Sulphur deprivation limits Fe-deficiency responses in tomato plants

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Abstract The aim of this work was to clarify the role of S supply in the development of the response to Fe depletion in Strategy I plants. In S-sufficient plants, Fe-deficiency caused an increase in the Fe(III)-chelate reductase activity, ^{59}Fe uptake rate and ethylene production at root level. This response was associated with increased expression of *LeFRO1* [Fe(III)-chelate reductase] and *LeIRT1* (Fe^{2+} transporter) genes. Instead, when S-deficient plants were transferred to a Fe-free solution, no induction of Fe(III)-chelate reductase activity and ethylene production was observed. The same held true for *LeFRO1* gene expression, while the increase in $^{59}\text{Fe}^{2+}$ uptake rate and *LeIRT1* gene over-expression were limited. Sulphur deficiency caused a decrease in total sulphur and thiol content; a concomitant increase in $^{35}\text{SO}_4^{2-}$ uptake rate was observed, this behaviour being particularly evident in Fe-deficient plants. Sulphur deficiency also virtually abolished expression of the nicotianamine synthase gene (*LeNAS*), independently of the Fe growth conditions. Sulphur deficiency alone also caused a decrease in Fe content in tomato leaves and an increase in root ethylene production; however, these events were not associated with either increased Fe(III)-chelate reductase activity, higher rates of ^{59}Fe uptake or over-expression of either *LeFRO1* or *LeIRT1* genes. Results show that S defi-

ciency could limit the capacity of tomato plants to cope with Fe-shortage by preventing the induction of the Fe(III)-chelate reductase and limiting the activity and expression of the Fe^{2+} transporter. Furthermore, the results support the idea that ethylene alone cannot trigger specific Fe-deficiency physiological responses in a Strategy I plant, such as tomato.

Keywords Ethylene · Iron deficiency · Iron uptake · Sulphur deficiency · Strategy I · Tomato

Abbreviations

DW Dry weight
NS Nutrient solution
SAM S-adenosylmethionine

Introduction

To cope with low Fe availability in the soil, higher plants have evolved two complex mechanisms: Strategy I (all plants, except Gramineae) and Strategy II (Gramineae) (Marschner et al. 1986; Römhild 1987). Strategy I plants can mobilize and take up Fe^{3+} ions from soil particles by acidification of the rhizosphere, probably driven by an increased plasma membrane H^+ ATPase activity (Dell'Orto et al. 2000), by enhancing the activity of a plasma membrane-bound Fe(III)-chelate reductase and by overexpression of a Fe^{2+} transporter of root epidermal cells (Eide et al. 1996; Robinson et al. 1999). Furthermore, some authors indicated ethylene as a possible regulator of the Fe-deficiency responses in these plants (Romera and Alcántara 2004).

Strategy II consists of the roots releasing high-affinity chelating compounds [phytosiderophores (PS)], which are able to form stable complexes with the micronutrient.

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Advances in the understanding of the metabolic responses to Fe deficiency have highlighted the key role of methionine in both Strategy I and Strategy II plants (Rudolph et al. 1985; Douchkov et al. 2002). Methionine is a S-containing amino acid required for the synthesis of protein and of S-adenosylmethionine (SAM), which is a common precursor of molecules as nicotianamine (NA), ethylene, polyamines and PS (Hesse and Hoefgen 2003). In higher plants, methionine biosynthesis involves two pathways: S assimilation and aspartate metabolism (Ravanel et al. 1998; Hesse and Hoefgen 2003). The level of free methionine is also assured by a recycling pathway known as the “Yang cycle”, which conserves the methylthio group for regeneration of methionine when SAM is used for synthesis of polyamines or ethylene (Yang and Hoffman 1984). However, it has recently been demonstrated that the level of free methionine is rate limiting for ethylene production in tomato fruit and that de novo synthesis of methionine is necessary to allow the evolution of higher rates of ethylene (Katz et al. 2006).

Nicotianamine is a common component for both strategies (Rudolph et al. 1985; Douchkov et al. 2002). In Strategy I plants, NA might function as a chelator of Fe in symplastic and phloem transport, whereas in Strategy II plants, NA has been shown to be a precursor for the biosynthesis of the mugineic acid family of PS.

