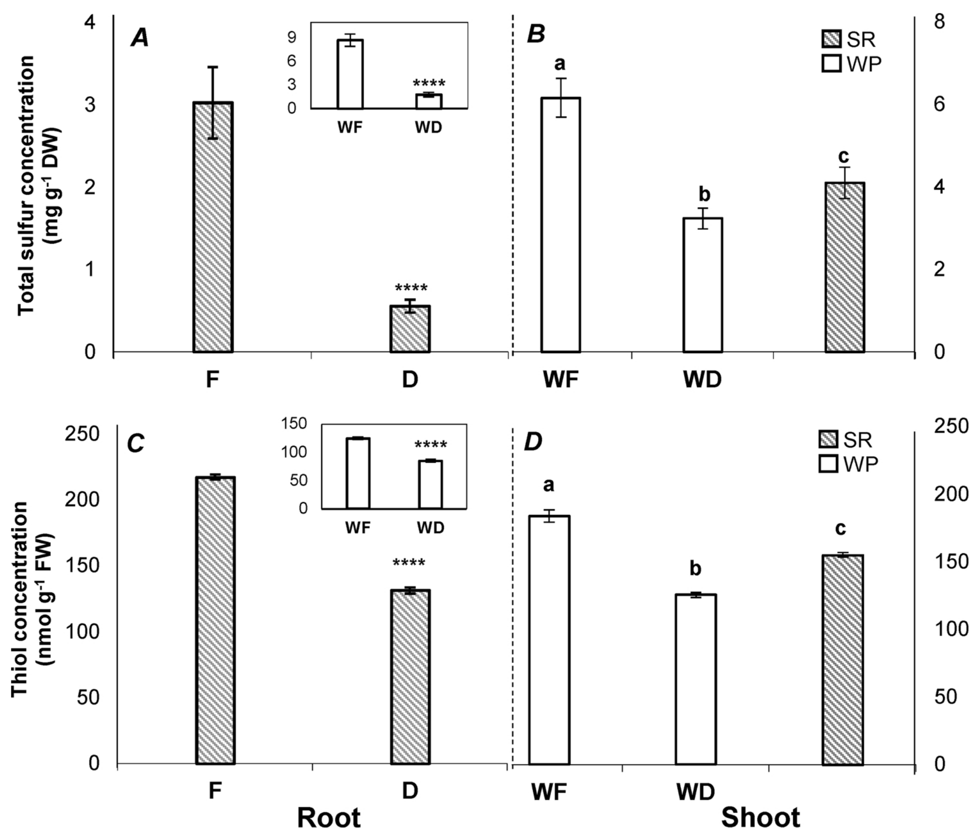

Fig. 3. Total sulfur (A and B) and thiol (C and D) concentration in roots and shoots of split- and not split-root tomato plants. Terms, treatments and statistics as in Fig. 2.

3.2. Accumulations of total S and thiols and activities of enzymes involved in S metabolism

As might be expected, in roots (insert in Fig. 3A and C) and shoots (Fig. 3B and D) from not-split plants under dual deficiency (WD) the concentration of both total S and thiols was significantly lower (approximately 80 and 30%, respectively) compared to that of the Fe-deficient plants (WF). Interestingly, in split root plants the different availability of sulfate in the two root compartments caused a different content of both total S (Fig. 3A) and thiols (Fig. 3C) between the two sides of root system, in spite of belonging to the same plant. As expected, the lowest levels of total S and thiols concentrations (-80% and -40%, respectively) were measured in the portion of the root system exposed to combined S and Fe deficiency (D) (Fig. 3B and D). It is worth to notice that in the shoot tissue of split-root plants the levels of the two parameters (total S and thiols concentrations) were significantly higher than those measured in the not-split-root plants exposed to the double deficiency (WD) (Fig. 3B and D).

To understand why total S and thiols concentration changed under single (F) and combined deficiency (D) stress we monitored the sulfate uptake and assimilation steps.

First, we investigated the modulation of the mechanisms underlying the S uptake process determining the transcript levels of the gene *SlST1.1* codifying for a high affinity sulfate transporter, whose expression is known to be modulated by the S availability. Indeed, transcript levels of *SlST1.1* in the roots were much higher for dual deficient (WD) plants than that of the Fe-deficient (WF) (insert in Fig. 4). Interestingly, *SlST1.1* expression was up-regulated also in the D portion of the root apparatus of split-root plants (Fig. 4). In particular, the level of *SlST1.1* transcripts in D roots of split-root plants was three-fold higher than in F half root system (Fig. 4).

