Early transcriptomic response to Fe supply in Fe-deficient tomato plants is strongly influenced by the nature of the chelating agent

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Abstract

Background: It is well known that in the rhizosphere soluble Fe sources available for plants are mainly represented by a mixture of complexes between the micronutrient and organic ligands such as carboxylates and phytosiderophores (PS) released by roots, as well as fractions of humified organic matter. The use by roots of these three natural Fe sources (Fe-citrate, Fe-PS and Fe complexed to water-extractable humic substances, Fe-WEHS) have been already studied at physiological level but the knowledge about the transcriptomic aspects is still lacking.

Results: The $^{59}$Fe concentration recorded after 24 h in tissues of tomato Fe-deficient plants supplied with $^{59}$Fe complexed to WEHS reached values about 2 times higher than those measured in response to the supply with Fe-citrate and Fe-PS. However, after 1 h no differences among the three Fe-chelates were observed considering the $^{59}$Fe concentration and the root Fe(III) reduction activity. A large-scale transcriptional analysis of root tissue after 1 h of Fe supply showed that Fe-WEHS modulated only two transcripts leaving the transcriptome substantially identical to Fe-deficient plants. On the other hand, Fe-citrate and Fe-PS affected 728 and 408 transcripts, respectively, having 289 a similar transcriptional behaviour in response to both Fe sources.

Conclusions: The root transcriptional response to the Fe supply depends on the nature of chelating agents (WEHS, citrate and PS). The supply of Fe-citrate and Fe-PS showed not only a fast back regulation of molecular mechanisms modulated by Fe deficiency but also specific responses due to the uptake of the chelating molecule. Plants fed with Fe-WEHS did not show relevant changes in the root transcriptome with respect to the Fe-deficient plants, indicating that roots did not sense the restored cellular Fe accumulation.

Background

Iron (Fe) is the micronutrient required in the largest amount by plants and plays a role in key metabolic processes such as respiration, chlorophyll biosynthesis and photosynthesis. This element is a component of the heme group and Fe-sulphur clusters and other binding sites; for its chemical proprieties it is involved in many redox reactions but it can also favour the generation of reactive oxygen species (ROS), which implies a precise control of its uptake, utilization and storage [1].

To counteract the low availability of Fe in soils, higher plants have developed two different strategies for its acquisition from the rhizosphere. The Strategy I (all higher plants except grasses) relies on the improvement of Fe solubility through the release of root exudates like protons (via an increase of activity of plasma membrane H$^+$-ATPase) and organic acids and phenolic compounds followed by a reduction of Fe(III) to the more soluble Fe(II) by a Fe(III)-chelate reductase (FRO) [2]. This reductive step is essential for the acquisition of micronutrient, since Fe(II) is taken up via the activity of a divalent cation transporter, Iron-Regulated Transporter (IRT) [1]. Strategy II is specific for grasses and is based on the biosynthesis and release of phytosiderophores (PS), which have a strong affinity for Fe(III),...
and on the uptake of the Fe-PS complexes by a specific transporter, Yellow-Stripe (YS) [1].

Physiological and molecular responses to Fe deficiency in Strategy I species have been extensively studied in Arabidopsis thaliana [3]. In this model plant, a set of 92 transcripts responsive to Fe deficiency was identified [4]. In tomato roots, a similar number of transcripts (97) was modulated in response to Fe deficiency [5]. More recently, through a co-expression analysis, a group of 180 genes potentially involved in the regulation of Arabidopsis responses to Fe shortage was detected [6]. Several works describing plant transcriptional responses to Fe-stress as a comparison between Fe sufficient and Fe deficient condition are present in literature [7–17]. However, no data are available on the modulations taking place during supply after a period of deficiency that is a condition reasonably occurring at the rhizosphere. In the recent years, this matter has been investigated at proteomic level in roots of Beta vulgaris [18] and in a Prunus hybrid [19], at metabolomic level in roots of Beta vulgaris [18], in the xylem sap and leaf extract of Strategy I plants [20].

In the rhizosphere the concentration of available Fe depends on the soil pH and on the presence of different types of natural ligands [2, 21–23], such as organic acids [24,25], flavonoids [26, 27], PS [28], microbial siderophores [29] and fractions of the humified organic matter [30, 31]. The acquisition mechanisms of Fe-chelates by Strategy I plants is considered to be based on the obligatory step of reduction [23], [32–34] even if recently their possibility to directly absorb Fe-PS has been envisaged [35]. Information about possible differences in the use efficiency of Fe-complexed to natural occurring chelates is still very scarce. It has been reported that fractions of low-molecular-weight water-extractable humic substances (WEHS) complexed with Fe(III) enhanced Fe deficiency responses when compared with natural (citrate) or synthetic [ethylenediaminetetraacetic acid (EDTA)] chelates [36]. Furthermore, a higher amount of $^{59}$Fe was accumulated in tomato plants treated with Fe-WEHS after 24 h in comparison to other Fe sources [23]. The higher acquisition of Fe from Fe-WEHS was related to a more efficient reduction, rhizosphere acidification and translocation [22, 23, 37].

Here we describe the transcriptional responses of Fe-deficient tomato roots after 1 h of supply with 1 μM Fe chelated to citrate, PS or WEHS. Results showed that the root transcriptional profile of plants supplied with Fe-WEHS is very similar to that of Fe-deficient plants being only two transcripts differentially expressed. The other two natural sources of Fe caused on the other hand a similar modulation of a common set of 289 transcripts. In addition, the Fe-citrate and Fe-PS complexes showed some specific responses as suggested by the modulation of 439 and 119 transcripts after supplying Fe-citrate or Fe-PS alone, respectively.

**Results and discussion**

Iron-$^{59}$Fe accumulation from natural Fe-sources by tomato roots

The capability of Fe-deficient tomato plants to utilize different natural Fe-sources was evaluated after 1, 4, or 24 h of treatment performing Fe-uptake experiments and using $^{59}$Fe complexed with WEHS, citrate or PS. In order to reproduce conditions closer to those where Fe-deficiency symptoms in crops usually appear [38], the uptake medium was buffered at pH 7.5 and each Fe source was used at 1 μM final Fe concentration.

Fig. 1a shows that after 1 h of supply, the concentration of $^{59}$Fe accumulated in tomato plants was comparable among all the three Fe treatments exhibiting values around 100 nmol $^{59}$Fe g$^{-1}$ DW root. Iron content markedly increased up to four folds after 4 h and from six to 18 folds after 24 h. In Fe-WEHS treated plants, the concentration of $^{59}$Fe taken up was significantly greater than the one measured in plants treated with $^{59}$Fe-citrate or $^{59}$Fe-PS at 4 and 24 h. Within each time point, there were no significant differences in Fe content in plants exposed to Fe-citrate and Fe-PS. Fe-sufficient plants (Fig. 1b) showed approximately one order of magnitude
lower $^{59}$Fe accumulation levels than Fe-deficient plants (Fig. 1a) suggesting that responses to Fe shortage are switched off. Also in this case Fe-WEHS treated plants accumulated the highest concentration of $^{59}$Fe both at 4 and 24 h.

In order to get information on the functionality of Fe-acquisition mechanisms working at the root level of plants supplied with different Fe-complexes, root Fe(III) reduction activity was measured after 1 h of treatment. Fig. 2 reports that the different type of Fe supply did not significantly modify the root Fe(III)-reduction activity.

Changes in tomato root transcriptome in response to the supply with different natural Fe sources
Root transcriptional profiles of tomato plants in response to 1 h supply with the three different Fe sources were characterized by a genome-wide microarray analysis.

Table 1 reports the numbers of upregulated and downregulated transcripts identified by Linear Models for MicroArray (LIMMA; adjusted p-value ≤ 0.05; $|\log_{2}(R)| ≥ 1$) [39] for each comparison of root transcriptional profiles. To have a further confirmation, the expression level of some differentially expressed transcripts was also tested by Real-time RT-PCR (Additional file 1: Table S1). The number of transcripts differentially expressed in response to Fe-citrate and Fe-PS supply was 728 and 408, respectively. Surprisingly, roots of tomato plants treated with Fe-WEHS showed only two differentially expressed transcripts (one upregulated and one downregulated) in comparison to the Fe-deficiency condition (Table 2) indicating an elevated similarity between these two transcriptional profiles (Additional file 1: Figure S1). This behaviour might explain the higher $^{59}$Fe content in Fe-WEHS-supplied tomato plants in comparison to Fe-citrate- and Fe-PS-supplied plants after 4 and 24 h (Fig. 1a).

