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Phosphate regulates malate/citrate-mediated iron uptake and transport in apple

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ABSTRACT

The accumulation of iron (Fe) in the apical meristem is considered as a critical factor involved in limiting the elongation of roots under low phosphate (Pi) conditions. Furthermore, the antagonism between Fe and Pi largely affects the effective utilization of Fe. Although the lack of Pi serves to increase the effectiveness of Fe in rice under both Fe-sufficient and Fe-deficient conditions, the underlying physiological mechanism governing this phenomenon is still unclear. In this study, we found that low Pi alleviated the Fe-deficiency phenotype in apples. Additionally, low Pi treatments increased ferric-chelated reductase (FCR) activity in the rhizosphere, promoted proton exocytosis, and enhanced the Fe concentration in both the roots and shoots. In contrast, high Pi treatments inhibited this process. Under conditions of low Pi, malate and citrate exudation from apple roots occurred under both Fe-sufficient and Fe-deficient conditions. In addition, treatment with 0.5 mM malate and citrate effectively alleviated the Fe and Pi deficiencies. Taken together, these data support the conclusion that a low Pi supply promotes organic acids exudation and enhances Fe absorption during Fe deficiency in apples.

1. Introduction

Iron (Fe) is widely involved in plant photosynthesis, respiration, and chlorophyll synthesis, and the demand for Fe is the largest among the essential micronutrients in plants [1]. Fe is easily oxidized to form poorly soluble compounds, making it difficult to be absorbed and utilized by plants [2], which severely limits crop quality and yields [3]. To cope with Fe deficiency, non-graminaceous monocot and dicot plants such as *Arabidopsis* acidify the soil environment by promoting H^+-ATPase AHA2 at the root plasma membrane to secrete protons around the rhizosphere [4]. Ferric reductase oxidase 2 (FRO2) increases the activity of ferric-chelated reductase (FCR), and the reduction products of Fe^{3+} to Fe^{2+} are absorbed by the action of high-affinity iron transporters (IRT1) [5]. Gramineous plants synthesize and secrete mugineic acid family phytosiderophores (MAs) to form an MA-Fe^{3+} complex, which is transported by YELLOW STRIPE 1 (YS1) and YELLOW STRIPE 1–like (YSL) transporters [6,7]. Traditionally, it was thought that plants have only one specific mechanism for iron absorption, but recent studies have shown that rice possesses two mechanisms [8,9]. Liu et al. (2019) showed that hydroponically grown rice mainly adopts the reduction mechanism to absorb Fe, while field-grown rice mainly achieves this via chelation [10].

The regulation of Fe homeostasis is mediated by transcription factors involved in Fe activation, absorption, and transport. The basic helix-loop-helix (bHLH) transcription factors play a major role in the response of plants to Fe deficiency [11]. In *Arabidopsis*, a small gene family encoding the N-terminal hemerythrin motif of the E3 ubiquitin ligase BRUTUS (BTS) and BRUTUS LIKE 1 and 2 (BTSL1 and 2) controls the expression of the bHLH transcription factor FE-DEFICIENCY INDUCED TRANSCRIPTION FACTOR (bHLH29/FIT) or POPEYE (bHLH47/PYE) [12–14], which in turn controls downstream Fe uptake, transport, and storage genes to help maintain Fe homeostasis [15–18]. In apples, MdbHLH104 is activated by the SUMO E3 ligase MdSIZ1 and then targets MdAHA8 to promote plasma membrane H^+ exocytosis [19,20]. The plant itself has an iron pool, and when the plants are unable to obtain Fe from the environment, the Fe-transport and reutilization mechanism forces them to obtain Fe^{3+} chelate from the iron pool and convert it to Fe^{2+}. A multi-specific vacuolar metal transporter NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 3 (NRAMP3) is involved in Fe transport in yeast and participates in Fe reutilization in *Arabidopsis* [21–23]. In apple plants, MdNRAMP3 has been proven to have similar functions to AtNRAMP3 [24,25]. During

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the transportation of Fe, a nodulin-like gene *VACUOLAR IRON TRANSPORTER 1* (*VIT1*) is down-regulated under the condition of Fe deficiency and participates in Fe distribution to regulate Fe homeostasis [26,27].

It is well known that nutrients interact with each other, and deficiency or excess of one can lead to imbalances in other nutrients [28]. For example, the accumulation of zinc (Zn) in *Arabidopsis* roots is negatively correlated with the Fe concentration, but this accumulation is not affected by the Fe concentration in shoots [29]. Zn inhibits the accumulation of Fe in apple leaves, and a deficiency of Fe causes excessive accumulation of manganese (Mn) [30]. A reduction in nitrogen (N) can restore the chlorotic phenotype of soybeans, whereas an increase in N restores the chlorosis phenotype. This phenomenon is thought to occur because NO_3^- affects the phosphorus (P) /Fe ratio of soybean plants [31]. The typical response to Pi deficiency is inhibited primary root elongation and increased density and length of lateral roots and root hairs [32–34]. Later studies indicated that these morphological changes were caused by Fe accumulation, and the co-deficiency of Fe and Pi overcame the limitation of *Arabidopsis* primary root elongation [35–37]. Recently, Zheng et al. (2009) pointed out that compared to a single Fe deficiency, Fe and Pi co-deficiency maintained the normal growth of rice at the seedling stage and caused the differential expression of Fe transport and mobilization genes [38]. From the 579 overlapping genes involved in the Fe or Pi response in *Arabidopsis* roots, Li and Lan (2015) found that Fe response genes such as *FRO2*, *IRT1*, and *CYP82C4* were significantly down-regulated in the absence of Pi but induced under conditions of co-deficiency of Fe and Pi [39]. These observations provide evidence for the interaction of Fe and Pi at the transcriptional level. In *Arabidopsis*, low Pi conditions can promote the *SENSITIVE TO PROTON TOXICITY1* (*STOP1*) transcription factor on the posttranscriptional levels, which stimulates the expression of the Al-activated malate transporter *ALUMINUM-ACTIVATED MALATE TRANSPORTER1* (*ALMT1*) and promotes malate efflux at the root apex [40]. In recent studies, it is generally accepted that malate forms a complex with Fe^{3+} after exudation. At the same time, Fe^{2+} is oxidized to Fe^{3+} with the participation of the Pi starvation response gene *LOW PHOSPHATE ROOT 1* (*LPR1*) to form more Malate-Fe^{3+} complexes [40,41]. Although there is currently no accurate evidence about the formation of malate-Fe^{3+} complexes in plants, malate-Fe^{3+} complexes among the external media are natural substrates that increase the rate of FCR activity [42,43]. However, malate promotion of Fe accumulation in the root apical meristem is achieved when Fe is sufficient, and whether this occurs under conditions of Fe deficiency is largely unknown, although a lack of Pi can increase the Fe concentration in *Arabidopsis* and significantly alleviate the Fe-deficiency phenomenon in rice leaves [38,44]. Additional important players involved in Fe uptake are the coumarins, as the biosynthesis and exudation of coumarins, especially catechol type coumarins, contribute to Fe acquisition under Fe-limiting conditions [45–48]. In *Arabidopsis*, the *PLEIOTROPIC DRUG RESISTANCE 9* (*PDR9*) transporter extrudes coumarins, but it relies on the FRO2 reductase to produce Fe^{2+} in response to Fe deficiency [46,49,50]. In the absence of Fe and Pi, the accumulation and exudation of coumarins, especially catechol type coumarins and their glucosides, are significantly reduced compared with –Fe + Pi treatment [51,52]. Malate or citrate is exuded under conditions of Fe or Pi deficiency, but whether this is similar to coumarins is currently unclear.

Organic acids are known to be involved in both aluminum (Al) toxicity and Pi deficiency tolerance. For example, under Al stress conditions, malate is exuded from *Arabidopsis* and wheat roots to chelate Al^{3+} [53,54]. STOP1 regulates the downstream genes *ALMT1* and *MULTI-DRUG AND TOXIC COMPOUND EXTRUSION* (*MATE*), which promote the secretion of malate and citrate, respectively [55]. Furthermore, the functions of ALMT1 and MATE in *Arabidopsis* are independent for each other, as knocking out one of these genes does not affect the plant's ability to secrete organic acids [56]. Under Pi stress conditions, malate and citrate is exuded from Soybean (Glycinemax) [57,58], while maize (Zeamays) and rice was mainly citrate [59,60]. Citrate is reported to play a major role in the chelating and transportation of Fe in the xylem of soybeans and tomatoes [61]. Recently, an *Arabidopsis* transporter of the MATE family, FERRIC REDUCTASE DEFECTIVE 3 (FRD3), has been shown to mediate Fe translocation by controlling the efflux of citrate from the root vasculature [62,63].

Among mature apple fruits, malic acid occupies a large proportion of the organic acids and is present at much higher concentrations than other organic acids, including citric acid [64]. However, research into the involvement of organic acids in apple plants in relation to stress resistance is far from complete. Importantly, the role of organic acids in regulating the steady-state of Fe and Pi has not been addressed. In this study, Pi restriction was applied to the study of Fe deficiency in apples, and it was observed that low Pi conditions significantly promoted Fe accumulation. At the same time, low Pi conditions promoted organic acid efflux from the roots, both in the presence and absence of Fe. Finally, transcriptome analysis was further employed to define this relationship between Fe and Pi.

