Phosphorus and iron deficiencies induce a metabolic reprogramming and affect the exudation traits of the woody plant Fragariaxananassa

(Article begins on next page)

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iris-AperTO

Abstract

Strawberries are a very popular fruit among berries, for both their commercial and economic importance, but especially for their beneficial effects for human health. However, their bioactive compound content is strictly related to the nutritional status of the plant and might be affected if nutritional disorders (e.g. Fe or P shortage) occur. To overcome nutrient shortages, plants evolved different mechanisms, which often involve the release of root exudates. The biochemical and molecular mechanisms underlying root exudation and its regulation are as yet still poorly known, in particular in woody crop species. The aim of this work was therefore to characterize the pattern of root exudation of strawberry plants grown in either P or Fe deficiency, by investigating metabolomic changes of root tissues and the expression of genes putatively involved in exudate extrusion. Although P and Fe deficiencies differentially affected the total metabolism, some metabolites (e.g. raffinose and galactose) accumulated in roots similarly under both conditions. Moreover, P deficiency specifically affected the content of galactaric acid, malic acid, lysine, proline, and sorbitol-6-phosphate, whereas Fe deficiency specifically affected the content of sucrose, dehydroascorbic acid, galactonate, and ferulic acid. At the same time, the citrate content did not change in roots under both nutrient deficiencies with respect to the control. However, a strong release of citrate was observed, and it increased significantly with time, being +250% and +300% higher in Fe- and P-deficient plants, respectively, compared with the control. Moreover, concomitantly, a significant acidification of the growth medium was observed in both treatments. Gene expression analyses highlighted for the first time that at least two members of the multidrug and toxic compound extrusion (MATE) transporter family and one member of the plasma membrane H+-ATPase family are involved in the response to both P and Fe starvation in strawberry plants.

Acidification, citrate, *Fragaria×ananassa*, gene expression, MATE, metabolomics, PM H+ATPases.

Issue Section: Research Paper

Introduction

Strawberries are a common fruit and are important for human health (Halvorsen *et al.*, 2006) due to their high content of phytochemicals (non-nutritive compounds which include oxygen radical scavengers such as vitamin C and a wide class of phenolic compounds). Consumption of strawberries is associated with a lower incidence of several chronic pathologies (Johnsen *et al.*, 2003; Vauzour *et al.*, 2010), due to their antioxidant (Diamanti *et al.*, 2014), anticancer (Stoner and Wang, 2013), and anti-inflammatory (Joseph *et al.*, 2014) biological properties. Environmental factors such as nutritional imbalances in the growing medium are able to affect considerably the composition of strawberries, particularly essential elements and phytochemical contents (Giampieri *et al.*, 2012). The effect of mineral nutrient imbalances on the quality of strawberries is limited. However, recently an enhancement has been reported in the bioactive fraction of phytochemicals in strawberries grown either in iron (Fe) or phophorus (P) deficiency (Valentinuzzi *et al.*, 2014).

With respect to nutritional shortage, it is widely known that P and Fe, in addition to nitrogen (N), are the most critical nutrients responsible for yield limitation of crops

in the world (Schachtman *et al.*, 1998; Zhang *et al.*, 2010); this effect is ascribable to the plant-available fraction of Fe and P in soil that is very often far lower than that required for an optimal plant growth. To overcome this nutritional problem, plants adopt different strategies including the release of low (organic acids, amino acids, sugars, phenolic acids, flavonoids, phytosiderophores, etc.) and high (polysaccharides, enzymes, etc.) molecular weight organic compounds, generally termed root exudates (Cesco *et al.*, 2012; Mimmo *et al.*, 2014). In this way, plants can significantly influence the chemical, physical, and biological characteristics of the surrounding soil (rhizosphere) and, in turn, the availability of P and Fe via an exudate-dependent solubilization from their poorly available soil sources (Colombo *et al.*, 2014; Terzano *et al.*, 2015) via acidification, reduction/complexation, and/or ligand exchange reactions (Cesco *et al.*, 2010; Terzano *et al.*, 2015). Independently from their chemical properties, exudates can be either passively released by roots (diffusates) due to the concentration gradient between the rhizosphere and root cells, or actively secreted (excretions) by the root tissue (Jones *et al.*, 2004; Bais *et al.*, 2006; Tomasi *et al.*, 2009). Nonetheless, to date the biochemical aspects related to the root exudation process, as well as its regulation, are still not well known (Mathesius and Watt, 2011), in woody plants in particular. The release of low molecular weight metabolites including citrate (Magalhaes *et al.*, 2007) involves specific transporter proteins belonging to the multidrug and toxic compound extrusion (MATE) family, which are encoded by the genomes of the majority of living organisms (Omote *et al.*, 2006). Plants have evolved a very high number of MATE genes (e.g. 58 and 40 orthologues in the *Arabidopsis thaliana* and rice genome, respectively) (Yazaki, 2005; Omote *et al.*, 2006), but only some of them have been characterized functionally. An example in this regard is represented by the root exudation of citrate in response to P deficiency in *Lupinus albus* where the release of this organic acid is associated with an overexpression of genes belonging to the MATE transporter family (Keerthisinghe *et al.*, 1998; Watt and Evans, 1999; Neumann and Martinoia, 2002; Uhde-Stone *et al.*, 2005). As regards Fe nutrition, it has been shown not only that a MATE transporter (FRD3) is involved in the micronutrient mobilization from the root apoplastic pool (Roschzttardtz *et al.*, 2011) but also that both citrate and MATE transporters are involved in the micronutrient distribution within plants (Takanashi *et al.*, 2013).