Next, we monitored the activities of enzymes involved in S metabolism: ATPS and OASTL (the first and the last enzyme involved in the S assimilatory pathway, respectively). the activity of both ATPS and OASTL was significantly increased (+25%) in the root portion exposed to S shortage (D condition) with respect to that only Fe-deficient (F condition) (Fig. 5A and C). On the other hand, we found that when not split-root plants were treated with dual deficiency (WD), only the root ATPS activity was increased (+45%, insert in Fig. 5A), whereas OASTL activity was significantly decreased (−27%, insert Fig. 5C) with respect to only Fe-deficient plants (WF). In shoots, the activity of OASTL was not significantly affected by different nutritional conditions (Fig. 5D), whereas the levels of ATPS activity was significantly augmented only in the shoots of split plants (+60% and +53% when compared with both WF and WD shoots, respectively) (Fig. 5B).

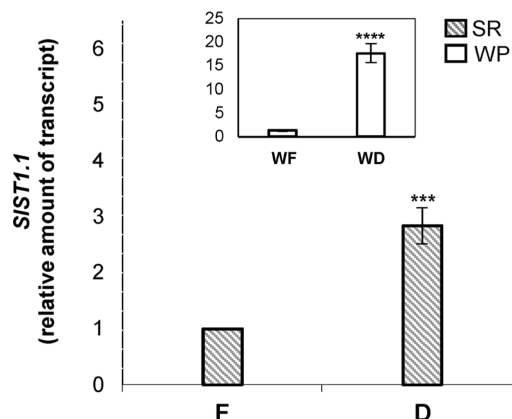

Fig. 4. Relative amount of transcript by qRT-PCR of *SlST1.1* gene in roots from split- and not split-root tomato plants. Terms, treatments and statistics as in Fig. 2.