The upregulated transcript in response to Fe-WEHS-supplied plants had the same behaviour in the plant subjected to the other two treatments (Table 2 and Fig. 3). It encodes a R2R3 MYB transcription factor (#1, Solyc06g005310.2.1; Table 2). The protein encoded by this tomato gene shows highest homology with Arabidopsis thaliana MYB48 (35 % of identity) that is not reported to be involved in responses to Fe-deficiency in that plant species. Until now, in Strategy I plants, the regulation of Fe-deficiency responses has been described to be controlled by bHLH transcription factors [1, 40]. Data here presented suggest that responses to Fe supply after a period of shortage could be driven by other transcription factors such as this MYB. The sole Fe-WEHS-specific transcript repressed encodes a putative amino acid transporter (#2, Table 2) of unknown function. However, both transcriptional modulations seem unlikely to be responsible for the different efficient use of Fe as Fe-WEHS source.

The root transcriptional profiles of Fe-deficient plants supplied with the three natural sources compared to that of Fe-sufficient plants (LIMMA; adjusted p-value ≤ 0.05; $|\log_{2}(R)| ≥ 1$) showed that 90, 1118 and 774 transcripts were after 1 h modulated in response to supply with Fe-WEHS, Fe-citrate and Fe-PS supplies respectively (Additional file 1: Table S2 and Figure S2, Additional file 2: Table S3). The number of differentially expressed transcripts between the Fe-WESH supplied plants and Fe-sufficient ones is similar (90 vs 97) to that identified in the previous transcriptional analysis comparing the transcriptome of Fe-sufficient and Fe-deficient roots [5]. It is therefore confirmed that the root transcriptional profile of Fe-deficient plants is very similar to that of plants supplied for 1 h with Fe-WEHS.

Responses to Fe-citrate and Fe-PS treatments
Differently from the transcriptional behaviour of Fe-WEHS, Fe-citrate and Fe-PS treatments vs Fe-deficient determined a modulation of a wider set of transcripts: 289 of them were in common, while 439 (~60 % of total differentially expressed transcripts of -Fe/Fe-citrate vs Fe-deficient comparison) and 119 (~30 % of total differentially expressed transcripts of -Fe/Fe-PS vs Fe-

![Fig. 2](image-url)
| #  | Probe_ID | Description | p-value, adj; Log2(R; Fe-citrate vs Fe) | p-value, adj; Log2(R; Fe-PS vs Fe) | p-value, adj; Log2(R; Fe-WEHS vs Fe) |
|----|----------|-------------|----------------------------------------|-----------------------------------|-------------------------------------|
| #1 | TC215712_723_40_S | R2R3-myb transcription factor, putative | 0.001 2.20 | 0.001 2.14 | 0.035 2.02 |
| #2 | TC194872_1016_38_S | Amino acid transporter, putative | Transcripts similarly affected by Fe-citrate, Fe-PS supply and Fe-WEHS | 0.035 -1.74 |
| #3 | TC191891_2590_35_S | Plasma membrane H^+ -ATPase | Transcripts affected by Fe-WEHS supply | 0.003 -1.72 |
| #4 | TC202455_704_34_X2 | Fructokinase-2 | 0.006 -1.39 | 0.008 -1.47 |
| #5 | TC215677_337_34_X2 | Fructose-bisphosphate aldolase | 0.008 -1.40 | 0.016 -1.29 |
| #6 | TC203759_474_40_S | Succinate dehydrogenase | 0.001 -1.67 | 0.003 -1.40 |
| #7 | TC205577_582_35_S | 2-Oxoglutarate dehydrogenase, putative | 0.002 -1.06 | 0.002 -1.08 |
| #8 | TC200117_1178_35_S | Methionine synthase | 0.004 -1.94 | 0.009 -1.72 |
| #9 | TC211903_86_41_S | SAM-dependent methyltransferase | 0.003 -1.40 | 0.013 -1.09 |
| #10 | TC212657_260_40_S | SAM-dependent methyltransferase | 0.011 -1.10 | 0.020 -1.06 |
| #11 | TC201480_474_36_S | Phenylalanine ammonia-lyase | 0.016 -1.12 | 0.018 -1.21 |
| #12 | TC207536_637_35_S | ABC transporter family protein | 0.002 -1.32 | 0.003 -1.23 |
| #13 | TC192092_3871_40_S | Cellulose synthase | 0.002 -1.34 | 0.001 -1.60 |
| #14 | TC192418_1233_40_S | Cellulose synthase catalytic subunit | 0.002 -1.72 | 0.003 -1.54 |
| #15 | TC214973_590_40_S | Cellulose synthase A catalytic subunit 3 | 0.004 -1.11 | 0.004 -1.23 |
| #16 | TC204385_218_36_X2 | UDP-apiose/xylose synthase | 0.003 -1.69 | 0.026 -1.11 |
| #17 | TC192860_1143_39_S | Expansin 1 protein | 0.017 1.28 | 0.026 1.26 |
| #18 | TC198812_683_37_S | Glucan endo-1,3-beta-glucosidase, putative | 0.017 1.05 | 0.010 1.31 |
| #19 | TC201525_569_35_S | Rho GTPase-activating protein At5g61530 | 0.002 -1.43 | 0.003 -1.34 |
| #20 | TC196357_464_36_S | ATP/GTP/Ca^{++} binding protein | 0.006 -1.60 | 0.022 -1.29 |
| #21 | TC211495_432_40_S | CBL-interacting protein kinase 1 | 0.004 -1.18 | 0.003 -1.45 |
| #22 | TC212764_568_34_X2 | Protein IQ-DOMAIN 14 | 0.003 -1.35 | 0.008 -1.20 |
| #23 | TC197849_292_41_X2 | Ras-related GTP binding protein | 0.016 -1.01 | 0.017 -1.10 |
| #24 | TC207137_449_35_S | RAS superfamily GTP-binding protein-like | 0.002 -1.38 | 0.007 -1.07 |
| #25 | TC196878_2001_40_S | Malic enzyme | 0.010 -1.20 | 0.013 -1.25 |
| #26 | TC191720_1243_40_S | NADH ubiquinone oxidoreductase-like | 0.012 1.29 | 0.027 1.17 |
| #  | Accession | Description                                                                 | Log2 fold change | p-value 1 | p-value 2 |
|----|-----------|-----------------------------------------------------------------------------|-----------------|-----------|-----------|
| #27| TC210154_386_41_X4 | Glutamate dehydrogenase                                                     | 0.005           | −1.45     | 0.016     |
| #28| TC192029_938_40_S | Putative basic helix-loop-helix protein BHLH7                               | 0.005           | 1.17      | 0.007     |
| #29| TC208592_1291_35_S | Triosephosphate isomerase, chloroplastic (TIM)                             | 0.004           | −1.27     | 0.008     |
| #30| TC194624_64_34_S  | 6-Phosphogluconate dehydrogenase                                           | 0.002           | −1.20     | 0.030     |
| #31| TC199057_182_40_S | Putative pyruvate dehydrogenase E1 beta subunit                            | 0.036           | −1.15     | 0.035     |
| #32| TC201985_646_40_S | Citrate synthase                                                            | 0.001           | −1.39     | 0.030     |
| #33| TC212309_491_35_S | Phosphoenolpyruvate carboxylase                                             | 0.048           | −1.03     | 0.003     |
| #34| TC193693_30_35_S  | NADH dehydrogenase, putative                                               | 0.003           | −1.21     | 0.003     |
| #35| TC195215_205_34_X2| NADH dehydrogenase, putative                                               | 0.003           | −1.41     | 0.003     |
| #36| TC193283_737_36_S | PHB2                                                                        | 0.001           | −1.19     | 0.003     |
| #37| TC212977_600_37_S | Nitrite reductase                                                           | 0.049           | 1.12      | 0.001     |
| #38| TC196100_60_35_S  | Plastid glutamine synthetase GS2                                            | 0.004           | −1.17     | 0.003     |
| #39| TC211800_873_40_S | Putative ferredoxin-dependent glutamate synthase 1                          | 0.006           | −1.00     | 0.003     |
| #40| TC197827_1154_40_S| Leucine-rich repeat/extension protein                                       | 0.030           | −1.13     | 0.003     |
| #41| TC203111_299_41_X2 | Extensin-like protein                                                        | 0.001           | −1.34     | 0.003     |
| #42| TC204863_245_40_S | Extensin-like protein Ext1                                                  | 0.016           | −1.15     | 0.003     |
| #43| TC216971_395_35_S | Extensin class 1 protein                                                    | 0.030           | 1.10      | 0.003     |
| #44| TC196973_752_38_X2| Pectinesterase                                                             | 0.019           | 1.11      | 0.003     |
| #45| TC210207_490_35_S | Pectinesterase                                                             | 0.004           | −1.18     | 0.003     |
| #46| TC193792_675_36_S | Putative glutathione S-transferase T5                                      | 0.023           | 1.48      | 0.003     |
| #47| TC202880_782_35_S | Glutathione S-transferase                                                   | 0.003           | −1.05     | 0.003     |
| #48| TC207401_351_40_S | Glutathione S-transferase/peroxidase                                        | 0.029           | 1.17      | 0.003     |
| #49| TC211832_300_41_X2| Glutathione-regulated potassium-efflux system protein kefB, putative       | 0.034           | 1.09      | 0.003     |
| #50| TC197773_1109_35_S| Peroxidase                                                                  | 0.032           | 1.08      | 0.003     |
| #51| TC209710_467_35_S | Peroxidase 16, putative                                                     | 0.012           | −1.17     | 0.003     |
| #52| TC192043_591_40_X3| 17.6 kDa class I heat shock protein (Hsp20.0)                              | 0.044           | 1.03      | 0.003     |
| #53| TC194246_668_40_S | Heat shock protein 70 (HSP70)                                               | 0.002           | −1.00     | 0.003     |
| #54| TC197122_92_35_S  | Hsp90 co-chaperone AHA1, putative                                           | 0.002           | −1.24     | 0.003     |
| #55| TC207719_568_36_S | Chaperone protein DNAj, putative                                            | 0.036           | 1.06      | 0.003     |
| #56| TC208736_54_40_S  | Chaperonin-60 alpha subunit                                                 | 0.044           | 1.05      | 0.003     |
Table 2  Differentially expressed transcripts cited in the Results and Discussion (Continued)