Taken together, this evidence suggests that sulphur availability might conceivably play a role in the Fe-deficiency responses due to the need to maintain an adequate level of methionine and its derivatives.

Interactions between S and Fe nutrition have been shown in Strategy II plants (Kuwajima and Kawai 1997; Astolfi et al. 2003, 2004, 2006a, b; Bouranis et al. 2003), indicating that S deficiency could limit Fe-deficiency response through a decrease in the production and release of PS.

This aspect is a matter of concern as S availability is limited in some soils and S-deficient areas are becoming widespread due both to reduced SO₂ atmospheric inputs caused by strict emission regulation and to changes in agronomic practices, such as use of high-analysis low-S fertilizers and declining use of S-containing fungicides (McGrath and Zhao 1995; McGrath et al. 1996).

The aim of this work was to clarify the role of S supply in the development of the response to Fe depletion in Strategy I plants. In particular, we investigated changes in several components of Strategy I response at root level of tomato plants, including ethylene production, Fe(III) reducing activity and ⁵⁹Fe uptake. In addition, the expression of the Fe(III)-chelate reductase (*LeFROI*) and Fe²⁺ transporter (*LeIRT1*) genes at root level was analysed. Due to the role of NA in Fe nutrition and its production from sulphur-containing compounds, nicotianamine-synthase gene (*NAS*) expression was also evaluated together with some param-

eters related to sulphur nutritional status (total sulphur and thiol contents, ³⁵SO₄ = uptake).

Materials and methods

Growing conditions

Tomato (*Solanum lycopersicum* L., cv. Gimar, kindly provided by Prof. Soressi, DABAC, University of Viterbo) was used as Strategy I model plant. Seedlings were hydroponically grown in plastic pots (six seedlings per pot) containing 2.5 l of nutrient solution (NS) (Zhang et al. 1991) for 7 days, being exposed to 1.2 mM sulphate and 40 μM Fe^{III}-EDTA. Half of the plants were then transferred for a further week to a S-free nutrient solution (NS). Thereafter, half of the plants deriving from the two S growth conditions (+S and −S) were transferred to a Fe-free NS.

In S-free NS, sulphate salts (K⁺, Mn²⁺, Zn²⁺, Cu²⁺) were replaced by appropriate amounts of the corresponding chloride salts (K⁺, Mn²⁺, Zn²⁺, Cu²⁺). NS was continuously aerated and changed every 2 days. Plants were grown in a growth chamber under 200 μmol photons m^{−2} s^{−1} PPF and 14 h/10 h day/night regime (27/20°C day/night temperature cycling; 80% relative humidity). Both leaves and roots were harvested 17 days after sowing.

Measurement of ethylene production

For measurement of ethylene production, the whole root system was excised 4 days after the onset of the Fe deprivation treatment and placed in a test tube (50 ml). The tubes were sealed with rubber caps and incubated in the dark at 24°C for 2 h. Gas samples (1 ml) were withdrawn from the tube through a syringe and analysed for ethylene by a gas chromatograph (Fraetovap 4200, Carlo Erba, Milano, Italia) equipped with a 80100 mesh alumina column. After ethylene analysis, the roots were weighed and the fresh weight was used to calculate the rate of ethylene production.

Measurements of ⁵⁹Fe uptake

To measure the capacity of the root apparatus to absorb ⁵⁹Fe from ⁵⁹Fe-hydroxide, roots of intact tomato plants were washed with micronutrient-free NS for 30 min and then transferred to beakers containing 200 ml of a freshly prepared micronutrient-free NS (uptake solution) buffered at pH 7.5 with 10 mM Hepes–KOH. ⁵⁹Fe-hydroxide was prepared as described by Cesco et al. (2000), by dissolving ⁵⁹FeCl₃ in water and then adding KOH 1 N to raise alkaline pH (specific activity 123 kBq μmol^{−1}Fe). The experiment was started by the addition of 1 ml of suspension containing

^{59}Fe -hydroxide (2 $\mu\text{mol Fe}$) to the uptake solution, and lasted for 24 h.