The root-mediated acidification of the rhizosphere is also of paramount relevance for plant nutrition; in fact, the bioavailability of many nutrients often sparingly soluble in agricultural soils, such as Fe and P, is strongly dependent on soil pH values (Hinsinger *et al.*, 2003). This phenomenon, connected with the activity of the plasma membrane (PM) H+-ATPase (Santi *et al.*, 2005; Santi and Schmidt, 2009), has been considered as an adaptive trait of plants to cope with P and Fe shortage (Hinsinger, 2001; Kobayashi and Nishizawa, 2012; Tomasi *et al.*, 2013). It is interesting to note that in cluster roots of P-deficient white lupin plants, a close link between the burst of citrate exudation and the PM H+-ATPase-dependent proton extrusion activity has been clearly demonstrated (Tomasi *et al.*, 2009). However, the root exudate patterns strictly rely on the metabolic changes occurring in plants under nutritional deficiencies. It is well known that both Fe and P strongly impact plant metabolism. Indeed, while Fe is an essential cofactor for enzymes belonging to both photosynthesis and respiration (Vigani *et al.*, 2013), P is an essential precursor of adenylation as well as being crucial for the modulation

of enzyme activity by phosphorylation/dephosphorylation processes (Zhang *et al.*, 2014).

As mentioned above, it is evident that, from a nutritional point of view, the effectiveness of the exudate-induced processes occurring in the rhizosphere could be of extreme relevance for a balanced plant growth, particularly for fruit crops such as strawberry where the quality of the fruit is strongly dependent on the nutrient availability. On the other hand, it is also very clear that the extent of this effectiveness depends greatly on (i) the metabolic changes occurring in plants and in turn on (ii) the types and the amount of exudates released by roots under variable conditions of plant nutrient supply. In strawberry plants, the yield and particularly their quality are strongly dependent on nutrient availability in the growing medium. However, relatively little is known about both the release of exudates and the mechanisms underlying the process, and also when they are affected by nutrient deficiencies. For these reasons, the aim of this work was to characterize (i) the pattern of root exudation of strawberry plants grown in either P or Fe deficiency; and (ii) the metabolomic changes occurring under both single P and Fe deficiencies. In addition, in order to shed light on the mechanisms underlying the release of root exudates, a molecular approach was undertaken and the expression of putative genes (i.e. MATE-like genes and PM H+-ATPases) involved in the release of both organic acids and protons was evaluated in relation to the root exudation process.

Materials and methods

Plant growth conditions and plants analysis

Strawberry frigo-plants (*Fragaria×ananassa* cv. Elsanta) were grown in hydroponic conditions in an aerated nutrient solution with the following composition: KH_2PO_4 0.25mM, $Ca(NO_3)_2$ 5mM, $MgSO_4$, 1.25mM, K_2SO_4 1.75mM, KCl 0.25mM, Fe(III)NaEDTA 20 μM, H_3BO_4 25 μM, $MnSO_4$ 1.25 μM, $ZnSO_4$ 1.5 μM, $CuSO_4$ 0.5 μM, $(NH_4)_6Mo_7O_{24}$ 0.025 μM. Plants were grown in individual black pots and nutrient solutions were prepared using distilled water at 5.5 μS m–1 [all nutrients at less than the limit of quantification (LOQ)]. Strawberry plants were grown in either a full nutrient solution (control), a zero Fe nutrient solution (–Fe), or a zero P (–P) nutrient solution. Seven strawberry plants were used for each treatment. The nutrient solution in the pots was renewed once a week. Plants were grown in a growth chamber under controlled conditions (day 14h, 24 °C, 70% relative humidity, 250 μmol photons m–2 s–1; night 10h, 19 °C 70% relative humidity). During the growing period, light transmittance of fully expanded leaves was determined using a portable chlorophyll meter SPAD-502 (Minolta, Osaka, Japan) and is presented as SPAD (single-photon avalanche diode) index values. Measurements were carried out twice a week on young leaves (at least two per plant), and five SPAD measurements were taken per leaf and averaged.

Nine weeks after the transfer to nutrient solution, strawberry plants were harvested, separating roots and shoots; fresh weight (FW) and dry weight (DW) of the tissues were measured and root/shoot ratios assessed.

Oven-dried samples (60 °C) of shoots and roots were acid digested with concentrated ultrapure HNO_3 (650ml l−1; Carlo Erba, Milano, Italy) using a single reaction chamber (SRC) microwave digestion system (UltraWAVE, Milestone, Shelton, CT, USA). Fe, P, Cu, Mn, Mg, S, Zn, and Ca concentrations were then determined by ICP-OES (Spectro CirosCCD, Spectro, Germany).

Metabolomic analysis of root of strawberry plants grown under Fe and P

deficiencies

For extraction, 50mg of ground material, homogenized with liquid nitrogen, was mixed with methanol containing ribitol and C13 sorbitol as internal standards. After mixing and incubation at 70 °C, water and chloroform were added to force a phase separation by centrifugation. Only the upper polar phase was used for further analysis and dried in a vacuum. The pellet was derivatized using methoxyamine hydrochloride (20mg ml–1 in pyridine) for methoxyamination, and *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) for silylation. To perform a retention time alignment later on, a mixture of alkanes (C10, C12, C15, C19, C22, C28, and C32) was added to the derivatization mix.

Metabolites were analysed using a GC-TOF-MS system (Pegasus HT, Leco, St Joseph, USA). Baseline correction was done by ChromaTOF software (Leco). For peak alignment and peak annotation, the TagFinder software tool (MPIMP Golm; Luedemann *et al.*, 2008) was used in combination with the Golm Metabolome Database (GMD; Kopka *et al.*, 2005). The metabolites were normalized using the internal standard and the fresh weight.

Collection of root exudates and their analysis

Root exudates were collected seven times each week starting from the appearance of the first symptoms of deficiency (at day 21). Plants were removed from the nutrient solutions and roots were washed several times with distilled water in order to remove any traces of nutrient solution. Plants were then transferred into smaller pots containing 100ml of distilled water (Valentinuzzi *et al.*, 2015). Root exudates were collected for 8h continuously, aerating the solution and covering the pots with aluminium foil to maintain the roots in the dark (Zancan *et al.*, 2006). After 8h, plants were removed and transferred to pots with fresh nutrient solution. Root exudate solutions were filtered at 0.45 µm (Spartan RC, Whatman), frozen at –20 °C, and lyophilized for the following analysis.

Total organic carbon (TOC) and total nitrogen (TN) of lyophilized samples were determined using a Flash EA 1112 elemental analyser (Thermo Scientific, Germany).