2. Materials and methods

2.1. Plant materials and growth conditions

Chinese crab apple (*Malus hupenensis*) seeds were mixed with wet sand at 4℃ for 45 days, and then sown in nutrient medium for 2 months. The M9T337 dwarf rootstock was treated with rooting medium (0.0025% 1/2 MS, 0.025% sucrose, 0.001 mM IAA and 0.006% agar powder; pH = 5.8) after rooting. The apple plants were transferred to vermiculite (main ingredients: 37–43% SiO_2, 9–17% Al_2O_3, 5–24% Fe_2O_3 11–23% MgO and 0.5–9% H_2O) for 1 week before treatment. The nutrient solution processed includes: 4 mM $Ca(NO_3)_2{\cdot}4H_2O$, 5 mM KNO_3, 1 mM NH_4NO_3, 4 mM $MgSO_4{\cdot}7H_2O$, 0.1 mM H_3BO_3, 0.15 mM $MnSO_4$, 0.05 mM $ZnSO_4$, 0.001 mM $Na_2MoO_4{\cdot}2H_2O$, 0.0001 mM $CuSO_4$, 0.0002 mM $CoCl_2$, 0.05 mM $FeSO_4{\cdot}7H_2O$, 0.05 mM EDTA-2Na, with or without 0, 0.125, 0.5, 1.25, or 5 mM KH_2PO_4, pH = 5.8. Each of the –Fe-treated nutrient solutions was simultaneously added to 300 μM ferrozine (Frz). Apple seedlings were grown in a greenhouse under a 16 h/8 h light/dark cycle at 25/20 ℃ (day/night).

Arabidopsis seedlings were transferred to conditions with (50 μM, +Fe) or without (0 μM, –Fe) $FeSO_4{\cdot}7H_2O$ and supplied with 0 (–Pi), 0.125, 0.5, 1.25, or 5 mM (high) KH_2PO_4 or 0, 0.1, 0.5, or 1 mM malate or citrate on MS medium for 7 day subsequent to 4 days of germination. For *Arabidopsis* experiments in vermiculite, *Arabidopsis* was transferred to vermiculite after germination in MS medium for 10 days. *Arabidopsis* seedlings were grown in a greenhouse under a 16 h/8 h light/dark cycle at 22/18 ℃ (day/night).

2.2. Chlorophyll content measurement

New apple leaves (1–3 new leaves growing from the top) and *Arabidopsis* leaves were placed in a dark state in 95% ethanol until the leaves were completely faded. Absorbance was colorimetrically calculated at 645 and 663 nm to determine the chlorophyll concentration. Chlorophyll content = $(20.8 \times A_{645} + 8.04 \times A_{663}) \times V \div M$. (A: absorbance, V: volume of alcohol, M: weight of leaves).

2.3. Fe and P content measurement

The apple or *Arabidopsis* roots and shoots (all organs other than the roots) were separated, dried in an 80℃ blast oven for two days and ground to a powder. The material was completely carbonized by the addition of 5 ml of H_2SO_4 overnight. Subsequently, 30% H_2O_2 was added while heating in a 300 ℃ digestion furnace to clarify. After filtration, the total Fe or P concentration was measured via inductively coupled plasma atomic emission spectroscopy (ICP-AES; Fisons ARL Accuris, Ecublens, Switzerland). Fe or P content = C × V / M. (C:

concentration, V: volume of filtrate, M: dry weight of root or shoot).

2.4. Soluble Fe content measurement

Soluble Fe content was measured as described in Lei et al. (2014) [23] with some changes. The roots and shoots of the apple or *Arabidopsis* plants were isolated, ground in liquid nitrogen, and extracted with ten volumes of deionized water. The samples were then centrifuged at 5000 × g for 10 min and the supernatant was collected. The Fe concentration was measured in the same manner as described above.

2.5. Root ferric-chelated reductase (FCR) activity measurements and staining

Root FCR activity was measured following a method reported by Lin et al. (2016) [65]. Apple roots treated for 1–7 days were immersed in measuring solution consisting of 0.5 mM $CaSO_4$, 0.1 mM 4-morpholineethanesulfonic acid, 0.1 mM Frz, and 100 mM Fe-EDTA (pH = 5.5). After a 2 h reaction in the dark, absorbance of the reaction solution was determined at 562 nm in an ultraviolet spectrophotometer.

For FCR activity staining, 0.7% agar powder was added to the assay solution, and the culture plate was placed at 25 ˚C for 1–2 h in the dark and photographed.

2.6. Solution pH measurement and rhizosphere proton secretion staining

The pH of the nutrient solution was measured at 1, 3, 5, and 7 days using a pH meter (PHS-3C; INESA Scientific Instrument Co., Ltd). Rhizosphere proton secretion staining was performed according to Zhao et al. (2016) with some changes [20]. Apple plant roots were placed in a mixed culture solution containing 0.01% bromocresol purple, 0.2 mM CaSO4 and 0.7% agar (pH = 6.5), and The photographs were taken after 45 min.

2.7. Organic acid content measurement

For the determination of organic acids, the protocol presented in Soga et al. (2001) [66] was used. Briefly, apple roots were ground to a powder with liquid nitrogen, dissolved in sterile water, and then centrifuged at 1000 ×g at 4℃ for 15 min. The organic acid content was determined via high-performance capillary electrophoresis (HPCE).

2.8. Rhizosphere organic acid efflux collection and measurement

The apple plants were treated in hydroponic nutrient solution for 4 days (pH = 5.8), and the nutrient solution was then dried to a powder in a -80℃ freeze dryer. The powder was dissolved with 5 ml of deionized water, the solution was then passed through a cation exchange resin and the filtrate was collected. After lyophilizing again, the sample was dissolved in 5 ml of deionized water and stored at -20℃. The organic acid efflux was analyzed via enzyme-linked immunosorbent assay (ELISA; Hengyuan, Shanghai, China). Organic acid was made into a solid-phase antibody, and horseradish peroxidase (HRP) was then combined with the organic acid antibody to form an antibody-antigen-enzyme-labeled antibody complex. After thorough washing, the TMB substrate was added to develop the color, with the TMB converted to blue under the catalysis of HRP and finally converted to yellow under the action of acid. The shade of the color positively correlated with the organic acid concentration in the sample. Finally, the absorbance was measured using a microplate reader at a wavelength of 450 nm. The organic acid concentration was calculated from a standard curve. Effluent malate content = (337.57 × A_{450} –9.0216) ×5 ×V / (134.09 × M). Effluent citrate content = (506.82 × A_{450} –17.148) ×5 ×V / (192.13 × M). (A: absorbance, V: volume of filtrate, M: fresh weight of apple root).

2.9. Gene-expression analysis via real time-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR experiments were conducted according to An et al. (2017) [67]. The RNA was extracted using the RNA plant plus reagent (Tiangen, Beijing, China). RT-qPCR assays were performed on an ABI7500 RT-qPCR system using the UltraSYBR mixture (SYBR Green I; Takara Bio, Shiga, Japan). The cDNA concentration was diluted to 3–8 $ng{\cdot}\mu l^{-1}$, and a 1 μl aliquot of diluted cDNA was employed for RT-qPCR. The $2^{-\Delta\Delta Ct}$ method was used to calculate the results. The results were normalized to *MdACTIN*. At least three biological replicates were run per sample. The primer sequences are detailed in Table S4.

2.10. Transcriptome experimental processing and data analysis

Apple plants were treated with 0.5 and 5 mM KH_2PO_4 for 5 days in the absence of Fe. The apple roots and shoots were taken from liquid nitrogen storage and then ground to a powder. The RNA extraction and transcriptome sequencing and analysis were conducted by OE biotech Co., Ltd. (Shanghai, China). Briefly, after extracting total RNA from the sample and digesting the DNA with DNase, the eukaryotic mRNA was enriched using magnetic beads with Oligo (dT), and the interruption reagent was added to break the mRNA into short fragments. Using the interrupted mRNA as a template, a six-base random primer was used to synthesize one-stranded cDNA. Then, a two-stranded reaction system was prepared to synthesize two-stranded cDNA, and the resulting double-stranded cDNA was purified using a kit. The purified double-stranded cDNA was then end-repaired, elongated with an A tail, and connected to the sequencing adapter. Then, the fragments were size selected and subjected to PCR amplification. After the constructed library was qualified using an Agilent 2100 Bioanalyzer, it was sequenced with a sequencer such as Illumina HiSeqTM 2500 or Illumina HiSeq X Ten to generate 125bp or 150bp double-ended reads. After passing a quality inspection, sequencing was performed using an Illumina sequencer. Databases referenced for transcriptome analysis included GeneID: 79501, Genbank: NM_001005484.1, HGNC: HGNC: 14825, and HPRD: 14974. The analysis was completed with the sequencing of the four groups (each containing 3 samples and 6 plants per sample) to generate the transcriptome. The genomic alignment of each sample was obtained by comparing reads to the reference genome. Based on the alignment results, protein-coding gene expression levels were analyzed. According to the expression level of the protein-coding gene in the different samples, the difference screening was performed, and a difference group was set up and 1.5 times was considered as significant differential expression.

To verify the accuracy of transcriptome data via RT-qPCR, apple materials extracted from the transcriptome (each group containing 3 samples with 6 plants per sample) were used. The methods for RNA extraction and gene expression analysis are the same as in 2.9.

2.11. Pi content measurement

For the determination of Pi content, refer to Zheng et al. (2009) [35]. Taking the roots and shoots fresh samples and homogenize in 5 M H_2SO_4. The homogenate was then diluted 40-fold with distilled water and mixed with malachite green reagent for 30 min. It was measured with a UV spectrophotometer at a wavelength of 650 nm. The Pi content was calculated from fresh weight and standard curve. Pi content = C × V / M. (C: concentration, V: volume of filtrate, M: fresh weight of root or shoot).