Uptake of Fe^{2+} was assayed as described by Zaharieva and Römheld (2000) with slight modifications. The radiolabelled stock solution was prepared by adding freshly made 10 mM ascorbate to an appropriate volume of $^{59}\text{FeCl}_3$ to reduce Fe^{3+} to Fe^{2+} . An appropriate volume of freshly prepared 10 mM FeSO_4 in 0.04 N HCl was subsequently added. The uptake solution (200 ml) contained 5 mM Mes–KOH (pH 5.5), 1 mM CaSO_4 , 250 μM $^{59}\text{FeSO}_4$ (specific activity of 144 kBq $\mu\text{mol}^{-1}\text{Fe}$) and 1 mM ascorbate. The solution was aerated by bubbling. Two tomato plants were transferred into each flask, after rinsing the roots with Fe-free nutrient solution for 30 min. The experiment was started by adding $^{59}\text{Fe}^{\text{II}}$ -sulfate to the uptake solution, and lasted for 1 h.

After the uptake periods, the plants from both radioactive experiments were transferred to a freshly prepared ^{59}Fe -free nutrient solution for 10 min and then harvested. Root extraplasmatic ^{59}Fe pool was removed by 1.2 g l^{-1} sodium dithionite and 1.5 mM 2,2'-bipyridyl in 1 mM $\text{Ca}(\text{NO}_3)_2$ under N_2 bubbling, according to the method described by Bienfait et al. (1985); the treatment was repeated three times. Roots and shoots were oven-dried at 80°C, weighed, ashed at 550°C and suspended in 1% (w/v) HCl for ^{59}Fe determination by liquid scintillation counting. The ^{59}Fe uptake rate, measured as $\mu\text{mol } ^{59}\text{Fe}$, is related to the whole plant (root + shoot) and is presented as per g dry weight of roots h^{-1} .

Measurements of $^{35}\text{SO}_4^{2-}$ uptake

Roots of intact tomato plants were washed with micronutrient- and sulphate-free NS for 30 min and then transferred to beakers containing 200 ml of a freshly prepared micronutrient- and sulphate-free NS; $^{35}\text{SO}_4^{2-}$ (specific activity 2.1 kBq $\mu\text{mol}^{-1} ^{35}\text{SO}_4^{2-}$) was added to give a final SO_4^{2-} concentration of 600 μM . The uptake solution was buffered at pH 6.0 with 10 mM Mes–KOH and the uptake period was 1 h. After the uptake, the plants were transferred to an ice-cold desorption solution containing 1 mM CaSO_4 and 10 mM Mes–KOH (pH 6.0) for 5 min. Roots and shoots were then oven-dried at 60°C, weighed, ashed at 550°C and suspended in 1% (w/v) HCl for $^{35}\text{SO}_4^{2-}$ determination by liquid scintillation counting. The $^{35}\text{SO}_4^{2-}$ uptake rate, measured as $\mu\text{mol } ^{35}\text{SO}_4^{2-}$, is referred to the whole plant (root + shoot) and is presented as per g dry weight of roots h^{-1} .

Determination of root ferric reductase activity

Fe(III)-EDTA reduction by roots of intact tomato plants was measured by using the bathophenanthrolinedisulfonate

(BPDS) reagent as described in Pinton et al. (1999). Briefly, roots of a single plant were placed in deionised water for 5 min. They were then transferred to an aerated assay solution consisting of 0.5 mM CaSO_4 , 0.25 mM Fe(III)–EDTA, 0.6 mM BPDS and 10 mM Mes at pH 5.5 adjusted by 1 M KOH, and incubated in the dark at 25°C for 30–60 min. The absorbance of the assay solution was recorded by a spectrophotometer at 535 nm and the amount of reduced Fe(III) was calculated by the concentration of the Fe(II)–BPDS₃ complex using an extinction coefficient of 22.1 $\text{mM}^{-1} \text{cm}^{-1}$.