After resuspension of the lyophilized samples in 1ml of ultrapure distilled water, organic acids were separated by high-performance liquid chromatography (HPLC) using a cation exchange column (Phenomenex-Rezex ROA), with an isocratic elution with 10mM H_2SO_4 as carrier solution at a flow rate of 0.6ml min−1. Organic acids were detected at 210nm using a Waters photodiode array detector (PDA 2998 Waters Spa, Italy).

Bioinformatics

The identification of *MATE-like* genes in the strawberry genome (using the *Fragaria vesca* genome v.1.1 hosted at Phytozome v.10, http://phytozome.jgi.doe.gov/pz/portal.html) was based primarily on amino acid sequence similarity between the MATE transporters of *Vitis vinifera*, *Arabidopsis thaliana*, *Glycine max*, *Hordeum vulgare*, *Lupinus albus*, *Medicago truncatula*, *Solanum lycopersicum*, *Nicotiana tabacum*, *Oryza sativa*, *Sorghum bicolor*, *Secale cereale*, *Lotus japonicas*, and *Zea mays* (Takanashi *et al.*, 2013). The same approach was used to identify PM H+-ATPase homologues on the basis of amino acid sequences of PM H+-ATPase of *Nicotiana plumbaginifolia* Viv., *O. sativa* L., *A. thaliana* (L.) Heynh. (Arango *et al.*, 2003), and *V. vinifera* (Pii *et al.*, 2014). The amino acid sequences were retrieved from public databases [http://www.ncbi.nlm.nih.gov/, http://www.uniprot.org/uniprot/, The Arabidopsis

Information Resource (TAIR), MSU, maizesequence.org, and Centro di Ricerca Interdipartimentale per le Biotecnologie Innovative (CRIBI) Grape Genome Browser http://genomes.cribi.unipd.it/grape/]. The predicted sequences for MATE and PM H+-ATPase in strawberry were identified through a BLASTP (Altschul *et al.*, 1997) search. BLASTP analysis was performed using each known protein, selecting the putative proteins encoded by the predicted coding sequences on the basis of the highest sequence homology value (the threshold value for sequence homology was set at 80%). A phylogenetic analysis was performed using the selected putative proteins for both the MATE and PM H+-ATPase gene families. The amino acid sequences of the previously mentioned dicot and monocot plants were aligned by the ClustalW ver. 2.1 algorithm (http://clustalw.ddbj.nig.ac.jp/). Phylogenetic trees were built using the Phylogenetic Interference Package program (PHYLIP; University of Washington, http://evolution.genetics.washington.edu/phylip.html), and they were visualized by the Phylodendron software (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html).

RNA extraction and real-time reverse transcription–PCR

Strawberry roots from three biological replicates were collected for each treatment (control, –P, and –Fe) at harvest (day 63). Total RNA was extracted using the InviTrap® Spin Plant RNA Mini Kit (Stratec Molecular, Germany), following the supplier's instructions.

Gene-specific primers were designed for the target genes as well as for the housekeeping genes (Supplementary Table S1 available at *JXB* online). Real-time reverse transcription–PCR (RT–PCR) experiments were carried out in biological triplicates and the reaction was performed using the SsoFast EvaGreen Supermix (Bio-Rad), a ready-to-use reaction cocktail containing all components, except the primers and template, and the Qiagen Rotor Gene Q real-time PCR system. Each reaction began with a 95 °C hold for 30 s followed by 40 cycles at 95 °C for 10 s and 55 °C for 20 s. Non-specific PCR products were identified by analysing dissociation curves. The amplification efficiency was calculated from raw data using LinRegPCR software (Ramakers *et al.*, 2003). The relative expression ratio value was calculated for treated samples relative to the corresponding untreated sample at the same time point according to the Pfaffl equation (Pfaffl, 2001). Standard error values were calculated according to Pfaffl *et al.* (2002).

Statistical analysis

The results are presented as means of at least three replicates ±standard error (SE). Statistical analysis was performed using Statgraphics (Statpoint Technologies, Inc., Warrenton, VA, USA). Data were analysed by analysis of variance (ANOVA), and means were compared using SNK's test at $P<0.01$ to determine the significance of differences found. For the metabolomic analysis, statistical analysis has been done using Excel and the Multi Experiment Viewer (MEV). Principal component analysis (PCA) was performed using the MetaGeneAlyse platform (www.metagenealyse.mpimp-golm.mpg.de; de Daub *et al.*, 2003). Data for the PCA are median centred and $\log_{10}$ transformed.

Results

Plant growth parameters and element concentrations

Table 1 shows the effect of Fe and P deficiency on strawberry growth parameters. Shoot biomass was significantly reduced by 40% in both nutrient shortages, whereas the root biomass was not affected. The root/shoot ratio was consequently

significantly increased (~50%) as a function of the nutrient deficiencies (Table 1). While P shortage did not have any effect on chlorophyll content, this was consistently affected in Fe deficiency. The chlorophyll content can be easily measured by hand-held instruments and expressed as SPAD units. ΔSPAD values (calculated as the difference between SPAD values at the 21st day of growth in hydroponics and values at harvest) showed a significant decrease (~16 SPAD index values) when comparing Fe-deficient leaves with those of control plants. This is even clearer when comparing the depth of colour of the different leaves (Fig. 1); Fe-deficient leaves in fact show the typical symptoms of intervenial chlorosis, whereas P-deficient leaves appear slightly darker than the control leaves and have purple-bluish veins. In addition, nutrient shortage also negatively affected leaf size (data not shown).

Table 1.

Fresh weight and shoot/root ratio of strawberries grown in a full nutrient (control), zero Fe (–Fe), and zero P (–P) solution

The SPAD values (ΔSPAD) calculated as the difference between the values determined at harvest and day 21 are also shown.

	n	Control	–P	–Fe
FW shoot (g per plant)	7	27.94±2.34 a	17.55±1.91 b	16.29±2.28 b
FW root (g per plant)	7	23.67±1.47 ns	24.30±1.30 ns	22.32±1.21 ns
Root/shoot ratio	7	0.85±0.04 a	1.39±0.06 b	1.37±0.05 b
ΔSPAD	7	-1.15±0.02a	2.48±5.49 a	– 15.62±0.69 b

FW, fresh weight; mean ±SE. Letters following the means indicate significant differences at $P<0.05$; ns, not significant; n is the number of samples.