2.12. Determination of anthocyanin content

For the determination of anthocyanin content, refer to An et al. (2017) [67]. Taking the top 1–3 fresh apple leaves and add 5 ml anthocyanin extract [95% ethanol contains 1.5 M HCl; 85:15 (v: v)] after

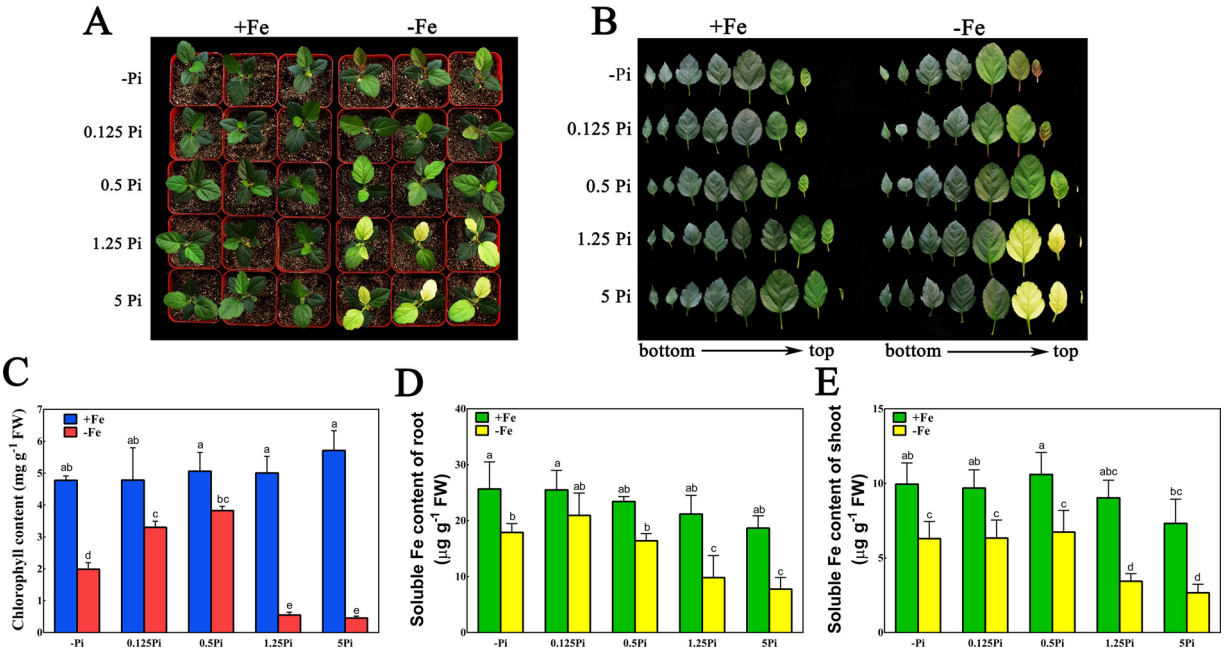

Fig. 1. Low Pi treatment alleviates Fe deficiency and increases Fe effectiveness. Apple seedlings treated with 50 μM Fe (+Fe) or 0 μM Fe (−Fe) in growth medium with 0, 0.125, 0.5, 1.25 and 5 mM KH_2PO_4 (Pi) for 14 days. (A) The phenotypes. (B) The appearance of the leaves from the bottom to the top. (C) Chlorophyll content of new leaves. (D) Soluble Fe content of the roots and (E) shoots. Changes in chlorophyll and Fe content are expressed as the mean and standard deviation (SD). Error bars represent standard deviations (n = 3 seedlings). Different letters represent significantly different values at $P < 0.05$.

grinding. The absorbance of each sample was measured at 530, 620 and 650 nm using a spectrophotometer (Shimadzu UV-2450, Kyoto, Japan). The anthocyanin contents were normalized according to the following formula: optical density (OD) = (A530 − A620) − [0.1 × (A650 − A620)]. One unit of anthocyanin content was expressed as a change of 0.1 × OD (units × 10^3 g^{-1} of fresh weight). Measurements were performed in triplicate.

3. Results

3.1. Low Pi conditions alleviate Fe deficiency

To explore the physiological response underlying Fe and Pi interactions, apple plants were cultured in vermiculite with (50 μM, +Fe) or without (0 μM, –Fe) $FeSO_4{\cdot}7H_2O$ and supplied with 0 (–Pi), 0.125, 0.5, 1.25 (normal), or 5 mM (high) KH_2PO_4. After 14 days under –Fe conditions, the 1.25 and 5 mM Pi-treated apple plants showed significant leaf chlorotic phenotypes (Fig. 1A, B) and the chlorophyll content of new leaves had decreased dramatically (Fig. 1C). However, the 0, 0.125, and 0.5 mM Pi-treated apple plants still maintained high levels of chlorophyll content in their leaves. In contrast, there was no significant difference between the different Pi treatments under the + Fe conditions (Fig. 1A-C). Interestingly, compared to the + Fe–Pi treatment, the petiole and young leaves of the –Fe –Pi-treated apple plants showed symptoms of P deficiency (Fig. 1A, B). At the same time, compared with –Fe +0.125Pi and –Fe +0.5Pi treatment, –Fe –Pi-treated apple plants showed lower chlorophyll content in new leaves (Fig. 1C). This indicated that the co-deficiency of Fe and Pi might accelerate the symptoms of P deficiency in apple plants. Additionally, *Arabidopsis* seedlings presented the same results as the apple plants using the same Pi treatment conditions, except for the accumulation of chlorophyll in the –Pi treatment group (Supplemental Fig. S1). Improving the efficiency of Fe utilization is an effective way to alleviate Fe deficiency. To investigate whether Pi affects the effectiveness of Fe, the soluble Fe content in the roots and shoots of the differently treated apple seedlings was determined, and the results showed that under Fe deficient conditions, the soluble Fe content of roots (Fig. 1D) and shoots (Fig. 1E) treated with 0, 0.125 and 0.5 mM Pi were higher than those treated with 1.25 and 5 mM Pi. However, no significant change was observed under Fe-sufficient conditions (Fig. 1D, E), indicating that low Pi concentrations might affect soluble Fe in roots and shoots to alleviate Fe deficiency in apples.

3.2. Low Pi conditions acidify the rhizosphere and promote Fe activation

FCR activation and proton extrusion are the typical means by which non-graminaceous monocot and dicot plants cope with Fe deficiency [4,5]. To further explore the impact of Pi on Fe deficiency, the FCR activity in the roots and the pH in the rhizosphere of apple plants treated hydroponically for 1–7 days were examined. The results showed that under conditions of Fe deficiency, treatments with 0, 0.125, or 0.5 mM Pi significantly enhanced FCR staining (Fig. 2A) and increased FCR activity (Fig. 2C) within 7 days when compared to the 1.25 and 5 mM Pi treatments. In addition, the 0, 0.125, and 0.5 mM Pi treatments also significantly enhanced the rhizosphere pH staining (Fig. 2B) and reduced the pH (Fig. 2D) under Fe deficient conditions. To investigate whether Pi affects the overall Fe content of apple plants, the total Fe contents were assessed, and the results indicated that treatment with 0.125 or 0.5 mM Pi significantly increased total Fe accumulation in apple roots and shoots in the absence of Fe when compared to the 1.25 and 5 mM Pi treatments. However, under Fe sufficient conditions, the treatment with different concentrations of Pi did not cause any significant changes in the total Fe content of the apple plants in either the roots (Fig. 3A) or shoots (Fig. 3B). Notably, this was in contrast to previous reports indicating that Pi deficiency can increase total Fe accumulation in the roots and shoots of *Arabidopsis* and rice under Fe-sufficient conditions [38,44,68,69].

3.3. Low Pi conditions promote the efflux of organic acids

In order to investigate whether a deficiency of Fe or Pi affects the accumulation of organic acids in apple roots, the organic acid contents of apple roots treated with –Pi were assessed. The results showed that malate and citrate were significantly induced by Pi deficiency within 7

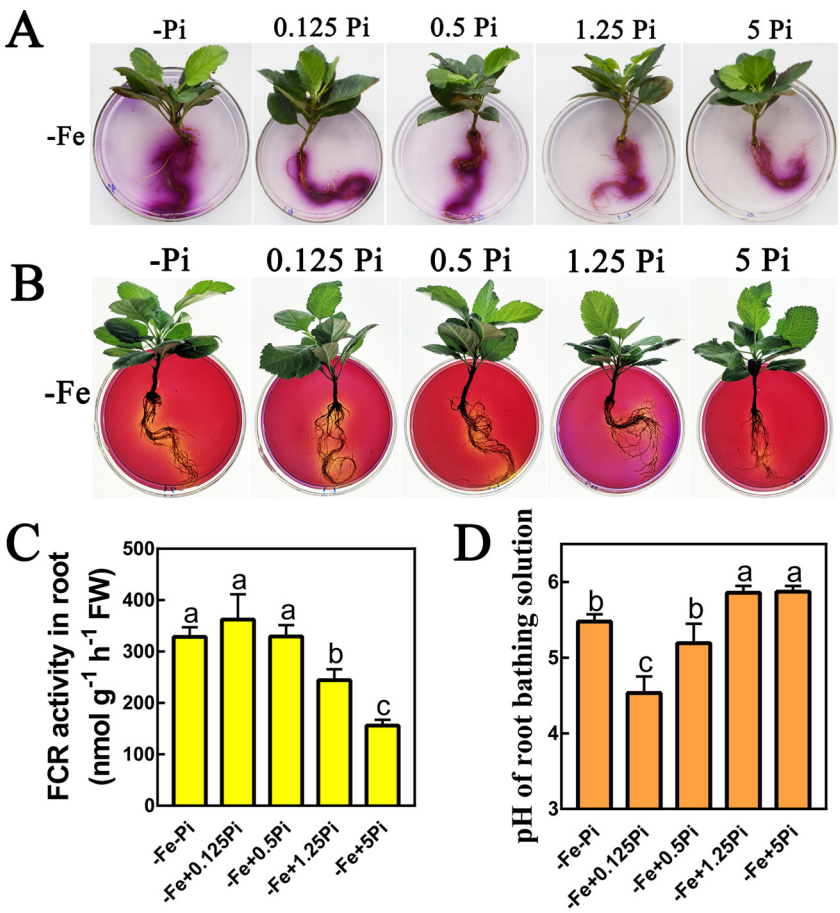

Fig. 2. Effects of different Pi treatments on root FCR activity and pH values. Apple seedlings treated with –Fe in growth medium with 0, 0.125, 0.5, 1.25, and 5 mM Pi for 5 days. (A) FCR activity staining, deep purple and light purple represent the high and low FCR activity, respectively. (B) Proton efflux staining, deep yellow and shallow yellow represent the high and low proton efflux, respectively. (C) FCR activity in roots treated with –Fe in the growth medium. (D) Rhizosphere pH under –Fe growth medium. Changes in FCR activity and pH values are expressed as the mean and SD. Error bars represent standard deviations (n = 3 seedlings). Different letters represent significantly different values at $P < 0.05$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