Total RNA extraction and RT-PCR analysis

Isolation of total RNA from the roots of tomato plants was performed using the Trizol[®] reagent system according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). One mg of DNase-treated RNA was reverse-transcribed by M-MLV (H-) Reverse Transcriptase (Invitrogen) to synthesise the first strand of cDNA. One microlitre of the oligo(dT)12-primed first strand cDNA samples was applied for PCR using reverse and forward primers designed by Li et al. (2004) to amplify *LeIRT1* and *LeFRO1* cDNA. Primers used were as follows: *LeIRT1*-1 primer, 5'-TGGCTGTGGCTGGAATCATGTTC-3' and *LeIRT1*-2 primer, 5'-AGAATTTTTTTTGCAACTCCCAATAGGT-3'; *LeFRO1*-1 primer, 5'-GGAGCCAGAGAAAATCAGTG-3' and *LeFRO1*-2 primer, 5'-CGAAGCCATAGGAGTTGC-3'. Reverse and forward primers to amplify *LeNAS* cDNA were designed on the basis of the published *LeNAS* gene sequence (accession no. TC178772) and were as follows: *LeNAS*-1 primer, 5'-TCACAGATCTTGTCCACACG-3' and *LeNAS*-2 primer, 5'-CATACGGCTGGACATGTCTG-3'. The level of the *18S* gene expression was used as control for quantification. PCR conditions were: 94 and 55°C for 45 s each and 72°C for 1 min for each cycle, plus a final extension time of 72°C for 7 min. The number of PCR cycles was adjusted to obtain detectable amounts of amplicons without reaching signal saturation, which was accomplished with 32 cycles for *LeIRT1*, *LeNAS* and *18S* (control) and 45 cycles for *LeFRO1*. All semi-quantitative RT-PCRs were performed in duplicate. RT-PCR amplification products were separated electrophoretically on 1% (w/v) agarose gels and stained with ethidium bromide.

Other measurements and statistics

The concentration of chlorophyll per unit area was estimated in attached leaves by a SPAD portable apparatus (Minolta Co., Osaka, Japan) using the first fully expanded leaf from the top of the plant.

To determine total S concentration, 1 g of each leaf or root sample was dried at 105°C and then ashed in a muffle

furnace at 600°C. The ashes were dissolved in 10 ml of 3 N HCl and filtered through Whatman no. 42 paper. In contact with BaCl₂, a BaSO₄ precipitate is formed which is determined turbidimetrically (Bardsley and Lancaster 1962).

Water-soluble non-protein thiol compounds were determined colorimetrically with DTNB following the procedure described in Badiani et al. (1997). Briefly, leaves and roots were homogenized in a solution containing 80 mM trichloroacetic acid, 1 mM ethylenediaminetetraacetic acid, 0.15% (w/v) ascorbic acid and 10% (w/v) PVP using 3 ml buffer per g as fresh weight roots. The DTNB reactive compounds were measured spectrophotometrically at 415 nm.

To determine Fe, Ca, Mg, Na and K content, leaf tissues were oven-dried at 80°C to constant weight and thereafter heated at 550°C for 6 h in a muffle furnace. The ashes were dissolved in 1 M HCl and analysed by inductively coupled plasma atomic emission spectrometry in an ICP-AES instrument (VISTA MPX, Varian, Torino, Italy).

Each reported value represents the mean $\pm$ SD of measurements carried out in triplicate and obtained from three independent experiments. Statistical analyses of data 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 < 0.05 .

Results

Plant growth and root morphology

Sulphur deficiency resulted in a significant decrease in fresh weight of both shoot and roots (–40 and –20%, respectively) (Fig. 1a, b). However, this phenomenon was mainly evident at leaf level with a consequent lowering of shoot/root ratio (from 4.62 to 3.18 in the +S and –S plants, respectively) (box in Fig. 1b). Also the imposition of Fe deficiency

significantly decreased the values of fresh weight of both leaves (–25% in both +S and –S plants) and roots (–30 and –20% in +S and –S plants, respectively). A similar behaviour was observed for dry weight (Fig. 1c, d).

As shown in Fig. 2, roots of S-sufficient plants developed several subapical swellings after imposition of Fe deficiency. Interestingly, subapical swellings formation did not occur upon Fe removal in plants grown in S-free nutrient solution.

The chlorophyll content was determined by chlorophyll meter readings (SPAD) that provide a sensitive and accurate index of plant response to the Fe treatment. Figure 3 shows that the levels of SPAD index were significantly decreased by S deficiency to 75% of the respective S-sufficient control plants, while being almost unaffected by Fe deficiency, probably because of the short period considered (4 days).