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Fig. 1.
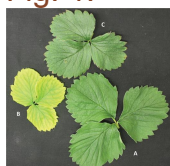
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Leaves of strawberry plants grown for 9 weeks in complete nutrient solution (A), Fe-free solution (B), and P-free solution (C). (This figure is available in colour at *JXB* online.)

At harvest, plant tissues were collected and analysed for their macro- and micronutrient composition. Both deficiencies caused imbalanced nutrient distribution in roots and shoots of strawberry plants (Table 2). For instance, P shortage, as expected, led to a decreased concentration of the element in both shoots and roots of P-deficient plants. Furthermore, it severely reduced the uptake of essential micronutrients such as Fe (–70%), copper (Cu; –40%), and manganese (Mn; –60%), and macronutrients such as calcium (Ca; –40%) and magnesium (Mg; –40%) when considering their concentration determined in the root tissues (Table 2). Iron shortage, on the other hand, enhanced the uptake of

bivalent nutrients: at least a 2-fold increase of Cu, zinc (Zn), and Mn in the shoots and an accumulation in the roots, especially of Zn and Cu, was observed. Regarding macronutrients, Ca was reduced by almost 70%, while Mg was increased by 50% when considering their concentrations detected in the roots (Table 2).

Table 2.

Macro- and micronutrients of shoots and roots of strawberries grown for 9 weeks in a full nutrient (control), zero Fe (–Fe), and zero P (–P) solution

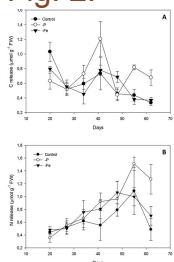|	Control		–P		–Fe	
	Roots	Shoots	Roots	Shoots	Roots	Shoots
Fe ($\mu g\ g^{-1}$ DW)	2262.22±345.15 a	79.96±7.34 a	857.67±190.71 b	73.86±6.58 a	395.99±127.49 c	38.46±2.56 b
Cu ($\mu g\ g^{-1}$ DW)	0.031±0.009 b	0.008±0.001 ns	0.018±0.004 b	0.008±0.001 ns	0.292±0.073 a	0.009±0.001 ns
Zn ($\mu g\ g^{-1}$ DW)	177.06±45.72 b	27.60±3.73 b	167.02±28.86 b	25.10±4.76 b	944.82±182.68 a	53.32±9.72 a
Mn ($\mu g\ g^{-1}$ DW)	63.30±13.12 a	79.51±10.25 b	29.75±11.02 b	68.60±12.17 b	60.35±5.16 a	143.27±19.89 a
P (mg g^{-1} DW)	4.42±0.77 a	4.87±0.83 a	1.12±0.19 c	1.94±0.50 b	3.02±0.51 b	4.65±0.13 a
Ca (mg g^{-1} DW)	9.99±1.94 a	14.58±1.33 ns	6.04±0.83 b	14.11±2.26 ns	2.22±0.14 a	15.64±1.17 ns
Mg (mg g^{-1} DW)	3.41±0.55 a	3.62±0.47 b	1.83±0.30 b	3.15±0.35 c	4.27±0.51 a	3.04±0.36 a
S (mg g^{-1} DW)	4.21±1.43 ns	1.78±0.48 ns	4.10±1.13 ns	1.57±0.42 ns	4.53±0.44 ns	1.55±0.28 ns

DW, dry weight; mean ±SE (*n*=3). Different letters within each plant tissue are significantly different at *P*<0.01 as measured by an LSD test.

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Characterization of the root exudation pattern

Figure 2A shows the total concentration of carbon (C) detected in the root exudates of strawberry plants, grown either in full nutrient, –Fe, or –P nutrient solution at different time periods. Independently of the treatment, the release of C-containing compounds decreased during the sampling period. However, on day 42, P and Fe deficiency seem to induce a transient exudation peak followed by a steep decrease. Conversely, N release increased with time (Fig. 2B), especially in P-deficient plants, reaching a concentration of 1.27 $\mu mol\ g^{-1}$ FW at harvest.

Fig. 2.

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Total organic carbon (A) and total nitrogen (B) determined in the root exudates collected from control, phosphorus-deficient (–P), and iron-deficient (–Fe) strawberry plants at 20, 27, 36, 42, 49, 56, and 63 d of growth;(mean ±SD, n=7). Two-way ANOVA results: carbon, treatment (ns), time ($P<0.05$), treatment×time (ns); nitrogen, treatment ($P<0.05$), time ($P<0.01$), treatment×time (ns).

Root exudates were further characterized by HPLC, but this revealed only the presence of citrate. Figure 3A shows the citrate released by strawberry roots of plants grown in full nutrient, Fe-free, and P-free nutrient solution, during the growing period. While control plants show an almost stable trend of exudation during the time period, the release of citrate steadily increased in P- and Fe-starved roots, with an exudative burst of citrate in both sets of nutrient-deficient plants at day 56 and 63.

Fig. 3.
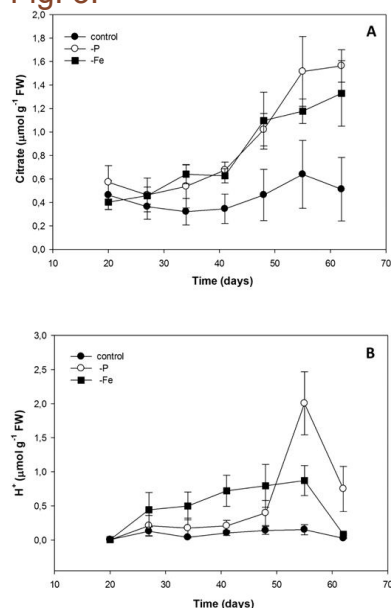
Citrate (A) and protons (B) released from control, phosphorus-deficient (–P), and iron-deficient (–Fe) strawberry plants at 20, 27, 36, 42, 49, 56, and 63 d of growth (mean ±SD, n=7). Two-way ANOVA results: citrate, treatment ($P<0.001$), time ($P<0.001$), treatment×time (ns); protons, treatment ($P<0.001$), time ($P<0.001$), treatment×time ($P<0.001$).