| #  | TC                 | Description                                           | Log2 Fold Change |
|----|--------------------|-------------------------------------------------------|------------------|
| 57 | TC214617_585_34_X2 | Hsp70-interacting protein 1                           | 0.002            |
| 58 | TC195735_752_37_S  | Avr9/Cf-9 rapidly elicited protein                    | 0.045            |
| 59 | TC196669_798_35_S  | Avr9/Cf-9 rapidly elicited protein 1                  | 0.040            |
| 60 | TC198633_775_40_S  | Avr9/Cf-9 rapidly elicited protein 231                | 0.019            |
| 61 | TC200277_609_40_S  | Avr9/Cf-9 rapidly elicited protein 194                | 0.032            |
| 62 | TC203605_414_40_S  | Avr9/Cf-9 rapidly elicited protein 75                 | 0.037            |
| 63 | TC204489_664_34_X2 | Avr9/Cf-9 rapidly elicited protein 20                 | 0.008            |
| 64 | TC207986_456_38_S  | Avr9/Cf-9 rapidly elicited protein 231                | 0.006            |
| 65 | TC208735_320_38_S  | Avr9/Cf-9 rapidly elicited protein 65                 | 0.006            |
| 66 | TC200524_503_40_S  | WRKY-type DNA binding protein                         | 0.035            |
| 67 | TC201566_1542_35_S | WRKY-like transcription factor                       | 0.040            |
| 68 | TC205993_1465_40_S | WRKY transcription factor 1                           | 0.023            |
| 69 | TC209196_761_40_S  | Double WRKY type transcription factor                | 0.014            |
| 70 | TC214887_802_40_S  | WRKY transcription factor-30                         | 0.006            |
| 71 | TC191592_2431_37_S | GRAS6                                                 | 0.006            |
| 72 | TC192009_1993_40_S | GRAS1                                                 | 0.038            |
| 73 | TC192616_2450_39_S | GRAS family transcription factor                      | 0.018            |
| 74 | TC193990_2097_35_S | GRAS9                                                 | 0.029            |
| 75 | TC195584_1695_40_S | GRAS4                                                 | 0.023            |
| 76 | TC208078_576_35_S  | GRAS4                                                 | 0.007            |
| 77 | TC213462_831_40_S  | GRAS2 transcription factor                            | 0.034            |

Transcript specifically affected by Fe-PS supply

| #  | TC                 | Description                                           | Log2 Fold Change |
|----|--------------------|-------------------------------------------------------|------------------|
| 78 | TC197535_663_40_S  | 3-Hydroxyacyl-CoA dehydrogenase                       | 0.004            |
| 79 | TC203351_729_35_S  | Fatty acid desaturase, putative                       | 0.004            |
| 80 | TC201677_436_40_S  | Acyl-CoA synthetase                                   | 0.002            |
| 81 | TC195028_1952_40_S | Putative phospholipase C                              | 0.026            |
| 82 | TC196917_1568_38_S | Delta(14)-sterol reductase                           | 0.026            |
| 83 | TC215747_470_40_S  | Phosphatidic acid phosphatase                         | 0.041            |
| 84 | TC195925_676_35_X2 | Ascorbate oxidase                                     | 0.049            |
| 85 | TC211305_518_35_S  | Oligopeptide transporter, putative                   | 0.044            |
| 86 | TC196465_645_40_X2 | Gibberellin 20 oxidase, putative                      | 0.029            |
| 87 | TC204594_438_40_S  | TGA10 transcription factor                            | 0.014            |
| 88 | TC196662_694_37_X2 | GRAS1                                                 | 0.041            |
| 89 | TC211460_599_40_S  |                                                        | 0.014            |
deficient) transcripts were Fe-citrate- and Fe-PS-specific, respectively (Fig. 3). These transcripts are related to specific responses that could be caused by the effect of different chelating agents on root plant metabolism. This hypothesis could be supported by the results obtained comparing the transcriptional profiles of Fe-deficient plant roots supplied for 1 h with Fe (Fe-citrate and Fe-PS) with Fe-sufficient plants (Additional file 1: Figure S2). This analysis revealed that about 52 % and 30 % of differentially expressed transcripts were specific for the -Fe/Fe-citrate vs Fe-sufficient and -Fe/Fe-PS vs Fe-sufficient comparisons, respectively (Additional file 1: Table S2, Figure S2).

Differentially expressed transcripts in the comparisons -Fe/Fe-citrate vs Fe-deficient and -Fe/Fe-PS vs Fe-deficient (Table 1), were manually annotated using terms of the biological process of the Gene Ontology (GO) [41] on the basis of BlastP analysis from UniProt database [42] (Additional file 3: Table S4). Eighty-five (11.67 %) and 64 (15.69 %) differentially expressed transcripts in response to Fe-citrate and Fe-PS treatment, respectively, encode proteins without homology with known proteins (“no hits found”). Table 3 shows that “biological process” (transcripts encoding protein involved in “unknown” biological process), “cellular process” and “metabolic process” are the more represented functional categories with similar percentage both for responses to Fe-citrate and Fe-PS supply. The other GO term categories showed similar percentages between the two treatments with the exceptions of “cellular component organization and biogenesis” that is more represented in the response to Fe-citrate relative to Fe-PS (3.53 % vs 1.77 %) while “lipid metabolic process” in the response to Fe-PS relative to Fe-citrate (2.00 % vs 0.92 %).