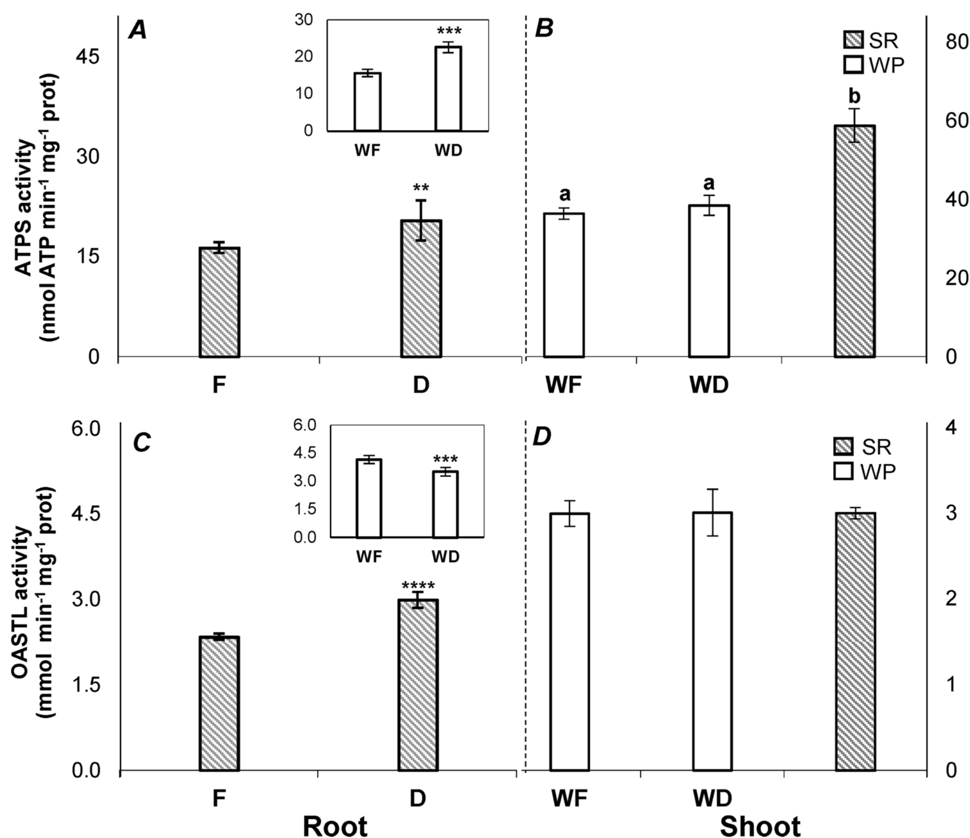

Fig. 5. ATPS (A and B) and OASTL (C and D) activity in roots and shoots of split- and not split-root tomato plants. Terms, treatments and statistics as in Fig. 2.

3.3. Iron homeostasis

Iron deficiency stress, sole or in combination with S starvation, affected the transcript levels of genes involved in Fe homeostasis. Changes in expression of *SlFER, SlFRO1* and *SlIRT1* were diverse. The expression levels of both *SlFER,* the ortholog in tomato of FIT (FE DEFICIENCY-INDUCED TRANSCRIPTION FACTOR) and *SlFRO1* (FERRIC REDUCTASE OXIDASE 1) were strongly up-regulated (to 60 and 2 folds, respectively) in the portion of the root system exposed to the dual deficiency (D side) with respect to the root portion only subjected to the Fe shortage (F side) (Fig. 6A and B). On the other hand, *SlIRT1* expression did not change between the two distinct portions of the root system (Fig. 6C).

In addition, we analyzed the root specific expression of the same key genes involved in Fe homeostasis also in not split-root plants. Surprisingly, no difference in the expression level of the *SlFER* and *SlFRO1* genes was measured in roots of not split-root plants between the treatment of sole Fe- (WF) and dual- (WD) deficiency (inserts in Fig. 6A and B). On the contrary, a higher amount of *SlIRT1* transcripts was measured in WD roots in comparison to WF ones (1.5-fold increase, insert in Fig. 6).

Having observed changes in expression of *SlFRO1* which encode for the FeIII-chelate reductase, we evaluated also the root FeIII-reducing capacity. We found a significant increase in *in vivo* assayed FeIII reducing capacity in D roots of split-root plants with respect to F ones (+40%) (Fig. 7). It has to be pointed out that this response pattern was very contrasting to that displayed by roots of not split-root plants exposed to combined Fe and S deficiency (WD), which indeed showed a strong reduction of the activity of this enzyme (−25%) (insert in Fig. 7).

It is well known that Fe deficiency induces production of organic acids (citric acid, malic acid) in plants [5] and it has been recently suggested that citric acid could also have a role as an important player in the regulation of adaptive responses to the combined S/Fe deficiency [13], in addition to the well-known role for the long-distance transport of Fe in plants [5].

Results here reported showed a strong increase of citrate concentration in D portion of the root system (+47%, with respect to F one) (Fig. 8A). In contrast, in not split-root plants citrate accumulation was significantly reduced in roots exposed to dual deficiency (WD) (−55%, with respect to WF roots) (insert in Fig. 8A). It is interesting to note that the citrate concentration was significantly but only slightly decreased (6%) in the shoots of WD plants with respect to WF ones (Fig. 8B), whereas in the shoots of split-root plants the concentration of this organic acid was more than halved (−55%) in comparison to the one measured in both WF and WD shoots of not split-root plants (Fig. 8B).

4. Discussion

It has been demonstrated that the enhanced sulfate uptake and assimilation rate are crucial to allow the onset of the typical responses to Fe deficiency in dicots (Strategy I) [4]. Contrariwise, the capability of these plants to cope with the Fe nutritional disorder is seriously hampered by the simultaneous imposition of S deficiency [4]. Following up on these previous findings, this work was addressed at studying the development of the typical root responses to Fe deficiency when the availability of S is not guaranteed homogeneously on the entire root system. In parallel, the mechanisms underlying S homeostasis necessary for the Fe shortage responses in plants sensing the S deficiency only in a specific root area but containing an adequate S content in the whole plant tissues, were also considered in order to decipher the long- or short-distance signaling triggering the onset of the plant response to the nutritional stresses. To this purpose, a split-root hydroponics system was used to grow the root system of each single tomato plant in two separated and independent compartments, both Fe-free, but only one