Plant total sulphur and thiol content, (³⁵S)-sulphate uptake rate

Total sulphur concentration was clearly much lower in S-deprived plants than in S-sufficient plants. Imposition of Fe deficiency significantly depressed sulphur content in the roots of S-sufficient plants (Fig. 4).

Concentration of non-protein thiols was also considerably decreased by S deprivation in both leaves and roots; however, no significant difference was observed between Fe-sufficient and Fe-deficient plants (Fig. 4).

S deficiency determined a significant rise in ³⁵SO₄^{2–} uptake rate, suggesting the involvement of a response mechanism to the nutrient limitation (Table 1).

Leaf ion (Fe, Ca, Mg, Na and K) concentration

The S-deficiency condition led to a sharp decrease in leaf Fe concentrations (–59%) with respect to plants that

Fig. 1 Shoot (a) and root (b) fresh weight and shoot (c) and root (d) dry weight of S-sufficient and S-deficient tomato plants grown for 4 days with (+Fe) or without (–Fe) 40 μ M FeIII–EDTA. Inserts show to root ratio. Data are mean $\pm$ SD of three independent replications. Significant differences between samples are indicated by different letters ($P < 0.01$, $n = 3$)

Fig. 2 Roots of S-sufficient and S-deficient tomato plants grown for 4 days with (+Fe) or without (–Fe) 40 μ M FeIII–EDTA. White circles indicate development of subapical swellings in S-sufficient Fe-deficient (+S – Fe) roots

Fig. 3 Values of SPAD index of S-sufficient and S-deficient tomato plants grown for 4 days with (+Fe) or without (–Fe) 40 μ M FeIII–EDTA. Data are mean $\pm$ SD of three independent replications. Significant differences between samples are indicated by different letters ($P < 0.01$, $n = 3$)

received adequate S supply (Fig. 5). Essentially the same behaviour was observed when S-sufficient plants were grown for 4 days in nutrient solution without added Fe. The lowest values were observed in plants without the addition of either S or Fe.

On the other hand, Ca, Mg, Na and K contents in shoots of tomato plants grown under S-deficiency conditions were not significantly different from those measured in S-sufficient control plants (Fig. 6). In contrast, all the analysed elements tended to increase in Fe-deficient plants and reached the highest levels in plants grown without either S or Fe (Fig. 6).

Root ethylene production

Ethylene production in tomato roots was significantly stimulated by S deficiency (60% higher than in the

S-sufficient control) (Fig. 7). Highest levels of ethylene production were recorded when S-sufficient plants were grown in Fe-deficient conditions (130% higher than the Fe-sufficient control). The imposition of the dual deficiency produced ethylene levels which were very close to the values observed for the S- and Fe-sufficient control.

Root Fe(III)-chelate reducing activity

Ferric-chelate reductase activity in roots of S-sufficient and S-deficient tomato plants was studied during 6 days of Fe starvation (Fig. 8).

Plants adequately supplied with S and Fe showed a low Fe(III)-chelate reducing activity; when Fe was omitted from the solution the level of activity rose to a maximum 4 days after starting the treatment. Thereafter, the activity declined to values comparable with those of the S- and Fe-sufficient control.

When grown in a S-deficient nutrient solution, tomato plants exhibited a Fe(III)-chelate reducing activity even lower than that of S-sufficient controls (Fig. 8).

Similar behaviour was observed when S and Fe were both omitted from the solution.

^{59}Fe uptake and translocation

The data in Table 1 show that Fe deficiency induced a rise in the capacity of S-sufficient plants to take up ^{59}Fe from $^{59}\text{Fe(III)-hydroxide}$. Fe deficiency also caused a strong enhancement of $^{59}\text{Fe}^{2+}$ uptake rates.