Transcripts commonly modulated by Fe-citrate and Fe-PS supply
The 289 transcripts commonly modulated after Fe-citrate and Fe-PS supply showed the same trend (235 downregulated and 54 upregulated transcripts, Additional file 3: Table S4). Excluding the peculiar behaviour of transcriptome in the presence of Fe-WEHS, this set of transcripts seems to represent the part of transcriptome responsive to the Fe-supply. Twenty upregulated transcripts and thirteen downregulated transcripts did not show any sequence homology with known proteins (“no hits found”). The distribution analysis of the main functional categories of transcripts with homology to known proteins showed that the more abundant terms “biological process”, “cellular process”, “metabolic process” and “biosynthetic process” were similarly represented in both downregulated and upregulated set of transcripts (Table 4). Differences were observed for “transport” with a higher fraction of a downregulated transcript dataset relative to the upregulated one (3.66 % vs 1.22 %) while other categories such as “carbohydrate metabolic process”, “catabolic process”, “cellular component organization and biogenesis” and “photosynthesis” were less represented in the downregulated transcript dataset (Table 4).

The downregulation of a plasma membrane H⁺-ATPase transcript (#3, Table 2) suggested that the acidification of the rhizosphere (component of Fe-acquisition machinery) is more quickly modulated than the expression of transcripts encoding FRO and IRT. Comparing the modulation of these common supply-specific transcripts with the results of the previous findings in tomato roots [5], we could observe that with the exception of transcripts related to Fe

![Fig. 3 Shared transcripts modulated in response to supply with the three natural Fe sources relative to Fe-deficient plants. Fe-deficient plants were supplied for 1 h with Fe-WEHS (-Fe/Fe-WEHS) or with Fe-PS (-Fe/Fe-PS) or with Fe-citrate (-Fe/Fe-citrate). As control, Fe-deficient plants were used (-Fe)](image)
### Table 3 Distribution in main functional categories of transcripts differentially expressed in response to Fe-citrate and Fe-PS supply respectively

| GO Class ID      | Definitions                                           | Counts | Fractions | GO Class ID      | Definitions                                           | Counts | Fractions |
|------------------|-------------------------------------------------------|--------|-----------|------------------|-------------------------------------------------------|--------|-----------|
| GO:0008150       | biological_process                                   | 178    | 27.34 %   | GO:0008150       | biological_process                                   | 122    | 27.05 %   |
| GO:0009987       | cellular process                                      | 118    | 18.13 %   | GO:0009987       | cellular process                                      | 80     | 17.74 %   |
| GO:0008152       | metabolic process                                     | 101    | 15.51 %   | GO:0008152       | metabolic process                                     | 76     | 16.85 %   |
| GO:0009058       | biosynthetic process                                  | 33     | 5.07 %    | GO:0009058       | biosynthetic process                                  | 27     | 5.99 %    |
| GO:0006139       | nucleobase, nucleoside, nucleotide and nucleic acid   | 24     | 3.69 %    | GO:0006139       | nucleobase, nucleoside, nucleotide and nucleic acid  | 18     | 3.99 %    |
|                  | metabolic process                                     |        |           |                  | metabolic process                                     |        |           |
| GO:0016043       | cellular component organization and biogenesis       | 23     | 3.53 %    | GO:0019538       | protein metabolic process                             | 16     | 3.55 %    |
| GO:0019538       | protein metabolic process                             | 22     | 3.38 %    | GO:0006810       | transport                                             | 13     | 2.88 %    |
| GO:0006810       | transport                                             | 20     | 3.07 %    | GO:0005975       | carbohydrate metabolic process                        | 11     | 2.44 %    |
| GO:0009056       | catabolic process                                     | 17     | 2.61 %    | GO:0009056       | catabolic process                                     | 11     | 2.44 %    |
| GO:0005975       | carbohydrate metabolic process                        | 13     | 2.00 %    | GO:0006629       | lipid metabolic process                               | 9      | 2.00 %    |
| GO:0006950       | response to stress                                    | 12     | 1.84 %    | GO:0016043       | cellular component organization and biogenesis       | 8      | 1.77 %    |
| GO:0007154       | cell communication                                    | 9      | 1.38 %    | GO:0007165       | signal transduction                                   | 6      | 1.33 %    |
| GO:0006091       | generation of precursor metabolites and energy        | 9      | 1.38 %    | GO:0007154       | cell communication                                    | 6      | 1.33 %    |
| GO:0007165       | signal transduction                                   | 8      | 1.23 %    | GO:0006950       | response to stress                                    | 6      | 1.33 %    |
| GO:0006464       | protein modification process                          | 8      | 1.23 %    | GO:0006464       | protein modification process                          | 6      | 1.33 %    |
| GO:0006259       | DNA metabolic process                                 | 6      | 0.92 %    | GO:0006091       | generation of precursor metabolites and energy        | 5      | 1.11 %    |
| GO:0006259       | lipid metabolic process                               | 6      | 0.92 %    | GO:0006259       | DNA metabolic process                                 | 4      | 0.89 %    |
| GO:0009628       | response to abiotic stimulus                          | 5      | 0.77 %    | GO:0006412       | translation                                           | 4      | 0.89 %    |
| GO:0009719       | response to endogenous stimulus                       | 5      | 0.77 %    | GO:0015979       | photosynthesis                                        | 3      | 0.67 %    |
| GO:0006412       | translation                                           | 5      | 0.77 %    | GO:0016265       | death                                                | 2      | 0.44 %    |
| GO:0015979       | photosynthesis                                        | 4      | 0.61 %    | GO:0009628       | response to abiotic stimulus                          | 2      | 0.44 %    |
| GO:0016265       | death                                                 | 3      | 0.46 %    | GO:0009607       | response to biotic stimulus                           | 2      | 0.44 %    |
| GO:0008219       | cell death                                            | 3      | 0.46 %    | GO:0009719       | response to endogenous stimulus                       | 2      | 0.44 %    |
| GO:0000003       | reproduction                                          | 2      | 0.31 %    | GO:0008219       | cell death                                            | 2      | 0.44 %    |
| GO:0009607       | response to biotic stimulus                           | 2      | 0.31 %    | GO:0040007       | growth                                               | 2      | 0.44 %    |
| GO:0007275       | multicellular organismal development                 | 2      | 0.31 %    | GO:0000003       | reproduction                                          | 1      | 0.22 %    |
| GO:0040007       | growth                                                | 2      | 0.31 %    | GO:0009791       | post-embryonic development                            | 1      | 0.22 %    |
| GO:0007049       | cell cycle                                            | 2      | 0.31 %    | GO:0009605       | response to external stimulus                         | 1      | 0.22 %    |
| GO:0009791       | post-embryonic development                            | 1      | 0.15 %    | GO:0009908       | flower development                                    | 1      | 0.22 %    |
| GO:0009653       | anatomical structure morphogenesis                    | 1      | 0.15 %    | GO:0007275       | multicellular organismal development                 | 1      | 0.22 %    |
| GO:0009605       | response to external stimulus                         | 1      | 0.15 %    | GO:0016049       | cell growth                                           | 1      | 0.22 %    |
| GO:0009908       | flower development                                    | 1      | 0.15 %    | GO:0019725       | cell homeostasis                                      | 1      | 0.22 %    |
| GO:0019748       | secondary metabolic process                           | 1      | 0.15 %    | GO:0007049       | cell cycle                                            | 1      | 0.22 %    |
homeostasis (e.g. those encoding FRO, IRT and Natural Resistance-Associated Macrophage Protein, NRAMP) most of the molecular mechanisms involved in the response to the Fe shortage (e.g. glycolysis, TCA cycle, methionine cycles, protein turnover, phenolic compound biosynthesis, root morphological adaptation and signalling) were modulated suggesting the restoration of sufficient nutrient condition. Specifically, we detected a negative modulation of transcripts encoding a phosphofructokinase (PFK; #4), a fructose-bisphosphate aldolase (FBP; #5) for glycolysis and a succinate dehydrogenase (SDH; #6) and a 2′-oxoglutarate dehydrogenase (OGDC; #7) for tri-carboxylic acid (TCA) cycle (Table 2). As far as methionine metabolism and cycle is concerned a methionine synthase (MS; #8) and two S-adenosylmethionine-dependent methyltransferase (SAMT) transcripts (#9 and #10) were repressed (Table 2). Furthermore, transcripts involved in protein turnover such as proteases and peptidases (Additional file 3: Table S4) were mainly negatively affected suggesting the readjusting of the protein metabolism related to the anaplerotic functions. The synthesis and transport of phenolic secondary metabolites appear to be negatively affected as highlighted by the downregulation of transcripts encoding a phenylalanine ammonia-lyase (PAL, #11) and an ATP-binding cassette (ABC) transporter [43] (#12, Table 2). A similar behaviour was observed for transcripts involved in the synthesis of cell wall components (cellulose synthases, CES, #13, #14 and #15 and UDP-apiose/xylose synthase, AXS, #16) while cell wall loosening and modification appeared to be positively influenced by the presence of the micronutrient as highlighted by the upregulation of transcripts encoding a phenylalanine ammonia-lyase (PAL, #11, Table 2) and a glucan endo-1,3-beta-glucosidase (#18, Table 2) [44–47]. Concerning the role of Ca2+ as secondary messenger during Fe shortage, the negative modulation of signal transduction machinery genes (Rho GTPase-activating protein 1, #19; ATP/GTP/Ca2+ binding protein, #20; mitochondrial Rho GTPase calcineurin B-like (CBL)-interacting protein kinase 1, #21; Protein IQ-DOMAIN 14, #22; Ras-related GTP binding protein, #23 and #24) is in agreement with the adjustment due to the restored nutrient conditions (Table 2).