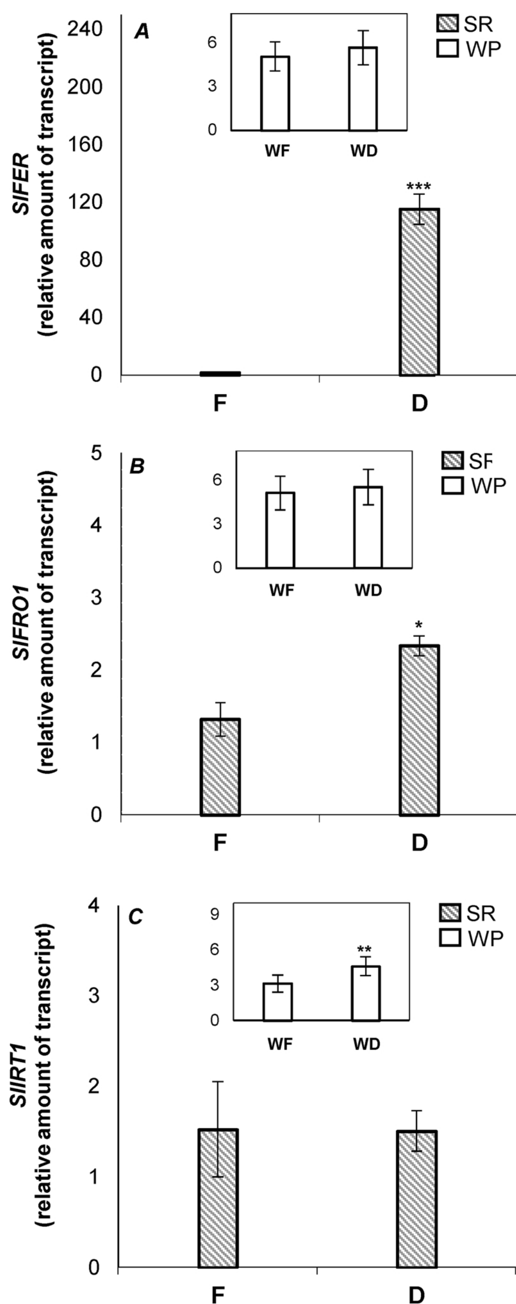

Fig. 6. Relative amount of transcript by qRT-PCR of *SlFER* (A), *SlFRO1* (B) and *SlIRT1* (C) genes in roots of split- and not split-root tomato plants. Terms, treatments and statistics as in Fig. 2.

root half was starved (combined S and Fe deficiency, D) while the other root half was supplied with sulfate (single Fe deficiency, F).

Stimulation of root growth, and following reduction of shoot:root ratio, is one of the earliest and distinct symptoms of plant S deficiency [3,19]. When the performance of hydroponically grown split-root plants was evaluated, no difference in root growth was observed between the two portions of the split root system, one part subjected only to Fe deficiency (F) and the other grown under dual deficiency of Fe and S (D) condition (Fig. 2A). In other words, the uneven S availability did not cause a different developmental rate of the root tissue. Interestingly, there was no difference in root system development also considering not-split (whole) plants between roots of plants exposed only to the Fe deficiency (WF) or to the combined Fe and S shortages (WD) (Fig. 2A, insert). On the other hand, shoot biomass production changed depending on the nutrient regime and the growing system. In

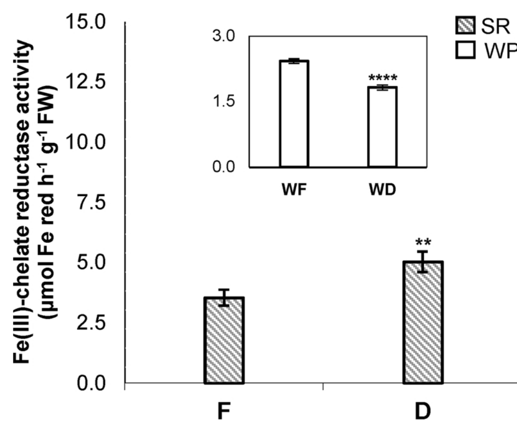

Fig. 7. FeIII-chelate reductase activity in roots of split- and not split-root tomato plants. Terms, treatments and statistics as in Fig. 2.

fact, the shoots of split-root plants displayed a reduced growth as compared to both shoots from not-split plants (WF and WD) (Fig. 2B) and leaf chlorosis when compared to shoots from Fe-deficient not-split plants (WF) (insert in Fig. 2B).