days (Fig. 4A), indicating that both malate and citrate could participate in the response to a lack of Pi in apples. Alternatively, under Fe deficient conditions, malate and citrate were synthesized in apple roots (Supplemental Fig. S2). Because the lack of Pi reduced the pH of the rhizosphere under Fe deficiency (Fig. 2B, D), we examined the exudation of organic acids in the nutrient solutions of apple plants treated under hydroponic conditions for 4 days. The results showed that under conditions of Fe deficiency, 0, 0.125 and 0.5 mM Pi treatments significantly increased malate efflux compared with 1.25 and 5 mM Pi treatments (Fig. 4B). The citrate efflux was only significantly different between the 0.5 mM Pi treatment and the 1.25 mM Pi treatment (Fig. 4C), while the citrate efflux treated with 0, 0.125, and 0.5 mM Pi still higher than that of 5 mM Pi treatment (Fig. 4C). Under Fe sufficient conditions, treatment with 0.125 and 0.5 mM Pi was increased the exudation of malate and citrate compared with the 1.25 mM Pi treatment (Fig. 4B, C). Notably, the amount of exudation of malate was increased under the condition of + Fe +5 mM Pi treatment compared with + Fe +1.25 mM Pi treatment (Fig. 4B, C), suggesting that a high Pi might inhibit Fe utilization and cause efflux of organic acids [30,31]. Taken together, these observations indicated that a reduction of Pi in the apple cultivation environment promoted the efflux of organic acids and contributed to the absorption and efficient use of Fe. Additionally, there was no significant difference between the extent and pattern of malate and citrate exudation in the presence of Fe or Pi deficiency in apples.

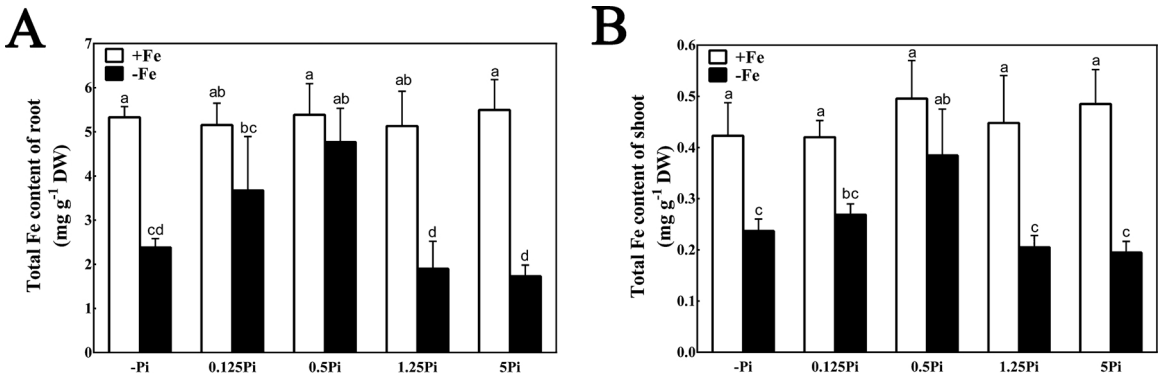

Fig. 3. Fe content in the roots and shoots of differently treated apple plants. Apple seedlings treated with + Fe or –Fe in growth medium with 0, 0.125, 0.5, 1.25 and 5 mM Pi for 14 days. (A) Total Fe content of the roots. (B) Total Fe content of the shoots. Changes in Fe content are expressed as the mean and SD. Error bars represent standard deviations (n = 3 seedlings). Different letters represent significantly different values at $P < 0.05$.

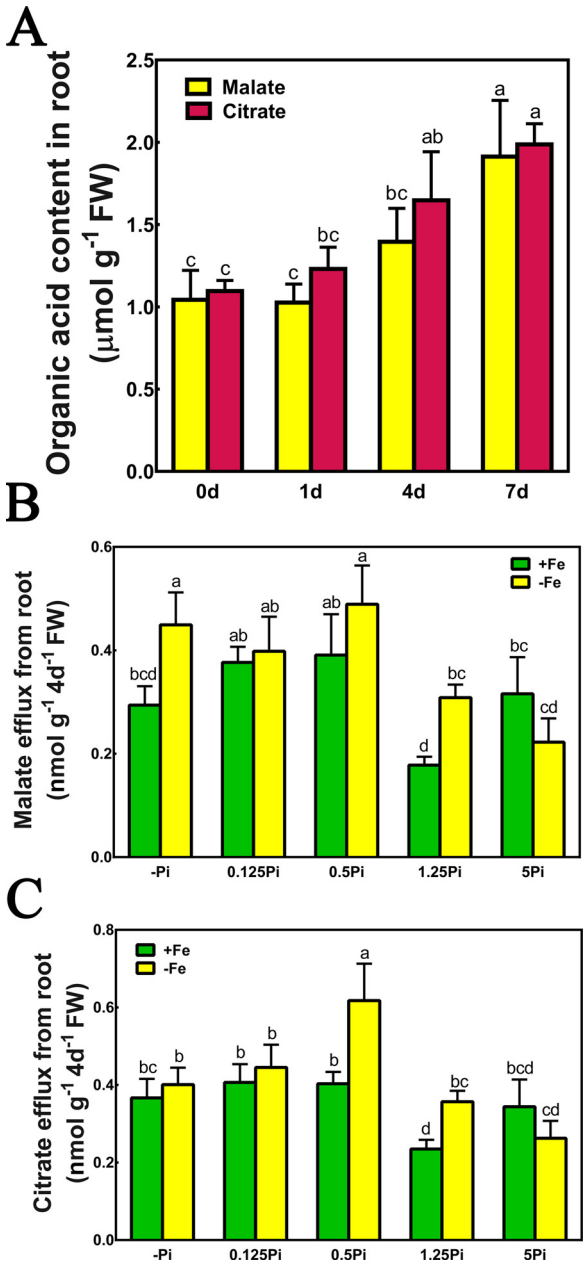

Fig. 4. Pi deficiency promotes malate and citrate accumulation and efflux in the roots. Apple seedlings treated with 0 mM Pi (−Pi) over 1–7 days. (A) Malate and citrate content in the roots under –Pi conditions. (B)Malate efflux from roots treated with + Fe or –Fe in the growth medium with 0, 0.125, 0.5, 1.25 and 5 mM Pi for 4 days. (C) Citrate efflux from the roots treated with + Fe or –Fe in the growth medium with 0, 0.125, 0.5, 1.25, and 5 mM Pi for 4 days. Changes in malate and citrate content are expressed as the mean and SD. Error bars represent standard deviations (n = 3 seedlings). Different letters represent significantly different values at $P < 0.05$.

3.4. Exogenous organic acid alleviates Fe deficiency caused by high Pi inhibition

Since malate and citrate efflux from apple roots in the absence of Fe (Fig. 4B, C), we tested their ability to alleviate Fe deficiency in *Arabidopsis*. *Arabidopsis* seedlings at 5 days after germination were separately transferred to + Fe or –Fe medium containing 1 mM Pi and 0, 0.1, 0.5, or 1 mM malate or citrate for 7 days. Under the condition of Fe deficiency, malate and citrate effectively alleviated the symptoms of Fe deficiency in *Arabidopsis* (Supplemental Fig. S3A, B) and increased the chlorophyll content of the leaves (Supplemental Fig. S3C), especially at 0.5 mM of malate and citrate, but there was no significant change under Fe-sufficient conditions (Supplemental Fig. S3). Subsequently, 0.5 mM of malate or citrate was applied to apple roots treated with –Fe +5 mM Pi for 14 days. The results showed that both malate and citrate could effectively alleviate the chlorosis (Fig. 5A, B) and increase the chlorophyll content of the leaves (Fig. 5C). In addition, determination of the Fe content indicated that malate or citrate increased the total Fe content and soluble Fe content in the roots (Fig. 5D, F) and shoots (Fig. 5E, G) of these plants, indicating that the application of malate or citrate could contribute to the accumulation and effectiveness of Fe in apple plants. To further investigate whether the effects of malate or citrate could play the same role in *Arabidopsis*, we cultivated *Arabidopsis* in –Fe +5 mM Pi-treated vermiculite, and then treated them with 0.5 mM malate or citrate for 14 days, respectively. The results show that 0.5 mM malate or citrate could effectively alleviate the Fe deficiency phenotype of *Arabidopsis* (Supplemental Fig. S4A), increase the chlorophyll content (Supplemental Fig. S4B), and the soluble Fe content in roots (Supplemental Fig. S4E) and shoots (Supplemental Fig. S4F), which was consistent with results in apples. Interestingly, although malate and citrate could increase the Fe content in the shoots of *Arabidopsis* for 14 days (Supplemental Fig. S4D), no significant increase in total Fe content in the roots was detected (Supplemental Fig. S4C). This indicated that malate and citrate can regulate the distribution of Fe in *Arabidopsis*, but the transfer rate of Fe may be higher than that of apple plants.