Together with the general behaviour described above suggesting the readjustment of metabolic pathways linked to Fe shortage to an adequate nutritional condition, our analysis revealed that other mechanisms are involved in this response.

It is known that the alternative pathway of pyruvate synthesis independent of pyruvate kinase (PK), which is involved in the supply of low-molecular weight organic acid to TCA cycle, is induced under Fe-deficiency [48]; this behaviour would allow to supply reducing power in plants where the functionality of the mitochondrial respiratory chain is limited [49, 50]. A malic enzyme (ME) transcript (#25, Table 2) was repressed in response to Fe supply, hence decreasing the substrate provision to the alternative metabolic cycle. Furthermore, the overexpression of the subunit I of the NADH:ubiquinone oxidoreductase transcript (NADH_UbQ_OxRdtase; #26, Table 2) suggests that Fe supply could restore the respiration chain activity. The repression of a transcript encoding a glutamate dehydrogenase (GDH; #27, Table 2) related to anaplerotic reaction of TCA [51] reinforces the hypothesis of a possible back regulation of TCA cycle during the supply.

Focusing on the transcript involved in molecular processes leading to protein synthesis (i.e. translation GO:0006411), protein folding (GO:0006457) and protein modification (i.e. protein phosphorylation GO:0006468, protein dephosphorylation GO:0006470; protein glycosylation GO:0006486) we observed a downregulation rather than an upregulation (Table 4). This suggests that the new protein synthesis and/or protein modification [52] necessary to respond to the micronutrient depletion are not required in the new restored nutrient condition. We also recorded a downregulation of transcripts involved in DNA and RNA metabolic processes (i.e. DNA topological changes GO:0006265; DNA replication initiation GO:0006270; DNA repair GO:0006281; transcription, DNA-templated GO:0006351; regulation of transcription, DNA-templated GO:0006355; RNA splicing GO:0008380) that could be in line with the decrease in protein synthesis. Despite that, other transcripts encoding transcription factors (regulation of transcription, DNA-templated GO:0006355) were

### Table 3 Distribution in main functional categories of transcripts differentially expressed in response to Fe-citrate and Fe-PS supply respectively (Continued)

| GO:00016049 cell growth | 1 | 0.15 % | Total | 451 | 100.00 % |
|--------------------------|---|---------|-------|-----|----------|
| GO:0009875 pollen-pistil interaction | 1 | 0.15 % |       |     |          |
| GO:0019725 cell homeostasis | 1 | 0.15 % |       |     |          |
| GO:0009856 pollination | 1 | 0.15 % |       |     |          |

The distribution in main functional categories on the basis of “biological process” terms was performed using CateGOrizer [79] setting Plant GO slim method and consolidated single occurrences. The analysis was performed using the GO terms of the 643 and 344 transcripts differentially expressed in response to Fe-citrate and Fe-PS respectively and showing homology to “known protein.”
upregulated by the treatment with Fe-citrate and Fe-PS (Table 4). Among these transcripts, one encodes for a bHLH (#28) and the other one for a R2R3-MYB transcript (#1). Interestingly this latter transcript is induced by all three Fe-sources (Table 2).

### Table 4 Distribution in main functional categories of transcripts modulated both during the Fe-citrate and Fe-PS supply

| GO Class ID | Definitions | Upregulated | Counts | Fractions | GO Class ID | Definitions | Downregulated | Counts | Fractions |
|-------------|-------------|-------------|--------|-----------|-------------|-------------|---------------|--------|-----------|
| GO:0008150  | biological process |             | 21     | 25.61 %   | GO:0008150  | biological process |       | 91     | 27.74 %   |
| GO:0009987  | cellular process |             | 15     | 18.29 %   | GO:0009987  | cellular process |       | 56     | 17.07 %   |
| GO:0008152  | metabolic process |          | 14     | 17.07 %   | GO:0008152  | metabolic process |       | 55     | 16.77 %   |
| GO:0009058  | biosynthetic process |        | 5      | 6.10 %    | GO:0009058  | biosynthetic process |       | 20     | 6.10 %    |
| GO:0005975  | carbohydrate metabolic process | | 4      | 4.88 %    | GO:0005975  | carbohydrate metabolic process |       | 14     | 4.27 %    |
| GO:0009056  | catabolic process |          | 4      | 4.88 %    | GO:0009056  | catabolic process |       | 12     | 3.66 %    |
| GO:0019538  | protein metabolic process |       | 3      | 3.66 %    | GO:0019538  | protein metabolic process |       | 12     | 3.66 %    |
| GO:0016043  | cellular component organization and biogenesis | | 2      | 2.44 %    | GO:0016043  | cellular component organization and biogenesis |       | 7      | 2.13 %    |
| GO:0006091  | generation of precursor metabolites and energy | | 2      | 2.44 %    | GO:0006091  | generation of precursor metabolites and energy |       | 7      | 2.13 %    |
| GO:0006139  | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process | | 2      | 2.44 %    | GO:0006139  | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |       | 6      | 1.83 %    |
| GO:0019579  | photosynthesis |          | 2      | 2.44 %    | GO:0019579  | photosynthesis |       | 6      | 1.83 %    |
| GO:0006950  | response to stress |        | 5      | 5.22 %    | GO:0006950  | response to stress |       | 5      | 5.22 %    |
| GO:0006629  | lipid metabolic process |       | 5      | 5.22 %    | GO:0006629  | lipid metabolic process |       | 5      | 5.22 %    |
| GO:0006142  | translation |          | 4      | 4.27 %    | GO:0006142  | translation |       | 4      | 4.27 %    |
| GO:0006259  | DNA metabolic process |       | 3      | 0.91 %    | GO:0006259  | DNA metabolic process |       | 3      | 0.91 %    |
| GO:0006901  | generation of precursor metabolites and energy | | 3      | 0.91 %    | GO:0006901  | generation of precursor metabolites and energy |       | 3      | 0.91 %    |
| GO:0006412  | translation |          | 2      | 0.61 %    | GO:0006412  | translation |       | 2      | 0.61 %    |
| GO:0006091  | cell communication |       | 2      | 0.61 %    | GO:0006091  | cell communication |       | 2      | 0.61 %    |
| GO:0019725  | signal transduction |       | 2      | 0.61 %    | GO:0019725  | signal transduction |       | 2      | 0.61 %    |
| GO:0007154  | cell communication |       | 2      | 0.61 %    | GO:0007154  | cell communication |       | 2      | 0.61 %    |
| GO:0007154  | cell communication |       | 2      | 0.61 %    | GO:0007154  | cell communication |       | 2      | 0.61 %    |
| GO:0006901  | lipid metabolic process |       | 2      | 0.61 %    | GO:0006901  | lipid metabolic process |       | 2      | 0.61 %    |
| GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    | GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    |
| GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    | GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    |
| GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    | GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    |
| GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    | GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    |
| GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    | GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    |
| GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    | GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    |
| GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    | GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    |
| GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    | GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    |
| GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    | GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    |
| GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    | GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    |
| GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    | GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    |
| GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    | GO:0007154  | lipid metabolic process |       | 2      | 0.61 %    |

The distribution in main functional categories on the basis of “biological process” terms was performed using CateGOrizer [79] setting Plant GO slim method and consolidated single occurrences. The analysis was performed using the GO terms of the 41 and 215 transcripts positively and negatively affected respectively in response to both Fe-citrate and Fe-PS and showing homology to “known protein.”