The reduction of biomass accumulation was greater in shoots from split-root plants than in those from whole plants grown in D condition (WD). On the other hand, no significant difference in chlorophyll content was observed between shoots from split-root plants and those from whole plants grown in D condition (WD) (Fig. 3).

As might be expected, S starvation induced a significant reduction of both total S and thiols concentrations in roots and shoots from not-split plants (Fig. 3). Interestingly, the same response (*i.e.* the lowest levels of total S and thiols concentrations) was found in split root plants in the portion of the root system exposed to combined S and Fe deficiency (D) (Fig. 3). It is worth to notice that in the shoot tissue of split-root plants the levels of the two parameters (total S and thiols concentrations) were significantly higher than those measured in the not-split-root plants exposed to the double deficiencies (WD) (Fig. 3B and D). This result clearly indicates that the supply of S, even if localized at only one side of the root, is likely to contribute to the nutritional S state of the aerial part of the plant. However, given that the levels of both parameters in shoots of split-root plants were significantly lower than those measured in not split-root plants exposed only to Fe deficiency (WF), it provides clear evidence that the localized application of sulfate was not able to adequately match S nutritional level similar to that of plants provided with sulfate uniformly available for the whole root system.

It is quite reasonable to expect that the reduced accumulation of total S and thiols in D side of root system of split-root plants could be most likely correlated with changes in S uptake and assimilation rate. Therefore, we investigated the modulation of the mechanisms underlying the S uptake process determining the transcript levels of the gene *SlST1.1* codifying for a high affinity sulfate transporter, whose expression is known to be modulated by the S availability [21,22,23].

The increased transcript levels of *SlST1.1* found in root tissues of not split-root plants exposed to the dual deficiency (WD) is a typical response to S deficiency. It is interesting to note that *SlST1.1* expression was up-regulated also in the D portion of the root apparatus of split-root plants (Fig. 4), suggesting a localized increased sulfate uptake capacity. On the other hand, withdrawal of S locally applied to one root portion (D) did not alter that *SlST1.1* expression, and thus S uptake capability, in the S-sufficient root portion (F) of the split-root system, as previously reported [24,25]. However, the increase in transcript abundance of *SlST1.1* in the roots under S deficiency was much higher for WD plants than that of the D side of split root plants (Fig. 4). It is possible that sulfate provision by the Fe deficient root half or the shoot mentioned above may be partly responsible for the inhibition of *SlST1.1* expression. Therefore, it remains a challenge to determine how plants sense

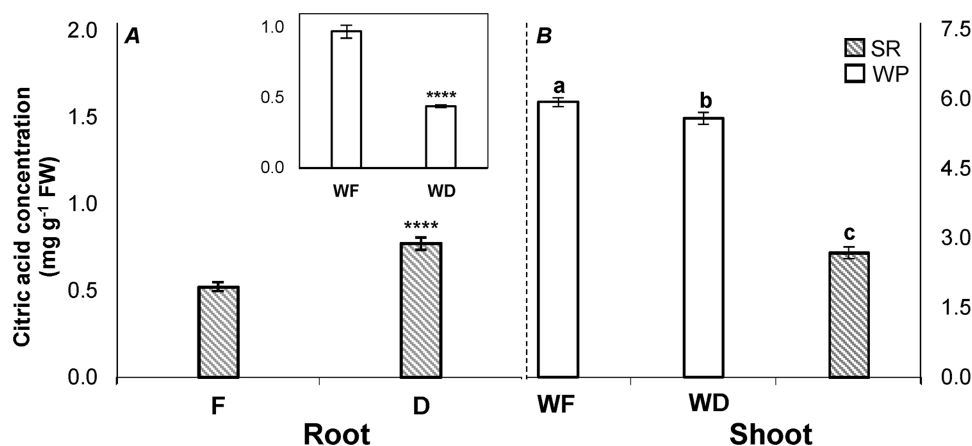

Fig. 8. Citric acid concentration in roots and shoots of split- and not split-root tomato plants. Terms, treatments and statistics as in Fig. 2.

changing environment and manage their different uptake rates of sulfate.