3.5. Exogenous organic acid alleviates Fe and Pi deficiency

The lack of P causes the plant's energy metabolism and transport of photosynthetic products to be blocked, which triggers large amounts of carbohydrates and anthocyanins to accumulate in the leaves, making the leaves darker [70,71]. In this study, the –Fe –Pi treated apple plants exhibited a strong P-deficient phenotype within 14 days when compared to the + Fe –Pi treated plants (Fig. 1A, B). To investigate whether exogenous organic acids could alleviate the effects of P deficiency in the absence of Fe, 0.5 mM of malate or citrate was used to treat –Fe –Pi apple roots for 14 days. The results showed that this treatment effectively alleviated the phenotype of both Fe and P deficiency in apple plants (Fig. 6A, B) and significantly increased the chlorophyll content (Fig. 6C), soluble Fe content, and total Fe content in the roots (Fig. 6D, F) and shoots (Fig. 6E, G). In order to investigate the effect of organic acids on Pi deficiency, we examined the accumulation of anthocyanins in apple leaves. The results showed that the amount of anthocyanins accumulated in apple leaves treated with –Fe –Pi, but significantly decreased in malate or citrate treatment (Fig. 6D). To further explore the effect of organic acids on the overall P content and inorganic phosphorus (Pi) content of apple plants, the Pi, and total P content was assessed, and the results showed that the 0.5 mM malate or citrate treatment significantly increased the Pi, and P content in the roots (Fig. 6I, K) and shoots (Fig. 6J, L). Taken together, these results indicated that a lack of Fe accelerated the symptoms of P deficiency, and the application of malate or citrate could alleviate the deficiency of Fe and also effectively alleviate the P deficiency and increases the accumulation of Fe and P in both the roots and shoots of the apple plants.

3.6. Differential expression analysis revealed changes in the transcriptional response of Pi treatment to Fe starvation

To explore the effect of the Pi supply on the expression of Fe-related genes under Fe-deficient conditions, whole transcriptome sequencing (RNA-seq) analysis was used to analyze the expression of Fe-related genes treated for 5 days under –Fe +0.5 mM Pi and –Fe +5 mM Pi conditions. We compared of transcript abundances between the –Fe +0.5 mM Pi and –Fe +5 mM Pi conditions to identify the differentially expressed genes [$-1.5 < \log FC > 1.5$; p-value < 0.05]. Principal

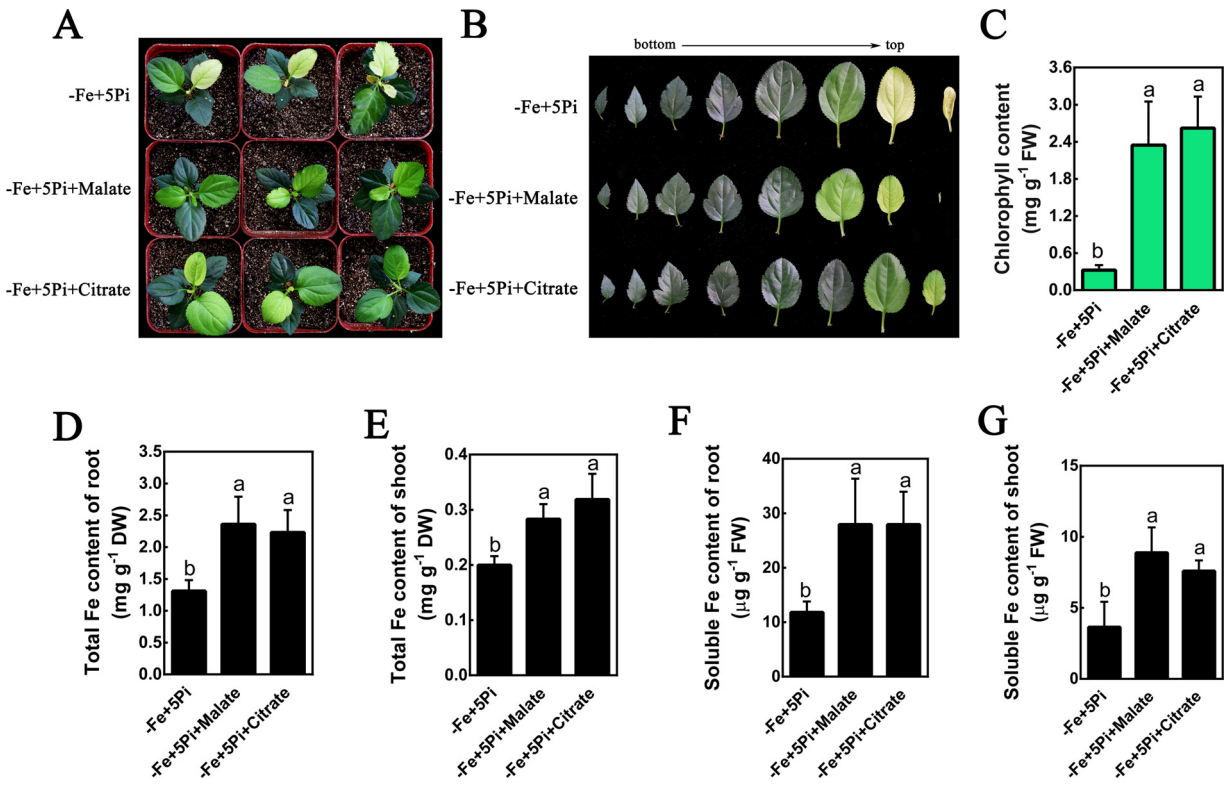

Fig. 5. Exogenous organic acid alleviates Fe deficiency caused by high Pi inhibition. Apple seedlings treated with –Fe in the growth medium with 5 mM Pi for 14 days. (A) The phenotypes. (B) The appearance of the leaves from the bottom to the top. (C) Chlorophyll content of new leaves. (D) Total Fe content of the roots and (E) shoots. (F) Soluble Fe content of the roots and (G) shoots. Changes in chlorophyll and Fe content are expressed as mean and SD. Error bars represent standard deviations (n = 3 seedlings). Different letters represent significantly different values at $P < 0.05$.

component analysis (PCA) showed that the difference in principal component 1 (PC1) reached 61.43% and 64.86% in the root (Fig. 7A) and shoot (Fig. 7B), respectively, indicating that the difference between the two groups was caused by the treatment. A total of 732 genes demonstrated altered expression between 5 mM and 0.5 mM Pi under conditions of Fe deficiency in the roots (385 up, 347 down; Fig. 7C; Supplemental Data S1 and S2) and 1253 in the shoots (591 up, 662 down; Fig. 7D; Supplemental Data S3 and S4). A large number of Pi response genes have been differentially expressed, including Pi starvation response genes: *MdLPR1* [A homologous gene of *AtLPR1*; Supplemental Data S1 and S2; for detailed gene naming, refer to "The Apple Genome" (https://iris.angers.inra.fr/gddh13/), the same as below], *MdSPX1*, and *MdSPX3*; Pi transporters: *MdPS2*, *MdPS3*, *MdPHT1;5*, *MdPHT1;7*, *MdPHT1;9*, *MdPHO1; H3*, *MdPHO2*; Pi homeostasis: *MdPAP17*, *MdCAX1* and *MdCAX3*; Pi starvation transcription factors: *MdWRKY6* and *MdWRKY42* (Table 1; Supplemental Data S1 and S3). It was further proved that differences in Pi treatment had an effect on gene expression levels.

To determine the biological processes, cellular components, and molecular functions associated with these genes with altered expression in the roots and shoots of apple plants in response to Pi treatments, we performed gene ontology (GO) enrichment analysis. Based on the top 30 GO terms, the differentially expressed genes from the roots were mainly concentrated to extracellular region, apoplast, cell wall, and organelle membrane. Iron ion homeostasis and hormone synthesis or metabolism among the biological processes as well as some heme binding, iron ion binding, oxidoreductase, and malate transmembrane transport activity among the molecular functions exhibited a higher degree of enrichment (Fig. 8). In the shoots, heme binding and iron ion binding among the molecular functions were differentially expressed in the extracellular region, cell wall, and intracellular membrane-bounded organelles (Fig. 9). To further explore the role of "classical" Fe deficiency response genes and malate or citrate that have been differentially expressed in transcripts, as based on GO enrichment analysis (Supplemental Data S5 and S6), we classified the transcriptionally dysregulated these genes according to their biological processes and molecular functions. The differentially expressed genes were mainly involved in iron ion homeostasis, iron ion starvation, iron transport, and transmembrane transport of malate or citrate in the roots (Fig. 10A, B) and shoots (Fig. 10C, D). This observation indicated that the Pi treatment affected multiple aspects of Fe absorption and utilization. Specifically, the Fe-activation gene *MdFRO2* [A homologous gene of *AtFRO2*; Supplemental Data S1 and S2; for detailed gene naming, refer to "The Apple Genome" (https://iris.angers.inra.fr/gddh13/), the same as below], Fe-transporter gene *MdOPT3*, Fe-homeostasis genes *MdCYP82C4* and *MdMTP10,* and bHLH transcription factor *MdORG2* were up-regulated in the roots, and some Fe-transporter genes, including *MdVIT1*, *MdFER4*, *MdMATE43*, and *MdZIP10*, and Fe-homeostasis genes, including *MdNEET* and *MdACO1*, were down-regulated (Table 2). In the shoots, most of the altered genes were up-regulated, including the Fe-transporter genes *MdNRAMP3*, *MdOPT3*, *MdZIP1/4/8*, and *DETOXIFICATION51* (*MdDTX51*), which belong to the MATE efflux family of proteins. In addition, the Fe-homeostasis core regulatory MdBTS, transcription factor *MdORG2*, and malate transporter *MdALMT2* genes were up-regulated. Similar to the roots, the Fe-transport negative regulators *MdVIT1*, *MdVIT4*, and *MdFER* were also down-regulated (Table 2). Additionally, RT-qPCR was used to verify the accuracy of the RNA-seq results (Table 2). This analysis confirmed the critical role of the low Pi treatment in regulating gene expression in relation to the Fe-deficiency response.