**Transcript specifically affected by Fe-citrate supply**

Among the 439 transcripts modulated exclusively by the Fe-citrate treatment, 233 were downregulated and 206 upregulated. Twenty-four downregulated and 26 upregulated transcripts did not show any sequence homology
with known proteins (“no hits found”). The distribution analysis of the main functional categories of transcripts with homology to known proteins showed that the more abundant “biological process”, “cellular process” and “metabolic process” were similarly represented both for downregulated and upregulated set of transcripts (Table 5). “Cellular component organization biogenesis“, “biosynthetic process” and “nucleobase, nucleoside, nucleotide and nucleic acid metabolic process” functional categories were more represented in the downregulated transcript dataset while “signal transduction” in the upregulated transcript dataset (Table 5).

The analysis of downregulated transcripts showed that in addition to those involved in carbohydrate metabolism and TCA cycle above discussed, other genes related to glycolysis (i.e. triose-phosphate isomerase, TIM, #29) and

| Table 5 Distribution in main functional categories of transcripts specifically affected by Fe-citrate supply |
|--------------------------------------------------|--------------------------------------------------|
| Upregulated                                      | Downregulated                                    |
| GO Class ID                                      | Definitions                                      | GO Class ID                                      | Definitions                                      |
| GO0008150 biological_process                     | 59 29.35 %                                       | GO0008150 biological_process                     | 93 28.53 %                                       |
| GO0009987 cellular process                        | 38 18.91 %                                       | GO0009987 cellular process                        | 59 18.10 %                                       |
| GO0008152 metabolic process                       | 31 15.42 %                                       | GO0008152 metabolic process                       | 48 14.72 %                                       |
| GO0019538 protein metabolic process               | 9 4.48 %                                         | GO0016043 cellular component organization and biogenesis | 17 5.21 % |
| GO0006950 response to stress                     | 7 3.48 %                                         | GO0009058 biosynthetic process                    | 17 5.21 % |
| GO0006810 transport                              | 7 3.48 %                                         | GO0019538 protein metabolic process               | 15 4.60 % |
| GO0009038 biosynthetic process                   | 6 2.99 %                                         | GO0006810 transport                               | 12 3.68 % |
| GO0006139 nucleobase, nucleoside, nucleotide and nucleic acid metabolic process | 6 2.99 % | GO0006139 nucleobase, nucleoside, nucleotide and nucleic acid metabolic process | 12 3.68 % |
| GO0016043 cellular component organization and biogenesis | 5 2.49 % | GO0009056 catabolic process                       | 9 2.76 % |
| GO0007165 signal transduction                    | 4 1.99 %                                         | GO0006950 response to stress                      | 5 1.53 % |
| GO0007154 cell communication                     | 4 1.99 %                                         | GO0005975 carbohydrate metabolic process          | 4 1.23 % |
| GO0009036 catabolic process                      | 4 1.99 %                                         | GO0007154 cell communication                      | 4 1.23 % |
| GO0009719 response to endogenous stimulus        | 3 1.49 %                                         | GO0006464 protein modification process            | 4 1.23 % |
| GO0006464 protein modification process           | 3 1.49 %                                         | GO0006412 translation                             | 4 1.23 % |
| GO0006928 response to abiotic stimulus           | 2 1.00 %                                         | GO0007165 signal transduction                     | 3 0.92 % |
| GO0005975 carbohydrate metabolic process         | 2 1.00 %                                         | GO0006091 generation of precursor metabolites and energy | 3 0.92 % |
| GO0015979 photosynthesis                         | 2 1.00 %                                         | GO0015979 photosynthesis                          | 3 0.92 % |
| GO0006629 lipid metabolic process                | 2 1.00 %                                         | GO0006259 DNA metabolic process                   | 2 0.61 % |
| GO0016265 death                                  | 1 0.50 %                                         | GO0009719 response to endogenous stimulus         | 2 0.61 % |
| GO0006259 DNA metabolic process                  | 1 0.50 %                                         | GO0006629 lipid metabolic process                 | 2 0.61 % |
| GO0019725 cell homeostasis                       | 1 0.50 %                                         | GO0000003 reproduction                            | 1 0.31 % |
| GO0006091 generation of precursor metabolites and energy | 1 0.50 % | GO0009628 response to abiotic stimulus            | 1 0.31 % |
| GO0008219 cell death                             | 1 0.50 %                                         | GO0009875 pollen-pistil interaction               | 1 0.31 % |
| GO0006412 translation                            | 1 0.50 %                                         | GO0019725 cell homeostasis                        | 1 0.31 % |
| GO0007049 cell cycle                             | 1 0.50 %                                         | GO0009856 pollination                            | 1 0.31 % |
| Total                                           | 201 100.00 %                                     | GO0009653 anatomical structure morphogenesis      | 1 0.31 % |
                                                                                     | GO0007275 multicellular organismal development   | 1 0.31 % |
                                                                                     | GO0019748 secondary metabolic process            | 1 0.31 % |
                                                                                     | Total                                            | 326 100.00 %                                     |

The distribution in main functional categories on the basis of “biological process” terms was performed using CateGOrizer [79] setting Plant GO slim method and consolidated single occurrences. The analysis was performed using the GO terms of the 180 and 209 transcripts positively and negatively affected respectively in response to Fe citrate and showing homology to “known protein”
pentose phosphate pathway (i.e. 6-phosphogluconate dehydrogenase, PGD, #30) were negatively affected (Table 2). A similar behaviour was observed for transcripts of the TCA cycles (pyruvate dehydrogenase E1 beta subunit transcript, PDC, #31; citrate synthase, CS, #32) and of the alternative pathway via PEPC (i.e. a transcript encoding PEPC, #33) (Table 2). Two NADH dehydrogenase (NDH) transcripts (#34 and #35) and another one showing homology to the tobacco prohibitin 2 (NtPHB2, #36), which is involved in stress tolerance stabilizing the mitochondrial function [53], were found to be repressed by Fe-citrate treatment. This might be explained as a specific regulation of TCA cycle and mitochondrial activity when Fe is supplied as Fe-citrate, bearing in mind that this organic acid might be absorbed by roots [54].

Other processes related to protein synthesis ("translational", "translational initiation" and "protein folding"; Table 5) and protein catabolism were mainly repressed (downregulated) in response to Fe-citrate treatment. On the other hand, in the same treatment the functional categories of protein modification processes (e.g. phosphorylation and proteolysis) were similarly represented both for downregulated and upregulated sets of transcripts (Table 5).

The supply with Fe-citrate caused the upregulation of a transcript encoding the Fe-containing enzyme nitrite reductase (NiR, #37, Table 2). This evidence might indicate the restoration of nitrate assimilation, which is known to be altered in Fe-deficient conditions [55]. Besides those involved in protein turnover (see above), other genes putatively related to N recycling were found to be downregulated, such as plastid GS (#38) and ferredoxin-dependent glutamate synthase 1 (GLU, #39).