As shown in Fig. 3B, a strong extrusion of protons is present in both sets of nutrient-deficient plants. As for citrate, control plants have a constant extrusion of protons, but a higher release was seen in deficient plants during the whole cultivation period. In particular, the highest level of extrusion is present in P-deficient plants at day 56, showing a 20-fold higher proton release compared with the controls (Fig. 3B).

Metabolic characterization of strawberry roots

A total of 151 metabolites were detected by GC-TOF-MS analysis, and 88 of them were identified ([Supplementary Table S4](#) at *JXB* online). Among the metabolites identified, 15 (16%) significantly changed in amount in Fe-deficient roots compared with the control, while 22 (23%) significantly changed in amount in P-deficient roots compared with the control. To get an overview of the metabolic changes, a PCA was performed ([Supplementary Fig. S1](#)). The first component

(PC1) clearly separated the P-deficient samples from Fe- deficient and control roots. A few identified metabolites are responsible for this separation, namely maltose, ornithine-1,5-lactam, quinic acid, and fructose-6-phosphate (F6P), together with some unknown metabolites. The second component (PC2) separated Fe-deficient from control samples. However, the P-deficient roots are also partially segregated by the PC2; samples representing either the –Fe condition or the control showed a tighter clustering as compared with those describing the –P condition. The relevant metabolite responsible for this allocation is succinic semialdehyde, which accumulated less in the deficient roots, especially in Fe- (0.46-fold) starved roots compared with the controls.

In particular, Fe-deficient root samples differed from the controls specifically by a significantly higher content (1.5- to 2-fold) of dehydroascorbic acid [oxidized form of the reactive oxygen species (ROS) scavenger ascorbic acid], trans-ferulic acid (phenolic acid metabolism), galactonic acid (oxidized form of galactose) sucrose, and thymidine (Fig. 4). On the other hand, treatment-specific changes for the P-deficient roots compared with their controls are the significant 1.8-fold increase of malic acid, the 3.9-fold increase of lysine, the 1.7-fold increase of galactaric acid (another oxidized form of galactose), and the 2.2-fold increase of butylamine (secondary metabolism, amines). Furthermore, these roots were characterized by significant decreases (0.25- to 0.65-fold) of ribonic acid (oxidized form of ribose, oxidative pentose pathway), sorbitol-6-phosphate (sugar metabolism), proline (known stress-related metabolite), psicose [uncommon monosaccharide, non-metabolizable fructose analogue (Rabot *et al.*, 2012), related to stress (Kano *et al.*, 2011), and found in potatoes (Weckwerth *et al.*, 2004) and in tea plants (Hasehira *et al.*, 2010), but first in wheat (Miller and Swain, 1960)], 4-aminobutanoic acid (GABA) (involved in stress signalling; Fait *et al.*, 2008), erythronic acid, and galactosamine (galactose metabolism) (Fig. 4).

Fig. 4.
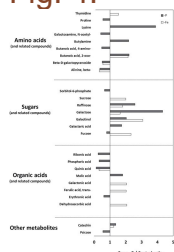
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Amino acids, sugars, organic acids, and their related compounds and other metabolites in roots of strawberry plants grown for 9 weeks in either Fe-free (–Fe) or P-free nutrient solution (–P). Metabolite changes are expressed as fold changes (treatment/control ratio). The figure shows metabolites that change significantly in –Fe or –P with respect to control samples (*$P<0.05$).

Characterization of transporter proteins and plasma membrane ATPases

Genes encoding putative MATE transporters were identified in the genome of *F. vesca* on the basis of protein sequence homology with members of the MATE family of *V. vinifera*, *A. thaliana*, *G. max*, *H. vulgare*, *L. albus*, *M. truncatula*, *S. lycopersicum*, *N. tabacum*, *O. sativa*, *S. bicolor*, *S. cereale*, and *Z. mays* ([Supplementary Table S2](#) at *JXB* online). *Fragaria vesca* MATE sequences were identified by running the BLASTP algorithm (Altschul *et al.*, 1997) in the strawberry genome database (www.phytozome.net). This approach allowed the retrieval of 48

protein sequences encoding putative MATE transporters in strawberry, and the phylogenetic analysis showed that five *F. vesca* proteins (Fv27005, Fv14671, Fv26473, Fv17086, and Fv13782) clustered in the MATE subfamily characterized for the transport of citrate in response to Fe and aluminium (Al) stresses. Fv27005 (hereafter referred to as FvMATE1) showed the closest phylogenetic relationship to GmFRD3a and GmFRD3b, both involved in the xylem loading of the Fe-citrate complex, and LaMATE, known to be expressed in the proteoid lupin roots under P, Fe, N, and Mn deficiencym and in Al stress (Fig. 5A) (Uhde-Stone *et al.*, 2005). Similarly, Fv14671, hereafter referred to as FvMATE2, displayed a high degree of similarity with AtMATE, ScFRDL2, and ZmMATE1 (Fig. 5A). Fv26473, Fv17086, and Fv13782, hereafter referred to as FvMATE3, FvMATE4, and FvMATE5, proteins still clustered with the above-mentioned MATE subfamily, albeit that they exhibited a lower degree of similarity with the other members of the cluster (Fig. 5A).

Fig. 5.
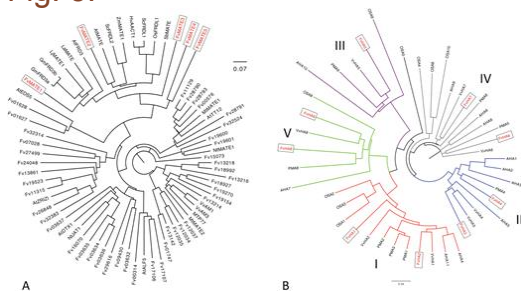
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Phylogenetic relationship of MATE-like genes (A) and PM H+-ATPases (B) in different plant species. The names of five MATE genes from strawberry (*F. vesca*) that clustered with the MATE subfamily characterized for the transport of citrate under Fe and Al stresses are boxed in (A). The names of the nine PM H+-ATPases distributed throughout the five subfamilies are boxed in (B). (This figure is available in colour at *JXB* online.)