Fig. 4 Total S and non-protein thiol content in leaves (**a** and **c**) and roots (**b** and **d**) of S-sufficient and S-deficient tomato plants grown for 4 days with (+Fe) or without (–Fe) 40 μ M FeIII–EDTA. Data are mean $\pm$ SD of three independent replications. Significant differences between samples are indicated by different letters ($P < 0.01$, $n = 3$)

Table 1 Uptake and translocation to the shoot of ^{59}Fe from $^{59}\text{Fe(III)}$ -hydroxide, and uptake of $^{59}\text{Fe}^{2+}$, in S-sufficient and S-deficient tomato plants grown for 4 days with (+Fe) or without (–Fe) 40 μ M FeIII–EDTA

	Treatment			
	+S+Fe	+S–Fe	–S+Fe	–S–Fe
^{59}Fe uptake rate from $^{59}\text{Fe(III)}$ -hydroxide (nmol g^{-1} root DW h^{-1})	5.57 ± 2.13^b	14.93 ± 4.12^a	4.86 ± 1.76^b	7.58 ± 1.70^b
As $^{59}\text{Fe}^{2+}$ ($\mu\text{mol g}^{-1}$ root DW h^{-1})	3.37 ± 0.03^c	18.27 ± 1.60^a	2.39 ± 0.57^c	10.30 ± 2.44^b
^{59}Fe translocation from $^{59}\text{Fe(III)}$ -hydroxide (nmol $^{59}\text{Fe g}^{-1}$ root DW h^{-1})	2.42 ± 1.32^b (43.4%)	5.65 ± 1.71^a (37.8%)	1.50 ± 0.94^b (30.8%)	1.93 ± 0.43^b (25.5%)
$^{35}\text{SO}_4^{2-}$ uptake ($\mu\text{mol g}^{-1}$ root DW h^{-1})	12.9 ± 1.3^b	12.0 ± 1.8^b	34.4 ± 6.9^a	47.7 ± 13.2^a

^{59}Fe uptake rate is referred to the whole plant (root + shoot) and reported per g dry weight of roots per hour. Uptake rates of $^{35}\text{SO}_4^{2-}$ are also reported. Data are mean $\pm$ SD of three independent replications

Significant differences between samples are indicated by different letters ($P < 0.05$, $n = 3$)

S deficiency prevented the development of a higher uptake rate in Fe-deficient plants, and also limited the development of a higher capacity to take up $^{59}\text{Fe}^{2+}$ in Fe-deficient plants (Table 1).

Expression of *LeIRT1*, *LeFRO1* and *LeNAS* genes in root tissue

Expression of genes involved in Fe acquisition, such as *LeIRT1* (Fe^{2+} transporter) and *LeFRO1* (FeIII-reductase) was analyzed as a function of Fe and S nutrition, utilizing specific primers as described by Li et al. (2004).

Fe deficiency caused an increase in the expression levels of *LeIRT1* gene; however, in S-deficient roots transcript amounts reached levels lower than those observed in S-sufficient plants either with or without Fe-supply (Fig. 9).

LeFRO1 transcript abundance was increased by Fe deficiency in S-sufficient plants, while being decreased by S-deficiency (Fig. 9).

Under unlimited S supply, *LeNAS* gene showed similar expression in Fe-sufficient and Fe-deficient roots (Fig. 9). S-deficiency virtually abolished *NAS* gene expression, independently of the Fe growth conditions.

Discussion

Iron-deficiency responses in Strategy I and II plants share common features: indeed, NA, a common component of both strategies, ethylene and PS are all synthesized from the S-containing amino acid methionine. This implies that reduced S availability might play a role in the development of an efficient response to Fe shortage in plants.

Interactions between Fe and S nutrition have been reported for strategy II plants. It has been demonstrated that S deficiency may limit the release of PS and Fe uptake in barley roots, thus leading to a reduced accumulation of Fe in the leaf tissue (Astolfi et al. 2006a). Furthermore, it has been shown that Fe deficiency caused an increase in $^{35}\text{SO}_4^{2-}$ uptake rates in maize and barley plants (Astolfi et al. 2004, 2006b).

This work was aimed at assessing the role of S availability in the development of the Fe-deficiency response in tomato, a Strategy I plant.

S deficiency resulted in a significant decrease in fresh and dry weight of tomato plants (Fig. 1). The shoot to root ratio calculated for fresh weight decreased as a result of a significant decrease in shoot fresh weight, reflecting a

Fig. 5 Iron content in leaves of S-sufficient and S-deficient tomato plants grown for 4 days with (+Fe) or without (–Fe) 40 µM FeIII-EDTA. Data are mean ± SD of three independent replications. Significant differences between samples are indicated by different letters ($P < 0.01$, $n = 3$)

classical response to S supply, as extensively reported in the literature (Robinson 1994) (box in Fig. 1).