The activity of ATPS and OASTL (the first and the last enzyme involved in the S assimilatory pathway, respectively) has been used as an indicator to evaluate changes of the S assimilation rate (Fig. 5). According to what has been described for the levels of *SlST1.1* transcripts, also the activity of both ATPS and OASTL was significantly increased in the root portion exposed to S shortage (D condition) with respect to that only Fe-deficient (F condition) (Fig. 5A and C). On the other hand, we found that when not split-root plants were treated with dual deficiency (WD), only the root ATPS activity was increased (insert in Fig. 5A), whereas OASTL activity was significantly decreased (insert Fig. 5C) with respect to only Fe-deficient plants (WF). Interestingly, the modulation of the two enzymes at the shoot level ascribable to the treatments appeared quite different: only ATPS activity was significantly augmented in the shoots of split plants (+60% and +53% when compared with both WF and WD shoots, respectively), whereas OASTL activity did not change irrespective of different nutritional conditions (Fig. 5B and D).

It is well known that the uptake and assimilation of most major nutrients including sulfate is governed by the nutritional status of the plant [23]. Accordingly, sulfate influx in roots, the expression of genes encoding different sulfate transporters and many enzymes involved in sulfur metabolism, as well as their activity, are regulated by signals that respond to the nutrient status of the plants [26].

The results of this work clearly show that the uneven distribution of a nutrient along the root system, does not only differently affect the response of the plant to the nutritional stress linked with the availability of this specific element, but can also affect the efficiency of the response to the shortage of other elements. It was found a positive modulation of the mechanisms underlying both the uptake and the assimilation of sulfate took anyway place in the S-deficient portion of root system (D side), although S was supplied to a portion of the root system (F side) thus reasonably guaranteeing the S-nutritional status of the whole plant. Accordingly, the higher accumulation of total S and thiols in shoots of split-root plants (+25% for both parameters) with respect to those from not-split root plants exposed to dual deficiency (WD) is most likely the result of both the S acquisition by the part of root supplied with the nutrient (F) and the satisfaction of the plant S nutritional needs (Figs. 4 and 5).

These findings suggest that S uptake, assimilation and accumulation are closely regulated in response to the local nutrient availability in the growth medium rather than as a consequence of the plant S nutritional status, since in split-root system, half root apparatus is in a S-deprived condition (D side) whereas the other receives S (F side), providing adequate amounts of the nutrient in the shoots. Thus, the increased S demand to cope with Fe starvation condition [4,6] might, at least in part, explain the phenomenon. For this reason, the modulation of the mechanism underlying the acquisition of Fe has been also evaluated in these growing conditions focusing on Fe-deficiency-induced gene expression patterns.

It is well known that Strategy I plants cope with Fe deficiency *via* the rhizosphere acidification and the reduction of sparingly soluble FeIII [27,28,29,30]. These steps involve two enzymatic activities located at the root surface, the plasma membrane (PM) H^+ATPase which catalyzes the active extrusion of protons in the rhizosphere and the FeIII -chelate reductase (FRO1) mediating the reduction of soil Fe^{3+} to soluble Fe^{2+}. Once reduced, Fe^{2+} is then taken up into root cells *via* the high-affinity Fe^{2+} transporter (IRT1) [31]. It has been also demonstrated that Fe shortage induces the ethylene production of roots playing an important role in the modulation of the FeIII-reducing capacity [32,33].

Our earlier work showed that S-deficient tomato plants were quite unable to cope with Fe-deficiency [4], since increases in both ethylene production and FeIII-chelate reductase activity were prevented. Moreover, the transcript levels of *LeFRO1* and *LeIRT1* genes, codifying for the reductase and for the Fe^{2+} transporter, respectively, were severely affected by the imposition of the dual (S and Fe) deficiency [4].

The variation of expression of Fe deficiency marker genes (*SlFER, SlFRO1, SlIRT1*) after quantitative RT-PCR analysis are summarized in the graphs given in Fig. 6. Both *SlFER,* the ortholog in tomato of FIT (FE DEFICIENCY-INDUCED TRANSCRIPTION FACTOR) and *SlFRO1* (FERRIC REDUCTASE OXIDASE 1) were strongly up-regulated in the D portion of the root system with respect to the root portion only subjected to the Fe shortage (F side) (Fig. 6A and B), whereas *SlIRT1* expression did not change between the two distinct portions of the root system (Fig. 6C).