The identification of the PM H+-ATPase gene in the *F. vesca* genome was carried out by exploiting the same approach described earlier for MATE transporters, using members of the PM H+-ATPase family of *A. thaliana*, *N. plumbaginifolia*, *O. sativa*, and *V. vinifera* as query sequences (Supplementary Table S3 at *JXB* online). This approach led to the isolation of nine putative coding sequences. The phylogenetic analyses showed that the putative strawberry PM H+-ATPases are distributed throughout the five subfamilies previously described. Specifically, Fv01281 (FvHA1) and Fv09568 (FvHA2) clustered in subfamily I, Fv08702 (FvHA3) and Fv17015 (FvHA4) clustered in subfamily II, Fv05497 (FvHA5) was in subfamily III, Fv30866 (FvHA6) and Fv15943 (VvHA7) grouped with subfamily IV, and Fv10846 (FvHA8) and Fv04924 (FvHA9) clustered within subfamily V (Fig. 5B). These results further confirm the hypothesis suggested by Arango and co-workers (2003) according to which the separation of the members of this gene family has probably occurred before the separation between monocotyledonous and dicotyledonous plants.

The gene-specific primers used for qRT–PCR analyses in the present work were designed using as template the nucleotide sequences retrieved from the *F. vesca* genome. Previous reports have already demonstrated that the diploid genome of wild strawberry *F. vesca* displays a very high sequence identity to the octaploid

genome of *F.×ananassa* (Rousseau-Gueutin *et al.*, 2009; Bombarely *et al.*, 2010; Guerrero-Molina *et al.*, 2015). The qRT–PCR analyses showed that Fe-deficient plants do not display any significant variation in the expression of *FaMATE1* and *FaMATE3* which are, on the other hand, down-regulated in P deficiency condition (Fig. 6A). Conversely, *FaMATE4* and *FaMATE5* were significantly induced by the nutrient stresses as compared with the control sample. In addition, according to the results presented in Fig. 6A, the gene *FaMATE2* specifically responded to Fe deprivation, whilst its expression was not detected in –P conditions. The analyses carried out on the members of the ATPase family of *F. vesca* showed that just four genes, namely *FaHA4*, *FaHA6*, *FaHA7*, and *FaHA8*, were expressed in the root tissue (Fig. 6B). Iron starvation increased the expression of three out of four genes, namely *FaHA6*, *FaHA7*, and *FaHA8,* whereas P deficiency up-regulated the expression only of *FaHA6*.

Fig. 6.
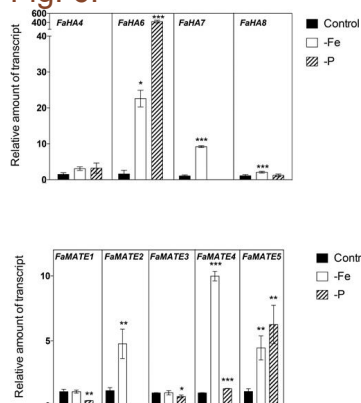
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Relative expression rate of strawberry MATE genes (A) and PM H+ATPase (B) in roots of plants grown for 9 weeks in control, Fe-free solution, and P-free solution. The expression level of each gene was normalized to the expression level of *FaUBIQ1*. Data are means ±SD of three independent replicates. The statistical significance was tested by *t*-test, comparing separately the gene expression in treated plants with the expression of the same genes in the control plants (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

Discussion

Strawberry quality, productivity, and yield are strongly dependent on the nutrient availability which in turn is closely related to rhizosphere processes such as root exudation (Schachtman *et al.*, 1998; Zhang *et al.*, 2010). Plant aboveground biomass was in fact influenced by Fe and P deficiency. Shoot biomass was significantly reduced by 40% with both nutrient shortages, whereas the root biomass was not affected. This might be explained by Thornley's model, since Fe and P deficiency affect the assimilation of N by plants, leading to a decrease in shoot growth (McDonald *et al.*, 1986). The root/shoot ratio was consequently increased by nutrient deficiencies, as already confirmed by earlier studies (Asher and Ozanne, 1967; Hunt, 1975; Fredeen *et al.*, 1989; Vance *et al.*, 2003). Regarding chlorophyll content, this was as expected significantly affected only in Fe-deficient plants. In fact, the chlorophyll content of –Fe leaves decreased by ~16 SPAD index units, showing the typical symptoms of Fe chlorosis (Fig. 1). This is consistent with what was observed in other studies (Terry, 1976; Pestana *et al.*, 2004), since Fe shortage causes a decrease of the photosynthetic pigments

(chlorophylls and some carotenoids) in the leaf (Abadía and Abadía, 1993). Furthermore, Fe and P deficiency very often cause micronutrient imbalances (Pestana *et al.*, 2012). In fact, as shown in Table 2, Fe shortage causes at least a 2-fold increase of Cu, Zn, and Mn in the shoots and an accumulation in the roots, especially of Zn and Cu. In particular, the high concentration of Zn could further interfere with chlorophyll metabolism by competing with Fe in the chlorophyll biosynthetic pathway (Singh, 2005), or influencing Fe reduction at the root level (Ambler *et al.*, 1970). The accumulation of divalent cations such as Cd, Cu, Zn, and Mn can be enhanced under Fe deficiency due to their chemical similarities (Pii *et al.*, 2015*a*), but with a differential distribution in the plant tissues (Cohen *et al.*, 1998). Even though it is not yet fully understood what governs this distribution, Fe deficiency induced the overexpression of an Fe^{2+} transporter (IRT1), which is also able to transport other divalent cations, such as, for instance, Mn, Zn, Cu, and Cd (Korshunova *et al.*, 1999).

A decrease in content of Fe was observed in P-deficient plants, a phenomenon which to our knowledge has seldom been documented. For instance, in P-deficient *Arabidopsis* plants the increase in Fe is accompanied by the induction of transcripts (mostly in leaves) of genes linked to Fe homeostasis (*NICOTIANAMINE SYNTHASE 3*; *NAS3*) or storage (*FERRITIN 1*; *AtFER1*) (Hirsch *et al.*, 2006). Other authors observed a co-ordinated suppression of the iron transporter *IRT1* in the roots and the induction of *AtFER1* in the leaves of *Arabidopsis* plants grown under P deficiency (Misson *et al.*, 2005).