Leaves from S-starved plants became uniformly chlorotic, as detected by SPAD readings (Fig. 3). The visual symptoms were accompanied by a strong decrease in total S content in the S-deprived tissues (Fig. 4a). However, we observed no further increase of leaf chlorosis upon Fe removal from the nutrient solution, probably because of a very short period (4 days) of Fe starvation.

Analysis of the leaf mineral composition showed that, consistent with the imposed treatment, Fe-deficient plants exhibited a marked decrease in leaf Fe content (Fig. 5). However, changes in Fe content in tomato leaves in the Fe- and in the S-starvation treatment were similar and, in particular, Fe content in S starvation leaves also decreased by nearly 60%. In our previous works (Astolfi et al. 2003, 2004), we found that maize and barley plants exposed to S starvation showed a lower Fe content than S-sufficient plants. It has been proposed that S starvation might decrease the accumulation of Fe in the leaf tissue by inhibiting Fe uptake primarily as a consequence of decreasing the PS release capacity of the roots (Astolfi et al. 2006a, b).

Strategy I plants do not release PSs to cope with Fe shortage, but one of the main characteristics of their Fe uptake system is the increase in root ethylene production (Romera and Alcántara 1994, 2004; Romera et al. 1999). Furthermore, NA is a common component for both strate-

gies (Rudolph et al. 1985; Douchkov et al. 2002). The evidence that both ethylene and NA are synthesized from methionine suggests that the observed decrease in leaf Fe content could probably be due to the inhibition of both uptake and translocation of Fe to the shoot, as a result of inadequate supply of reduced S to maintain the ethylene and NA biosynthetic pathway. This idea is indeed suggested by the evidence that the total sulphur content was decreased by growth under Fe deficiency condition in tomato roots, whereas thiol compound levels were kept constant in roots of plants grown in the absence of Fe (Fig. 4).

To evaluate if changes in S supply could be associated with the capacity of tomato plants to cope with Fe shortage, various components of the Strategy I mechanism were considered: root morphological changes and ethylene production, ferric reductase activity and uptake rate of Fe. Furthermore, the expression of the Fe(III)-chelate reductase (*LeFRO1*) and Fe^{2+} transporter (*LeIRT1*) genes at the root level was analysed. Due to the role of NA in Fe nutrition and its production from sulphur-containing compounds, NAS expression was also evaluated together with some parameters related to sulphur nutritional status (total sulphur and thiol contents, $^{35}\text{SO}_4$ = uptake).

When grown for 4 days in a Fe-depleted nutrient solution, S-sufficient tomato plants showed a classical response to Fe deficiency (Römheld and Marschner 1981; Marschner et al. 1986; Römheld 1987), as evidenced by increased Fe(III)-chelate reducing capacity (Fig. 8), Fe uptake rate (Table 1) and root ethylene production (Fig. 7). These changes were accompanied by the appearance of subapical root swellings and proliferation of abundant root hairs (Fig. 2). Furthermore, the expression of both *LeFRO1* and *IRT1* was greatly induced in roots in response to Fe deficiency (Fig. 9).

However, when plants were grown in S-deficient conditions, the response to Fe deficiency was strongly depressed: increases in ethylene production (Fig. 7), Fe(III)-chelate reductase activity (Fig. 8) and reduction based ^{59}Fe uptake (Table 1) were prevented; moreover, expression of the *LeFRO1* gene was barely detectable (Fig. 9). S deficiency also limited the development of a higher activity of the Fe^{2+}

Fig. 6 Ca, Mg, Na and K content in leaves of S-sufficient and S-deficient tomato plants grown for 4 days with (+Fe) or without (–Fe) 40 µM FeIII-EDTA. Data are mean ± SD of three independent replications. Significant differences between samples are indicated by different letters ($P < 0.01$, $n = 3$)