In addition, Fe-citrate caused the modulation in either directions of transcripts involved in the cell wall metabolism (i.e. extensin, EXT, #40, #41, #42 and #43; pectinesterase, PE, #44 and #45), in oxidative stress (#46, #47, #48, #49, #50 and #51) and encoding heat-shock proteins (#52, #53, #54, #55, #56 and #57) (Table 2). These results suggest that the modulation of these processes might be related on one side to the changing of Fe nutritional status, and on the other side to the presence of citrate.

Interestingly, many transcripts involved in the regulation of plant defence response such as Avr/Cf-9 rapidly elicited (ACRE) genes (#58, #59, #60, #61, #62, #63, #64 and #65) and those encoding WRKYs (#66, #67, #68, #69 and #70) were induced by the Fe-citrate supply (Table 2). The activity of these transcripts could be related to the avoidance of Fe toxicity. The involvement of ACRE genes in the response to Al-toxicity in rice roots [56] and the role of a WRKY rice protein in response to excess of Fe [57] has been reported. A similar role in response to Fe toxicity could be ascribed to the upregulation of transcripts belonging to GAI, RGA, RCS (GRAS) gene family (#71, #72, #73, #74, #75, #76 and #77, Table 2). GRAS proteins play a role in the regulation of root growth, nodulation signalling and response to environmental stresses [58]; furthermore, members of this gene family are involved in disease resistance and mechanical stress response in tomato [59].

Transcript specifically affected by Fe-PS supply

One hundred and nineteen transcripts were specifically modulated in tomato roots by Fe-PS treatment (Fig. 3; Additional file 3: Table S4), 82 and 37 in a negative and in a positive way, respectively. Twenty-one downregulated and ten upregulated transcripts did not show any homology to known proteins (“no hit found”, Additional file 3: Table S4). The distribution in main functional categories highlighted that for the Fe-PS specific transcripts the most abundant categories are "biological process", "cellular process", "metabolic process", "protein metabolic process" and "nucleobase, nucleoside, nucleotide and nucleic acid metabolic process" (Table 6). Transcripts related to "lipid metabolic process" are mainly negatively affected (Table 2) such as those encoding a 3-hydroxyacyl-CoA dehydrogenase (HADH, #78), a fatty acid desaturase (FADS, #79), an acyl-CoA synthetase (ACS, #80) and a phospholipase C (PLC, #81) while only a lipid metabolism-related transcripts, encoding a delta(14)-sterol reductase (Delta-14-SR, #82) was upregulated. In addition to the phospholipase C (PLC) transcript, the treatment with Fe-PS repressed the expression of another transcript encoding a phosphatidic-acid-phosphatase (#83, Table 2) suggesting that this Fe source can specifically affect the phospholipid-based signal, which is involved in plant environmental responses [60, 61]. It has been shown that the repression of plant PLCs is related to the response to toxic metals, such as Al3+ and Cd2+, that implies limiting ROS generation and lipid peroxidation [61, 62]. Iron-PS might negatively affect the phospholipid-based signal that controls responses to Fe, possibly through the reduction of ROS as suggested by the downregulation of an ascorbate oxidase (AO) transcript (#84, Table 2). This AO gene is involved in ascorbic acid biosynthesis in tomato [63] playing an important role as an antioxidant and protecting plant cells during oxidative damage by scavenging free radicals and ROS. On the basis of these results, it might be speculated that Fe is present within the root cells as Fe-PS complex and that this Fe-form could limit Fe-induced ROS production. Indeed there is some evidence that Strategy I plants can directly take up Fe-PS complexes [35]. The idea that tomato roots could at least in part adsorb the Fe-PS complexes is supported by the observation that a transcript encoding an oligopeptide transporter “Yellow stripe-like protein” (#85, Solyc03g031920.2.1)
was repressed after 1 h of Fe-PS treatment. The rapid back-regulation of this putative Fe-PS transporter would indicate a secondary role in Fe nutrition of tomato plants, while possibly having a role in preventing oxidative damages in the early stages of Fe supply.

A gene encoding the gibberellin 20 oxidase (GA20OX, #86, Table 2), previously hypothesized acting in tomato root morphological changes in response to Fe deficiency [5], was one of those specifically downregulated by Fe-PS supply.

Furthermore, the Fe-PS treatment specifically modulated transcripts encoding transcription factors in a negative (i.e. TGA10, #87; GRAS, #88; bHLH JAF13, #89) and in a positive way (Myb-like protein, #90; Homeobox-leucine zipper protein, #91) (Table 2). GRAS transcripts were positively modulated in response to Fe-citrate treatment while, in the case of Fe-PS, one GRAS transcript was downregulated. Our results suggest that some transcription factors could play a role in the response to Fe supply common to different Fe sources, such as the R2R3 MYB tran-

Table 6 Distribution in main functional categories of transcripts specifically affected by Fe-PS supply

| Upregulated | GO Class ID | Definitions | Counts | Fractions | GO Class ID | Definitions | Counts | Fractions |
|-------------|-------------|-------------|--------|-----------|-------------|-------------|--------|-----------|
| GO:0008150  | biological_process | 19 | 27.94 % | GO:0008150  | biological_process | 32 | 29.36 % |
| GO:0008152  | metabolic process | 14 | 20.59 % | GO:0008152  | metabolic process | 20 | 18.35 % |
| GO:0009987  | cellular process | 11 | 16.18 % | GO:0009987  | cellular process | 19 | 17.43 % |
| GO:0009058  | biosynthetic process | 5 | 7.35 % | GO:0009058  | biosynthetic process | 6 | 5.50 % |
| GO:0019538  | protein metabolic process | 5 | 7.35 % | GO:0006139  | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process | 5 | 4.59 % |
| GO:0006139  | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process | 2 | 2.94 % | GO:0006629  | lipid metabolic process | 4 | 3.67 % |
| GO:0006464  | protein modification process | 2 | 2.94 % | GO:0006810  | transport | 3 | 2.75 % |
| GO:0005975  | carbohydrate metabolic process | 2 | 2.94 % | GO:0006464  | protein modification process | 3 | 2.75 % |
| GO:0009056  | catabolic process | 2 | 2.94 % | GO:0005975  | carbohydrate metabolic process | 3 | 2.75 % |
| GO:0006810  | transport | 1 | 1.47 % | GO:0006810  | transport | 2 | 1.83 % |
| GO:0007165  | signal transduction | 1 | 1.47 % | GO:0007165  | signal transduction | 2 | 1.83 % |
| GO:0007154  | cell communication | 1 | 1.47 % | GO:0007154  | cell communication | 2 | 1.83 % |
| GO:0006091  | generation of precursor metabolites and energy | 1 | 1.47 % | GO:0006950  | response to stress | 2 | 1.83 % |
| GO:0006412  | translation | 1 | 1.47 % | GO:0009058  | biosynthetic process | 2 | 1.83 % |
| GO:0006629  | lipid metabolic process | 1 | 1.47 % | GO:0009056  | catabolic process | 2 | 1.83 % |
| Total       | 68 | 100.00 % | GO:0006259  | DNA metabolic process | 1 | 0.92 % |
|             | 109 | 100.00 % | GO:0009719  | response to endogenous stimulus | 1 | 0.92 % |

The distribution in main functional categories on the basis of “biological process” terms was performed using CateGOrizer [79] setting Plant GO slim method and consolidated single occurrences. The analysis was performed using the GO terms of the 27 and 61 transcripts positively and negatively affected respectively in response to Fe-PS and showing homology to “known protein” Zamboni et al. BMC Genomics (2016) 17:35 Page 13 of 17

and pathways selectively modulated in response to each Fe-source.

Conclusions

Our results suggest that the root transcriptional response to Fe supply depends on the nature of the ligand (WEHS, citrate and PS). The supply with Fe-WEHS, which has been demonstrated to be able to enhance Fe acquisition responses in Strategy I plants [23, 36], did not cause relevant changes in the root transcriptome with respect to the Fe-deficient plants, indicating that roots did not sense the restored cellular Fe accumulation. This result could explain the higher Fe concentration observed after 4 and 24 h in tomato plant tissues supplied with Fe-WEHS as compared to the other Fe-sources. This behaviour is confirmed by a faster and more efficient Fe allocation in the leaf tissue [37]. As a result, Fe-WEHS supply would favour a better distribution of Fe within the plant.