Other than changes in the ionome of roots, P and Fe deficiencies strongly affect the entire metabolism. The main metabolic changes are summarized in Fig. 7. Indeed, five metabolites showed a higher concentration under both nutrient deficiencies than under control conditions: raffinose, galactinol, galactose, catechin, and 2-oxo-butanoic acid. Additionally, on the one hand, metabolites specifically up-regulated under P deficiency were galactaric acid, malic acid, lysine, proline, and butylamine; while those specifically up-regulated under Fe deficiency were sucrose, dehydroascorbic acid, galactonic acid, and trans-ferulic acid. On the other hand, metabolites specifically down-regulated under P deficiency were sorbitol-6-phosphate, erythronic acid, and GABA; while no metabolites were specifically down-regulated under Fe deficiency. Interestingly, fucose was down-regulated under P deficiency and up-regulated under Fe deficiency. Metabolites such as galactose (and derivates) and fucose are monosaccharides involved in the composition of the cell wall. A differential change in their content under P and Fe deficiencies suggests that the cell wall composition might be differentially affected under such nutrient deficiencies, in agreement with other studies (Fernandes *et al.*, 2013; Maejima *et al.*, 2014). However, galactose, along with raffinose (both metabolites were up-regulated under Fe and P deficiencies), belongs to the raffinose family of oligosaccharides (RFOs) group that plays several roles in plants. Indeed, the increase in RFOs could act as a long-distance Fe deficiency signal via phloem sap transport (Rellán-Álvarez *et al.*, 2010). Furthermore, such compounds have hydroxyl radical scavenging activities, and it has been suggested that a large increase in the relative amounts of RFOs could be required for antioxidant activities (Nishizawa *et al.*, 2008; Van Den Ende and Valluru, 2009; Rellán-Álvarez *et al.*, 2010). Under Fe deficiency, the increase in RFO concentration could help to alleviate any ROS damage produced (Rellan-Alvarez *et al.*, 2010). Other compounds with antioxidant activities such as

dehydroascorbic acid accumulate in Fe-deficient strawberry roots, whereas catechin accumulates under both nutrient deficiencies. Other than the increase in the catechin content, P and Fe deficiencies determined a decrease in quinic acid content, while trans-ferulic acid increases only in Fe-deficient roots (Fig. 7). These metabolites are related to the shikimate pathway. Such a pathway is essential for the synthesis of phenolics, which represent important molecules characterizing the root exudate pattern of several plants. Indeed, under biotic and abiotic stress, an increase in the content of phenolics in plant tissues, roots, and root exudates has been observed (Cesco *et al.*, 2010). In particular, several plant species release phenolics from roots under Fe deficiency (Mimmo *et al.*, 2014). It has been suggested that phenolics could implement plant Fe acquisition by (i) mediating the mobilization of root apoplastic Fe; (ii) improving Fe solubility in the rhizosphere mainly due to their reducing and chelating properties; and (iii) their allelopathic activity influencing the rhizosphere microbial communities to produce siderophores and auxin (Jin *et al.*, 2008).

Fig. 7.
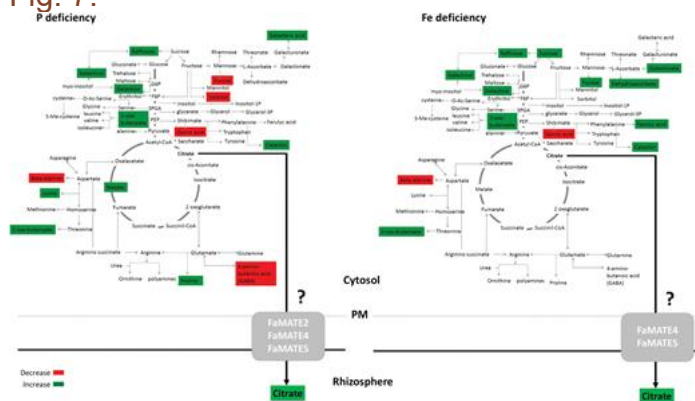
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Representation of metabolic reprogramming of strawberry roots under P (left) and Fe (right) deficiencies. Here the main metabolites that change in content under nutrient deficiencies are reported. The metabolites that changed significantly in nutrient-starved with respect to control plants are boxed. (This figure is available in colour at *JXB* online.)

However, despite P and Fe being essential elements to keep mitochondrial metabolism working, only a few changes in the content of tricarboxylic acid (TCA) cycle intermediates occurred in both Fe- and P-deficient roots. Under Fe deficiency, no significant changes in the content of organic acids of the TCA cycle occurred, while only malic acid accumulated significantly under P deficiency (1.8-fold compared with the control, $P=0.011$).

Several reports showed that P deficiency and Fe deficiency lead to an accumulation of citrate and malate in root tissues (Kania *et al.*, 2003; Mimmo *et al.*, 2014). Under P deficiency, citrate accumulation was observed in lupin cluster roots as a consequence of a down-regulation of metabolic activities related to citrate catabolism (Kania *et al.*, 2003), while under Fe deficiency citrate accumulation was observed in several plants, since it is an Fe(III) chelator and is thought to play a relevant role in xylem Fe transport in non-graminaceous plants (Rellán-Álvarez *et al.*, 2010). In Fe-deficient strawberry roots, citrate clearly tends to accumulate (4-fold, $P=0.06$), but not significantly with respect to the control conditions. As reported in the literature, nutrient shortage is very often overcome