Fig. 7 Ethylene production from roots of S-sufficient and S-deficient tomato plants grown for 4 days with (+Fe) or without (–Fe) 40 μ M FeIII–EDTA. Data are mean $\pm$ SD of three independent replications. Significant differences between samples are indicated by different letters ($P < 0.05$, $n = 3$)

Fig. 8 Time course of changes in root Fe(III)-chelate reductase activity in S-sufficient and S-deficient tomato plants grown for 4 days with (+Fe) or without (–Fe) 40 μ M FeIII–EDTA. *Inserts* changes in root Fe(III)-chelate reductase activity at the fourth day of Fe starvation. Data are mean $\pm$ SD of three independent replications. Significant differences between samples are indicated by different letters ($P < 0.01$, $n = 3$)

transporter (Table 1) and expression of the *LeIRT1* gene (Fig. 9). These data show that Fe(III)-chelate reductase activity was much more influenced by S-deficiency than Fe^{2+} uptake. A limited response to Fe deficiency in plants subjected to both S and Fe deficiency might be related to a lower demand for the micronutrient as a consequence of reduced growth rate; however, it is noteworthy that these plants were able to develop an even higher $^{35}\text{SO}_4^{2-}$ uptake rate than that shown by S-deficient Fe-sufficient plants (Table 1). Furthermore, the concentration of several cationic nutrients was not affected by S deficiency and even increased when plants were simultaneously subjected to Fe and S deficiency (Fig. 6).

Ethylene has been indicated as a component of the complex regulation mechanism for the onset of the Fe-defi-

Fig. 9 RT-PCR expression analysis of iron transporter gene *LeIRT1*, ferric reductase gene *LeFRO1* and nicotianamine synthase gene *LeNAS* in roots of S-sufficient and S-deficient tomato plants grown for 4 days with (+Fe) or without (–Fe) 40 μ M FeIII–EDTA. An 18S PCR product was used as a cDNA calibration control

ciency response (Schmidt 2003; Romera and Alcántara 2004). The evidence that both ethylene and NA are synthesized from methionine (Hesse and Hoefgen 2003) suggests that the observed decrease in leaf Fe content might be due to the inhibition of both uptake and translocation of Fe to the shoot, as a result of inadequate supply of reduced S to sustain the ethylene and NA biosynthetic pathway. *NAS* expression was not significantly modified by Fe starvation in S-sufficient plants (Fig. 9), thus confirming a previous observation by Ling et al. (1999). On the other hand, *NAS* transcripts were virtually undetectable in S-deficient plants, irrespective of the Fe nutritional treatment. This effect might be related to the low Fe content of S-starved plants.

It is interesting to note that the lack of increase in ethylene production in S-deficient, Fe-deficient plants was paralleled by a lack of activation of the Fe(III)-chelate reductase activity, while Fe^{2+} transport rate was enhanced by Fe deficiency in S deficient plants, although to a lesser extent than in S-sufficient plants. This result would indicate that the two components of the Fe-deficiency response [Fe(III)-reduction and Fe^{2+} transport] are differently sensitive to (or regulated by) ethylene levels.

S deficiency alone also caused chlorosis and a decrease in Fe content of leaf tissue. In these plants a higher ethylene production with respect to S-sufficient plants was also observed. It was reported that ethylene production is increased by ammonia accumulation in plant tissue (Feng and Barker 1993). Our results seem to support this hypothesis: as the severity of S deficiency increased, protein synthesis would likely decline leading to ammonia accumulation in the plant (Nikiforova et al. 2005) and ethylene production would increase.

However, this behaviour was not accompanied by an activation of the Fe-deficiency response mechanisms. Lower Fe accumulation might be related to an altered NA synthesis, while the lack of activation of the Fe(III)-chelate reductase could likely be ascribed to an adequate Fe status of the roots. Increased ethylene production has been observed in response to different nutritional deficiencies (Romera and Alcántara 2004 and references therein), but not necessarily related to an adaptive mechanism. Lucena et al. (2006) also showed that application of the ethylene precursor ACC to Fe-sufficient plants determined little or no induction of Fe(III)-chelate reductase (*FRO*) gene in *Arabidopsis*, thus suggesting that the increase in ethylene production alone is not sufficient for activation of the plasma membrane-bound enzyme.

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