The transcriptional behaviour of tomato roots with the other two natural Fe-sources, Fe-citrate and Fe-PS, underlined that the supply responses are fast and based on a back regulation of molecular mechanisms modulated
under Fe deficiency. We also observed some responses specific for each of the two natural Fe sources suggesting a transcriptional response in roots to the molecule used to chelate the micronutrient. Considering transcripts specifically regulated by Fe-citrate, we could hypothesize that citrate is also absorbed by roots causing a further negative regulation of the TCA cycle and influencing mainly cell wall metabolism and the response regulation to stress. Iron-PS specific responses seem to be mainly based on a negative regulation of lipid metabolism and phospholipid-based signal that control ROS responses in the presence of heavy metals.

**Methods**

Water extractable humic substances (WEHS) were isolated as reported by Pinton et al. [64] and Fe-WEHS complexes were prepared as described by Cesco et al. [31] by mixing 5 μg organic carbon (Corg) of WEHS fraction for each μmol of FeCl₃. A thorough chemical characterization of the fractions is described elsewhere [23].

Phytosiderophores (PS) were collected in the root exudate of Fe-deficient barley plants as described by Tomasi et al. [22]. Iron-PS and Fe-citrate were prepared accordingly to von Wirsén et al. [65] by mixing an aliquot of Fe-free-PS or citrate (10 % excess of the chelating agent) with FeCl₃. For radiochemical experiments, ⁵⁹FeCl₃ was utilized at the specific labeling activity of 144 kBq μmol⁻¹ Fe (Perkin Elmer, Monza, Italy).

**Plant material and growth conditions**

Tomato seedling (*Solanum lycopersicum* L., cv. ‘Marmande superprecoce’, DOTTO Spa, Italy) were first germinated for 6 days on filter paper moistened with 1 mM CaSO₄ and consequently grown for other 14 days in a continuously aerated nutrient solution (pH adjusted at 6.0 with 1 M KOH) as reported by Tomasi at al. [22]. Iron-PS and Fe-citrate were prepared accordingly to von Wirsén et al. [65] by mixing an aliquot of Fe-free-PS or citrate (10 % excess of the chelating agent) with FeCl₃. For radiochemical experiments, ⁵⁹FeCl₃ was utilized at the specific labeling activity of 144 kBq μmol⁻¹ Fe (Perkin Elmer, Monza, Italy).

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concentration of the Fe(II)-BPDS₃ complex formed, using an extinction coefficient of 22.1 mM⁻¹ cm⁻¹.

RNA extraction and microarray analyses
Transcriptional analysis was carried out using a Combi-matrix chip [68], produced by the Functional Genomics Lab., University of Verona [69]. The chip (TomatoArray2.0) carries 25,789 nonredundant probes (23,282 unique probes and 2507 probes with more than one target) randomly distributed in triplicate across the array, each comprising a 35–40-mer oligonucleotide designed using the program oligoarray 2.1 [70]. The source of sequence information included tentative consensus sequences (TCs) derived from the DFCI Tomato Gene Index [71] Release 12.0 and expressed sequence tags. Eight bacterial oligonucleotide sequences provided by CombiMatrix, 8 probes designed on 8 Ambion spikes and 40 probes based on Bacillus anthracis, Haemophilus ducreyi and Alteromonas phage sequences were used as negative controls. Complete description of the chip is available at the Gene Expression Omnibus [72] under the series entry (GPI13934).

Total RNA was isolated using the SpectrumTM Plant Total RNA kit (Sigma-Aldrich) and quantified by spectrophotometry using NanoDrop™ 1000 (Thermo Scientific). RNA quality was evaluated using Agilent 2100 Bioanalyzer (Agilent). Total RNA (1 μg) was amplified and labelled using the RNA ampULSe kit (Kreatech). After checking the quantity and quality of antisense (aRNA) by spectrophotometry using NanoDrop™ 1000 (Thermo Scientific) and the quality subsequent labelling, 4 μg of labelled aRNA was hybridized to the array according to the manufacturer’s recommendations [68]. Pre-hybridization, hybridization, washing and imaging were performed according to the manufacturer’s protocols. The array was scanned with an Axon GenePix® 4400A scanner (MDS Analytical Technologies).

Analysis of raw data was performed using the open source software of the Bioconductor project [73, 74] with the statistical R programming language [75, 76]. Background adjustment, summarization and quantile normalization were performed using limma package. Probes expressed in all three biological replicates were considered otherwise probes were removed. Differentially expressed probes were identified by linear models analysis [39] using limma package and applying Bayesian correction, adjusted p-value of 0.05 and a FC ≥ 2. All microarray expression data are available at the Gene Expression Omnibus [72] under the series entry (GSE69419). The data obtained by Fe-deficient and Fe-sufficient plants used in the experiments presented in Zamboni et al. [5] were used as control and submitted with the GEO code: GSE31112. Differentially expressed transcripts between Fe-deficient plants supply for 1 h with the three natural Fe sources and Fe-deficient plants were grouped in main functional categories according to the “biological” terms of the Gene Ontology [30] assigned to each tomato TC or EST (Release 12.0) on the basis of the results of BlastP analysis [77] against the UniProt database [42] (Additional file 3: Table S4). Genes without significant BlastP results were classified as “no hits found” (E-value < 1e-8; identity > 40 %).

Real-time RT-PCR experiments
Five hundreds nanograms of total RNA (isolated as previously described) of each sample was retrotranscribed using 1 pmol of Oligo d(T)23VN (New England Biolabs, Beverly, USA) and 10 U M-MulV RNase H⁺ for 1 h at 42 °C (Finzymes, Helsinki, Finland) following the application protocol of the manufacturers. After RNA digestion with 1 U RNase A (USB, Cleveland, USA) for 1 h at 37 °C, gene expression analyses were performed by adding 0.16 μL of the cDNA to the realtime PCR complete mix, Fluocycle™ sybr green (20 μL final volume; Euroclone, Pero, Italy), in a DNA Engine Opticon Real-Time PCR Detection (Biorad, Hercules, USA). Specific primers (Tm = 58 °C) were designed to generate 80–150 bp PCR products. Three genes were used as housekeeping to normalize the data: LeEF1a, coding for 1-alpha elongation factor (X14449), LeH1, coding for histone protein (AJ224933) and LeUbi3, coding for a ubiquitin protein (X58253). Each Real-Time RT-PCR was performed 4 times on 3 independent experiments; analyses of real-time result were performed using Opticon Monitor 2 software (Biorad, Hercules, USA) and R [74–76], with the qpcr package [78]. Efficiencies of amplification were calculated following the authors’ indications. Sequences of forward and reverse primers and efficiencies were reported in Additional file 1: Table S5 gene.

Additional files

Additional file 1: Figure S1. Cluster heat map of gene expression data.

Additional file 2: Table S3. Differentially expressed transcripts resulted by the comparison of root transcriptional profiles of Fe-deficient plants supplied for 1 h with Fe-WEHS, Fe-citrate and Fe-PS with root transcriptional profile of Fe-deficient plants. Probe ID, adjusted p-value and Log₂(R) were reported for each transcript. (XLS 174 kb)

Additional file 3: Table S4. Functional annotation of differentially expressed transcripts resulted by the comparison of root transcriptional profiles of Fe-deficient plants supplied for 1 h with Fe-citrate, Fe-PS and Fe-WEHS with root transcriptional profile of Fe-deficient plants. Probe ID, description, species, identity, score, e-value, Uniprot entry and biological
GO term were reported. The adjusted p-value and Log2(R) were also reported for each transcript. (XLSX 130 kb)

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AZ and LZ made a substantial contribution to data collection and interpretation and manuscript drafting. NT participated in the project’s design, data analysis and manuscript revision. LA critically revised the manuscript. RP contributed to data interpretation and critically revised the manuscript. 2V contributed to data interpretation and manuscript writing. SC participated in the project’s design and coordination and critically revised the manuscript. All authors read and approved the final manuscript.

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