3.7. Fe is a key factor affecting Pi response gene expression

Compared with –Pi + Fe treatment, –Pi –Fe treated apple plants

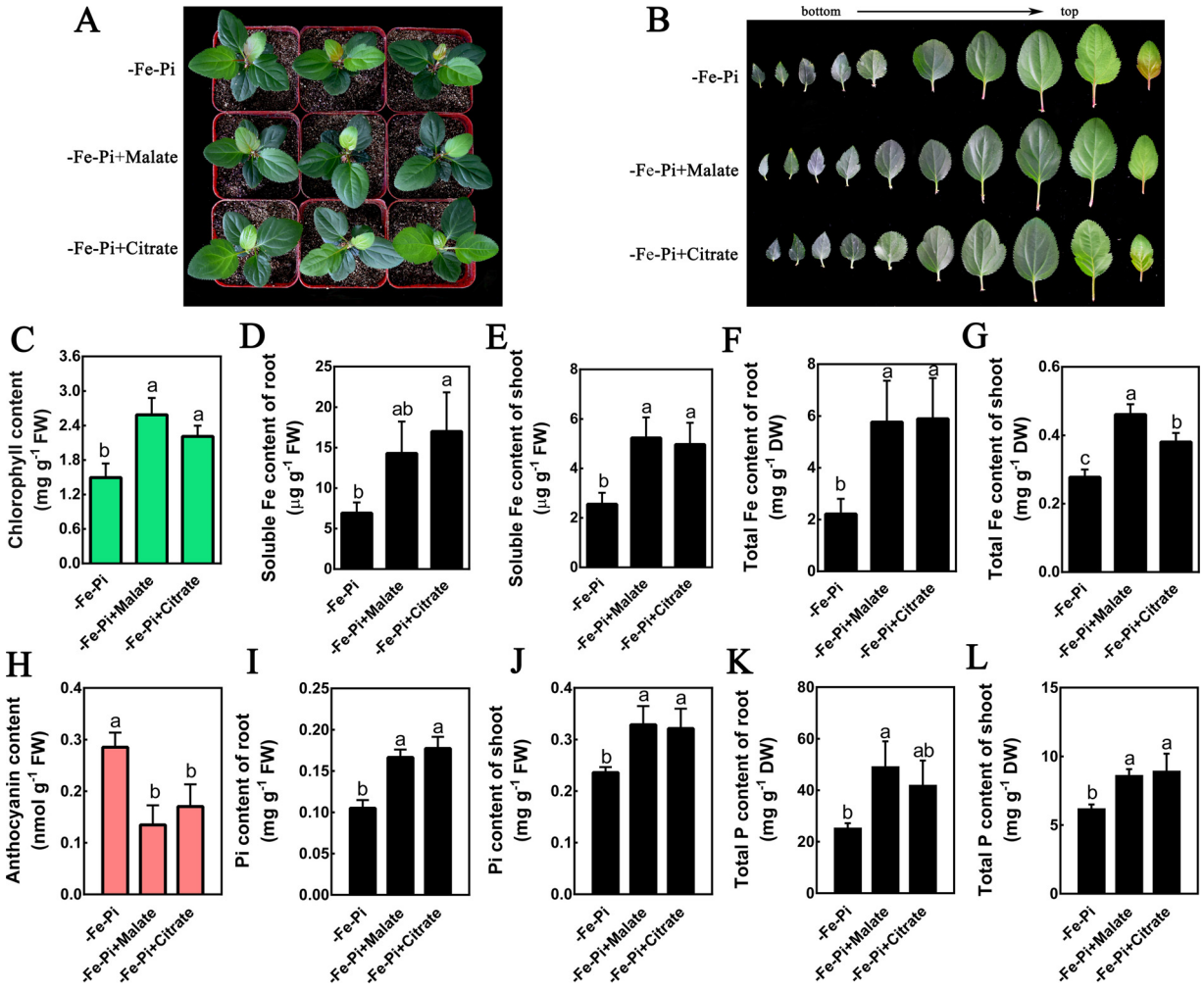

Fig. 6. Exogenous organic acid alleviates Fe and Pi deficiency. Apple seedlings treated with –Fe–Pi in the growth medium for 14 days. (A) The phenotypes. (B) The appearance of the leaves from the bottom to the top. (C) Chlorophyll content of new leaves. (D) Soluble Fe content of roots and (E) shoots. (F) Total Fe content of roots and (G) shoots. (H) Anthocyanin content of the leaves. (I) Pi content of roots and (J) shoots. (K) Total P content of roots and (L) shoots. Changes in chlorophyll and Fe, P, and Pi content are expressed as the mean and SD. Error bars represent standard deviations (n = 3 seedlings). Different letters represent significantly different values at $P < 0.05$.

were more likely to show symptoms of Pi deficiency (Fig. 1). To further investigate the effect of Fe on Pi deficiency, we tested the expression of Pi response-related genes in the roots of Table 1. The results showed that the expression of many Pi-responsive genes (including Pi starvation genes: *MdSPX1* and *MdSPX3*; Pi transporters: *MdPHO2*, *MdPHT1;5*, *MdPHT1;9*, *MdPS2* and *MdPS3*) was up-regulated within 6 h after –Pi + Fe treatment, but the expression was significantly decreased under the conditions of –Pi –Fe treatment for the corresponding period (Fig. 11). The homologous genes of *MdSPX1* and *MdPHO2* are basically consistent with their expression (Supplemental Fig. S5). *AtLPR1* is an important gene for the participation of Pi starvation in *Arabidopsis* [32], The expression of *MdLPR1* was up-regulated at 3 h after –Pi + Fe treatment, but obviously down-regulated after –Pi –Fe treatment (Fig. 11C). This indicates that *MdLPR1* may also participate in Pi deficiency response at the transcription level.In summary, our results indicated that Fe deficiency results in the down-regulation of Pi-related genes, proving that Fe plays an important role in Pi starvation response.

4. Discussion

4.1. Reducing the interaction between Fe and Pi is an effective means to alleviate Fe deficiency

It is well known that the interaction between Fe and Pi is important for the absorption and utilization of plant nutrients. Although the mechanism by which Fe accumulates in the apical meristem to counter P deficiency has been reported in detail [35,41,44,69,72,73], the mechanism of how Pi functions in response to Fe deficiency is still unclear.

Pi inhibits Fe uptake mainly due to the formation of Fe-Pi complexes. When the Pi content is increased to a certain level, a large amount of Fe-Pi complexes will be formed, which causes the symptoms of Fe deficiency even in the case of normal Fe concentrations [74]. Fe and Pi also have antagonistic effects in plants, and high levels of Pi affect Fe transport [75]. In order to reduce the inhibitory effect of Fe-Pi complexes on Fe absorption, we reduced the Pi concentration in the culture environment, which effectively alleviated Fe deficiency in apples (Fig. 1). It has been shown that reducing the supply of Pi under Fe deficiency is indeed an effective means of overcoming the formation of Fe-Pi complexes. However, although –Fe –Pi-treated apple plants demonstrated alleviated symptoms of Fe deficiency to some extent, they faced the problem of Pi deficiency. This shows that, under Fe-deficient