by exudate-induced rhizosphere processes such as the release of C- and N-containing organic compounds (Mimmo *et al.*, 2014). Therefore, total C and N release might represent a rough estimation of the exudation activity (Fig. 2).
In the present experiment, a decrease in C-containing compounds was observed, even if an exudation peak was found after 42 d of cultivation in both P and Fe deficiency. In fact, it is well known that plants trigger the release of C-containing compounds as organic acids (malate and citrate) in response to P deficiency (Raghothama, 1999; Vance *et al.*, 2003) and Fe deficiency in Strategy I plants (Gerke *et al.*, 1994; Jones *et al.*, 1996). The exudation of N-containing compounds instead increased during plant growth, particularly in P-deficient plants. Less is known about the release of these types of compounds in P-deficient plants, even though recent studies observed an enhanced amino acid release in soybean plants grown under P deficiency (Tawaraya *et al.*, 2014) and in cucumber grown under Fe deficiency (Pii *et al.*, 2015*b*). Amino acids might be involved in stress signalling functions (Carvalhais *et al.*, 2011), even though their role still needs to be fully elucidated. Changes in the amino acid content have been also observed in strawberry roots under both Fe and P deficiencies.
Even though the total C release decreased with time, it is interesting to note that at day 49 there is an exudation burst of citrate in plants deficient in both types of nutrient (Fig. 3A). To the authors' knowledge, this is the first time that this carboxylate has been detected in root exudates of fruit crops such as strawberry plants. This behaviour is consistent with the typical strategy adopted by plants to overcome P deficiency (Jones, 1998). The release of carboxylates can in fact increase the solubilization and mobilization of barely available soil P by ligand exchange reactions (Jones *et al.*, 1996). Therefore, citrate did not accumulate in root tissues under P and Fe deficiencies probably because it is released as root exudate.
It is known that carboxylates are actively released by plants through secondary active transport mediated by protein carriers located at the plasma membrane, and the exudation of citrate most probably relies on the activity of MATE transporters (Magalhaes *et al.*, 2007). On the basis of sequence homology, this group of proteins can be subdivided into two separate families displaying different substrate specificity (Takanashi *et al.*, 2013). Forty-eight genes encoding putative MATE transporters were identified in the *F. vesca* genome, and the phylogenetic analysis showed that five members of this family clustered in the MATE subfamily characterized for the transport of citrate in response to Fe and Al stresses. The analyses of gene expression revealed that three (*FaMATE2*, *FaMATE4*, and *FaMATE5*) out of the five putative MATE transporters were differentially expressed in Fe-starved plants. According to the phylogenetic analysis, *FaMATE2* displayed a high degree of homology with *AtMATE*, *ScFRDL2*, and *ZmMATE1*, which have been shown to be involved in Al tolerance mechanisms, mainly relying on citrate transport (Maron *et al.*, 2010; Yokosho *et al.*, 2010). Indeed, Al tolerance mechanisms are based on the release of organic acids (oxalate, malate, and citrate) which can form high affinity complexes with Al^{3+} to protect plant roots (Ma *et al.*, 2001). However, the release of citrate has also been demonstrated to be a mechanism adopted by plants to solubilize Fe from unavailable sources (Jones, 1998). On the other hand, *FaMATE4* and *FaMATE5* that were also induced in P deficiency conditions did not display a close sequence similarity to characterized members of the MATE subfamily, but their induction in nutrient starvation condition

suggests a putative involvement of these genes in the response to these abiotic stresses.

Strawberries belong to the Strategy I plants (Marschner and Römheld, 1994) that, together with the release of carboxylates, acidify the rhizosphere by proton extrusion (Bienfait *et al.*, 1989; Alcántara *et al.*, 1991; Tomasi *et al.*, 2009), increasing the solubility of Fe-bearing soil minerals. Consistent with these assumptions, a strong acidification of the growth medium was observed in –Fe plants (Fig. 3B). However, the highest level of proton extrusion was detected in P-deficient plants after 56 d of cultivation and was maintained afterwards. This could be correlated to the simultaneous high citrate exudation burst occurring at the same time, since the release of citrate is associated with proton exudation (Tomasi *et al.*, 2009). With PM H+-ATPase being the main factor responsible for proton extrusion by plants, the aim of the present study was to identify the genes involved in this process in strawberry roots. The identification of the PM H+-ATPase gene in the *F. vesca* genome was carried out by a sequence homology approach, leading to the isolation of nine candidate genes. In order to evaluate the involvement of these molecules in the acidification of the strawberry rhizosphere coping with nutrient deficiencies, the expression of genes encoding putative PM H+-ATPases was determined. The increased H+ extrusion in Fe-deficient plants might be ascribable to the increased expression of *FaHA6*, *FaHA7*, and *FaHA8*. *FaHA6* and *FaHA7* clustered in the PM H+-ATPase subfamily IV which also encompasses *OSA4*, *OSA5*, and *OSA6* that are known to be expressed in the root tissue and are most probably involved in mineral nutrient acquisition in rice plants (Zhu *et al.*, 2009; Zeng *et al.*, 2012). On the other hand, *FvHA8* clustered within the PM H+-ATPase subfamily V together with *AHA7* that is expressed in Fe deficiency, and its role is crucial for the formation of Fe deficiency-induced root hairs (Santi and Schmidt, 2009). In addition, it is worth noting that *FaAH6* is the only member of the *F. ananassa* ATPase family induced by P starvation. Previous studies carried out in *L. albus* demonstrated that under conditions of P shortage, the exudation of citrate aiming at P mobilization was associated with an increase in expression of the *LHA1* PM H+-ATPase gene, as well as with an increased amount of H+-ATPase protein (Tomasi *et al.*, 2009).

Conclusions

In conclusion, the results presented here have provided for the first time a comparison between the effect of Fe and P deficiencies on strawberry roots from metabolism to the carboxylate exudation mechanism (Fig. 7). It was observed that both Fe and P deficiencies affect root metabolism similarly and cause the accumulation of the same metabolites (i.e. RFOs). Furthermore, citrate is released in strawberry root exudates under both nutrient deficiencies. In particular, this release is significantly higher in –P and –Fe plants compared with controls 3 weeks after the appearance of symptoms of deficiency on leaves. At the same time, a substantial acidification of the growth medium was observed in the same treatments. Moreover, phylogenetic analyses allowed the identification of five strawberry proteins which clustered in the MATE subfamily involved in citrate transport, and nine putative PM H+-ATPases. The analyses of gene expression has highlighted for the first time that at least two members of the MATE transporter family and one member of the PM H+-ATPases family are involved in the response to both P and Fe starvation in strawberry plants.

Acknowledgements

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