Surprisingly, no difference in the expression level of the *SlFER* and *SlFRO1* genes was measured in roots of not split-root plants between the treatment of sole Fe- (WF) and dual- (WD) deficiency (inserts in Fig. 6A and B). On the contrary, a higher amount of *SlIRT1* transcripts was measured in WD roots in comparison to WF ones (insert in Fig. 6C). Furthermore, the up-regulation of *SlFRO1* expression was associated by significant increase in *in vivo* assayed FeIII reducing capacity found in D roots of split-root plants with respect to F ones (Fig. 7). Thus, these results seem to indicate that, in split-root plants, unexpectedly, a more efficient response to the Fe deficiency is taking place in the portion of the root system subjected to the combined deficiency (D side, Fig. 7). It has to be pointed out that this response pattern was very contrasting to that displayed by roots of not split-root plants exposed to combined Fe and S deficiency (WD), which indeed showed a strong reduction of the activity of this enzyme (−25%) (insert in Fig. 7), as was observed in previous experiences [4]. The expression patterns of Fe deficiency marker genes (*SlFER, SlFRO1, SlIRT1*) led us to suggest that the two

different steps of Strategy I mechanism (uptake and reduction) could be regulated by different mechanisms among which Fe deficiency is not the sole regulating factor. In particular, it is likely that *SlIRT1* might be controlled by regulatory mechanisms more complex with respect to *SlFRO1*.

The strong induction of transcription of *SlFRO1* gene followed by a significant increase of the enzyme activity observed in the portion of root system exposed to the dual deficiency (D) makes plausible to argue the intervention of other signal mechanisms regulating the development of the adaptive responses, mediating the expression of genes and consequently the activity of enzymes.

Indeed, very recently it has been hypothesized that intracellular citrate could act as a signaling molecule affecting the expression level of several genes [13].

It is well known that Fe and S closely interact for the building of Fe–S clusters, whose assembly takes place mainly in the mitochondria [11]. Therefore, mitochondria might play a pivotal role in the regulation of Fe and S interaction. The Fe deficiency-induced alteration in the synthesis of mitochondria-derived carboxylic acids, such as citric acid [6], and the evidence that such molecules have already been identified as important players of metabolite signalling in several organisms, further support this hypothesis [34,35,36,37]. Thus, it is possible this also occurred in our study: the increased citrate accumulation in D portion of the root system (Fig. 8) was found to be positively correlated with Fe(III) reducing capacity (Fig. 7), as well as reduction in citrate accumulation in roots of not split root plants exposed to dual deficiency (WD) (insert in Fig. 8) correlated with lower Fe(III) reducing capacity in the same plants (insert in Fig. 7). However, it cannot be ruled out the possibility that the changes in citrate concentration could be attributed to a redistribution of the Fe tissue concentration. Further studies are required and are ongoing in order to fully elucidate the mechanisms behind such differential accumulation of citrate between WD and D plants.

In conclusion, the split-root system developed in this work also integrates a better knowledge of how and to what extent the root system is able to adjust nutrient acquisition capacity to meet variations in shoot demand or in external nutrient availability, an aspect that requires special attention in the frame of plant mineral nutrition.

However, it remains a challenge to determine how the interplay between S and Fe is regulated and how plants sense environmental nutrient fluctuations and manage their different uptake, translocation, assimilation, and signaling. Indeed, although it is well known that Fe deficiency response can be hampered by S starvation [4], results of the present work indicated that the supply of S in specific portion of the root system (F side) can allow the onset of the Fe deficiency response also in those root zones not adequately supplied with S (D side). More importantly, being the Fe response over-activated in such root zones, where, on the contrary, it should be limited on the basis of what described in literature [4] this phenomenon suggests that a local response signal can be ruled out.

Therefore, the interaction S/Fe must include a complex signaling pathways to be further studied. However, the results led us to conclude that this may be partly related to the role of citric acid in the cross talk of signalling pathways required for the adaptation of the plants to combined deficiency, as recently hypothesized [13].

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