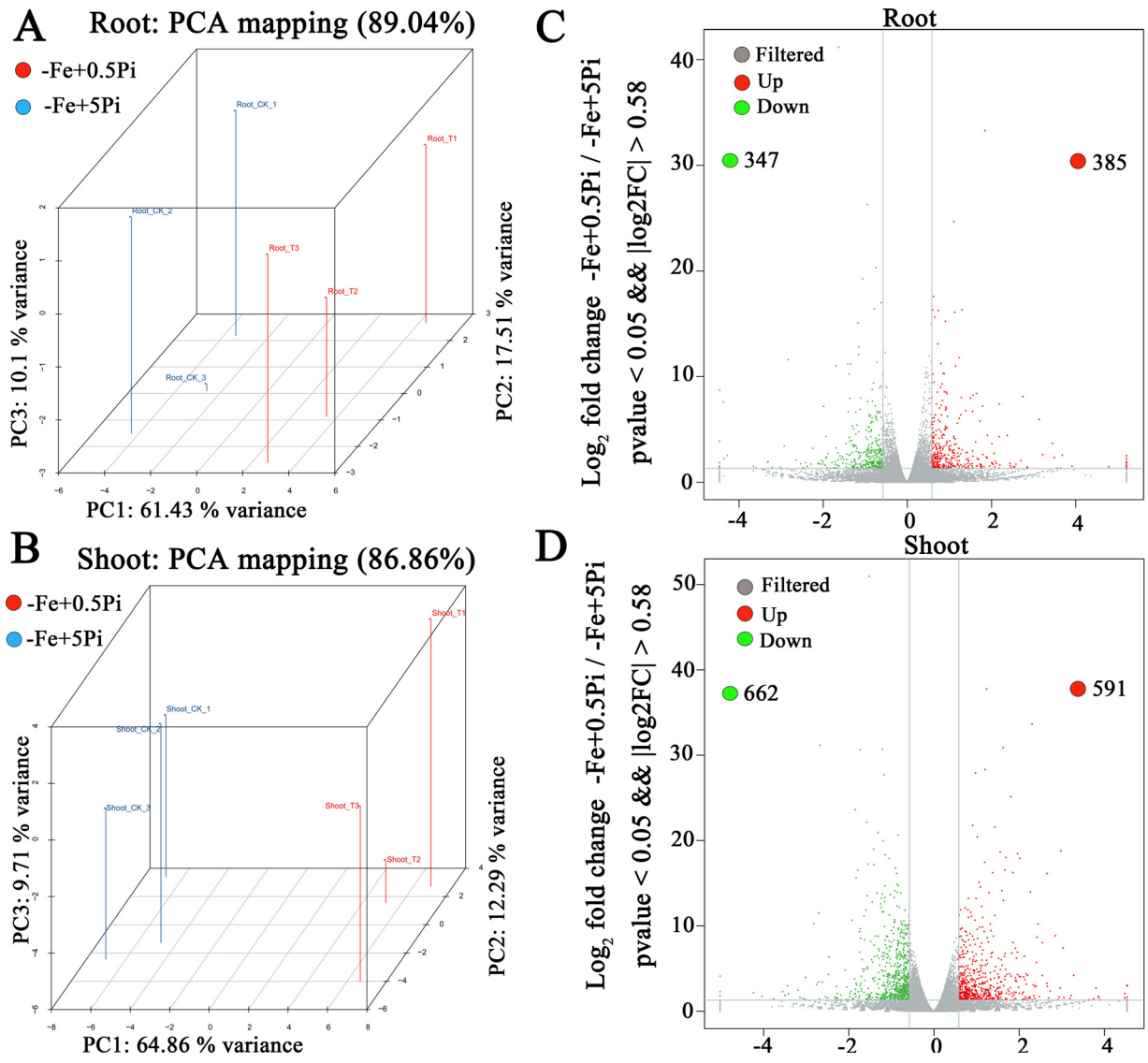

Fig. 7. Overview of transcript changes occurring under different treatments. Apple seedlings treated with –Fe +0.5 mM Pi and –Fe +5 mM Pi in the growth medium for 5 days. Principal component analysis (PCA) of the changes in transcript abundance in the roots (A) and shoots (B) under different treatments. Volcano map indicates up- and down-regulation of differentially expressed genes [−1.5 > logFC (–Fe +0.5Pi/ –Fe +5Pi) > 1.5; P < 0.05] in the roots (C) and shoots (D) in response to –Fe conditions. The histogram shows the number of up- and down-regulated transcripts in the roots and shoots. The red color in the volcano map indicates that the gene is up-regulated, and the green color represents down-regulated genes (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 1
Transcriptomic analysis of Pi biological process and molecular function regulated gens in the root and shoot in the Pi treated apple plants under Fe deficiency. [L (Like); -1.5 > logFC (-Fe+0.5Pi/-Fe+5Pi) > 1.5; n = 3 samples, 6 plants per sample; P < 0.05].

Root			Shoot		
Gene ID	Gene name	Fold	Gene ID	Gene name	Fold
MD06G1233500	*MdPAP17*	11.03	MD04G1151600	*MdCAX1*	5.49
MD17G1175900	*MdPS2*	4.06	MD12G1165800	*MdCAX1 L1*	3.12
MD07G1115200	*MdSPX3*	3.20	MD16G1222100	*MdPHO1*	2.66
MD02G1031100	*MdSPX1 L1*	2.98	MD09G1006500	*MdGDPD1*	2.52
MD09G1006500	*MdGDPD1*	2.86	MD02G1031100	*MdSPX1 L1*	2.28
MD07G1046300	*MdPHT1;5*	2.43	MD11G1074900	*MdCAX3*	2.22
MD15G1172700	*MdSPX1*	2.20	MD09G1111200	*MdWRKY6*	1.91
MD07G1253900	*MdG3PP1(MdPS3)*	1.68	MD03G1070900	*MdCAX3 L1*	1.90
MD13G1026600	*MdLPR1*	1.57	MD10G1184700	*MdPHT1;7*	1.58
MD06G1004700	*MdPHT1;9*	1.56	MD05G1312700	*MdPHO2 L1*	0.65
MD10G1294500	*MdPHO2*	0.66	MD16G1029700	*MdLPR1 L1*	0.58
MD11G1213500	*MdWRKY42*	0.55			
MD05G1312700	*MdPHO2 L1*	0.52			
MD00G1200800	*MdPHO1;H3*	0.47			

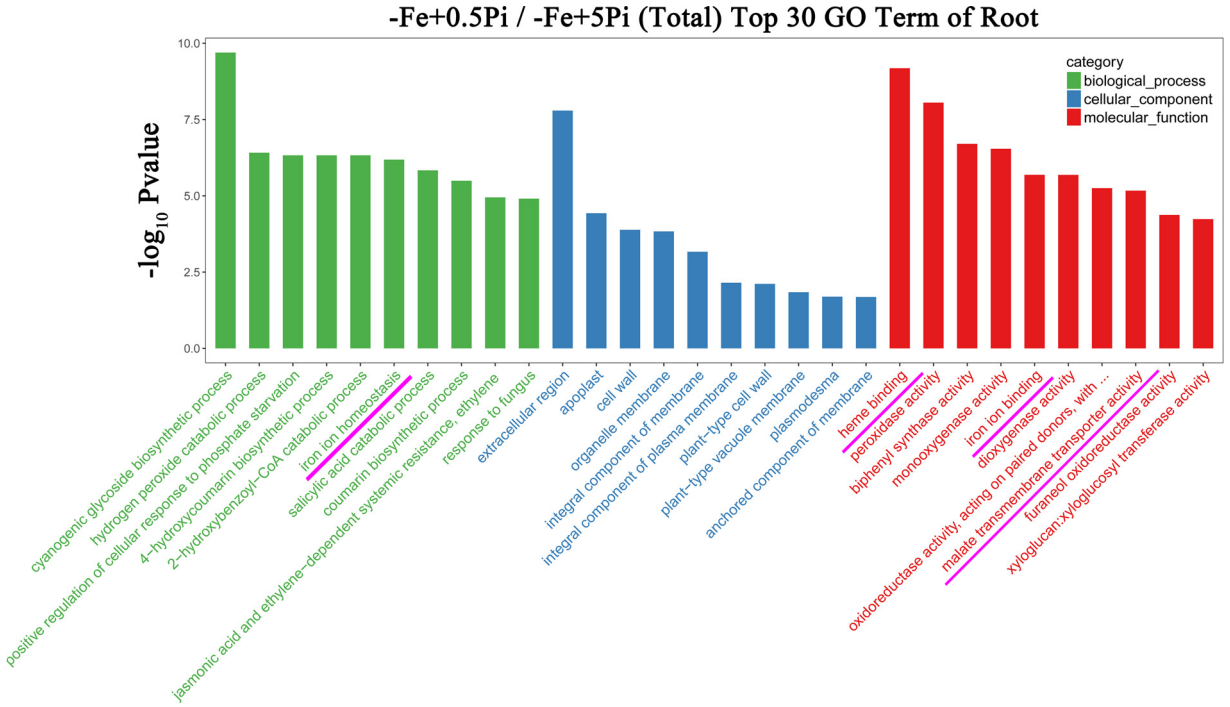

Fig. 8. Gene ontology (GO) enrichment analysis of the differentially expressed Fe genes in the roots. Apple seedlings treated with –Fe +0.5 mM Pi and –Fe +5 mM Pi in the growth medium for 5 days. The top 30 represent three classifications corresponding to the number of differential genes screened greater than 1.5, and each of the 10 items ranked by the -log10 P-value corresponding to each entry was sorted from large to small.

conditions, an appropriate amount of Pi is still necessary to maintain the growth of apple plants. Fe also plays an important role in Pi deficiency. Low Pi stimulates the expression of *AtFER1* and *AtNAS3* in the shoots and promotes the distribution of Fe [44]. Bournier et al., point out that phosphate starvation response 1 (AtPHR1) and its homolog AtPHR1-like 1 (AtPHL1) can bind to the promoter of *AtFER1* and regulate its expression in an Fe-independent manner, thus playing a crucial role in regulating the homeostasis of Fe and Pi [76]. Fe response genes such as *FRO2*, *IRT1*, and *CYP82C4* are significantly down-regulated in the absence of Pi, but they are induced under conditions of co-

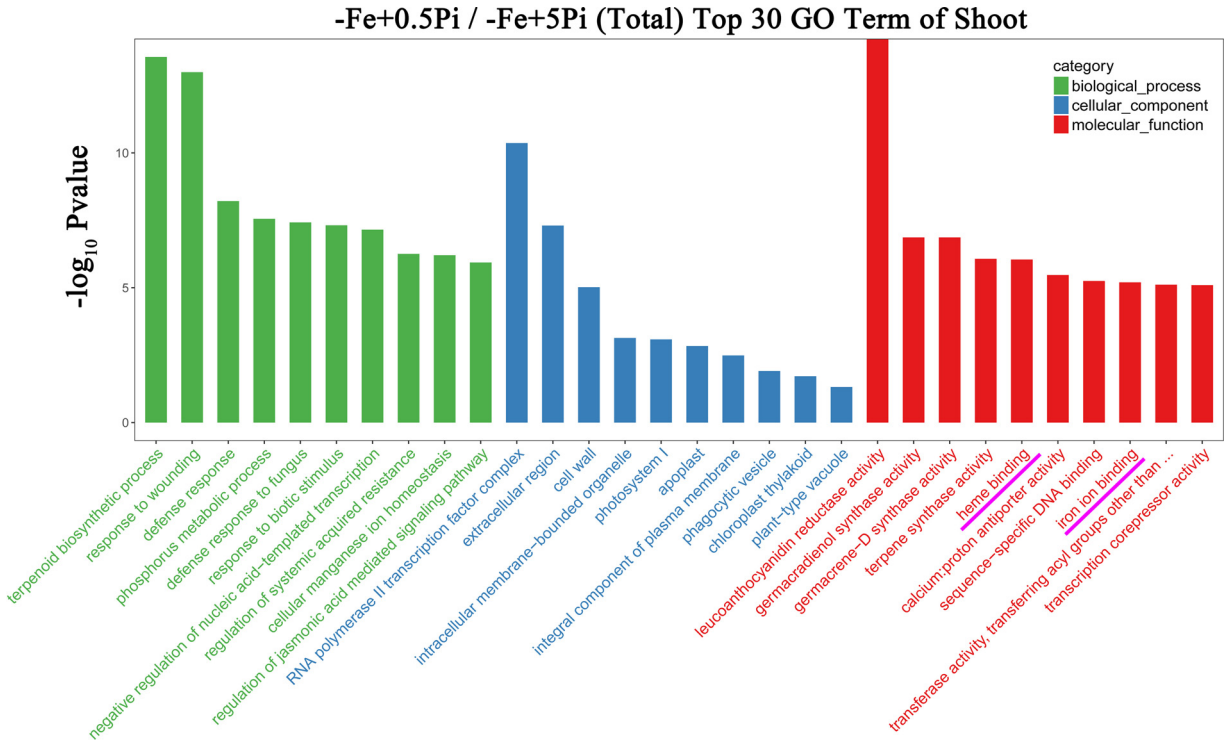

Fig. 9. Gene ontology (GO) enrichment analysis of the differentially expressed Fe genes in the shoots. Apple seedlings treated with –Fe +0.5 mM Pi and –Fe +5 mM Pi in growth medium for 5 days. The top 30 represent three classifications corresponding to the number of differential genes screened greater than 1.5, and each of the 10 items ranked by the -log10 P-value corresponding to each entry was sorted from large to small.

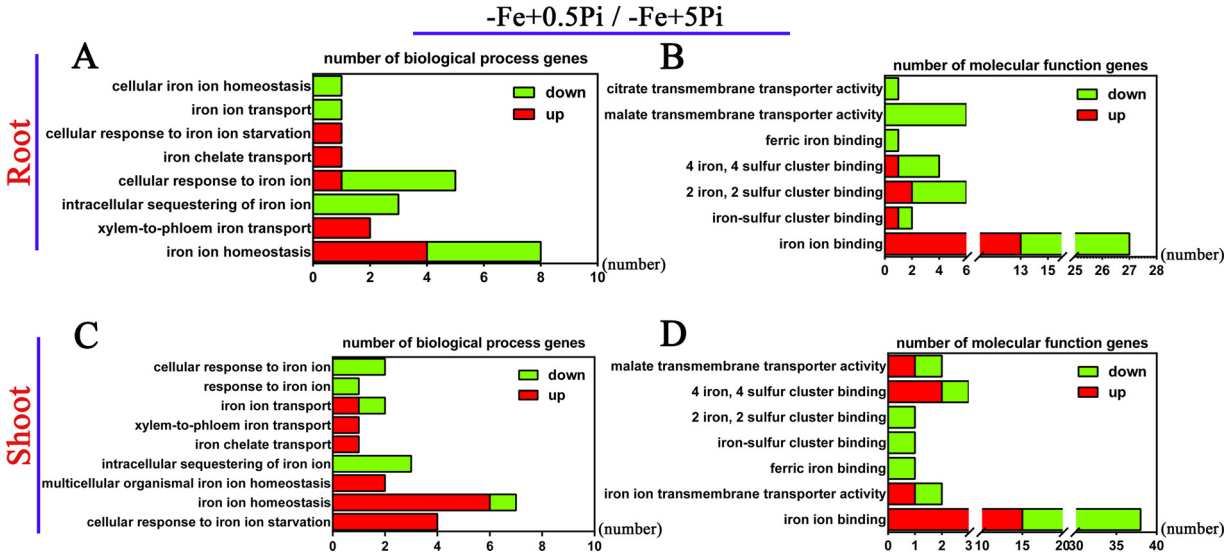

Fig. 10. Functional distribution of the differentially expressed Fe genes in the roots and shoots. Apple seedlings treated with –Fe +0.5 mM Pi and –Fe +5 mM Pi in growth medium for 5 days. Classification qualitative statistics of the transcriptionally dysregulated Fe-response genes based on biological processes in the roots (A) and shoots (C), and molecular functions in the roots (B) and shoots (D). The red color in the histogram indicates that the gene is up-regulated, and the green color represents down-regulated genes (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

deficiency of Fe and Pi [39]. This shows that Fe is necessary for the down-regulation of Fe response genes during Pi deficiency. In our study, compared to –Pi + Fe, –Pi –Fe treatments showed earlier Pi deficiency symptoms (Fig. 1). In addition, a large number of Pi starvation response genes were down-regulated under –Pi –Fe treatment (Fig. 11; Supplemental Fig. S5), indicating that Fe also plays a vital role in coping with Pi deficiency.

4.2. The decrease of Pi content in the rhizosphere helps to improve the reducing ability of Fe

Under the condition of Pi deficiency, the accumulation of Fe is considered to be mainly achieved by the continuous conversion of the malate-Fe^{3+} complex to Fe^{2+}, which is absorbed by the plant. In this study, we identified another avenue by which this could be achieved, as low Pi treatments increased the FCR activity and proton efflux in apple plants in response to Fe deficiency (Fig. 2). This indicated that low Pi treatments enhanced the reduction of Fe^{3+} around the rhizosphere, thereby promoting the formation of Fe^{2+} and allowing it to enter the apple root cells effectively. Under conditions of Fe deficiency, there are two main pathways for proton efflux: one is to promote the expression of AHA family genes, such as *Arabidopsis AHA2* [77,78], thereby releasing H^+. The second method is the release of organic acids, such as citrate [1,68,79]. In the absence of Fe or Pi, malate and citrate accumulated within 7 days and were released from the apple roots (Fig. 4; Supplemental Fig. S2). To further explore the mechanism by which low Pi alleviated Fe-deficiency in apple plants, transcriptome analysis was employed. The results indicated that *MdFRO2* was up-regulated in –Fe +0.5 mM Pi treated apple roots compared with the –Fe +5 mM Pi treated roots. In addition, the Fe transporters *MdVIT1*, *MdFER*, *MdNRAMP3*, and *MdOPT3* were differentially expressed in the roots or shoots (Table 2). Combined with the data presented in Fig. 1–3, these observations confirmed the important role of low Pi treatments in the absorption and transport of Fe.

Table 2
Transcriptomic and RT-qPCR analysis of Fe biological process and molecular function regulated gens in the root and shoot in the Pi treated apple plants under Fe deficiency. [L (Like); -1.5 > logFC (-Fe+0.5Pi/-Fe+5Pi) > 1.5; n = 3 samples, 6 plants per sample; P < 0.05].

Root				Shoot			
Gene ID	Gene name	Fold	RT-qPCR	Gene ID	Gene name	Fold	RT-qPCR
MD01G1068200	*MdFRO2*	1.92	2.12	MD14G1086500	*MdORG2 L1*	2.02	3.51
MD15G1033800	*MdCYP82C4*	3.63	3.28	MD14G1086600	*MdORG2*	3.25	3.78
MD08G1191400	*MdOPT3*	1.94	1.89	MD14G1228100	*MdBTS*	1.90	2.39
MD05G1360700	*MdOPT3 L1*	1.56	1.78	MD16G1039900	*MdBTS L1*	1.51	3.95
MD14G1086600	*MdORG2*	1.53	1.90	MD01G1068200	*MdFRO2*	2.33	2.36
MD10G1211700	*MdMTP10*	1.55	2.61	MD02G1180400	*MdNRAMP3*	1.98	3.30
MD16G1016500	*MdVIT1*	0.46	0.15	MD08G1191400	*MdOPT3*	2.30	3.01
MD16G1016600	*MdVIT1 L1*	0.63	0.45	MD15G1282700	*MdDTX51*	13.54	8.37
MD12G1178200	*MdFER4*	0.19	0.26	MD03G1155500	*MdALMT2 L1*	3.20	3.50
MD01G1187500	*MdNEET*	0.64	0.42	MD10G1227800	*MdZIP1*	2.14	2.68
MD15G1205100	*MdACO1*	0.56	0.85	MD08G1061900	*MdZIP4*	1.65	2.43
MD02G1311900	*MdMATE43*	0.39	0.50	MD09G1140500	*MdZIP8*	1.51	2.28
MD05G1255500	*MdZIP10*	0.45	0.43	MD16G1016500	*MdVIT1*	0.47	0.57
MD03G1155200	*MdALMT2*	0.60	0.36	MD10G1024000	*MdVIT4*	0.37	0.66
MD07G1153600	*MdALMT10*	0.45	0.64	MD07G1226500	*MdFER*	0.59	0.85
MD14G1135700	*MdALMT10 L1*	0.49	0.59	MD08G1195400	*MdPUMP5*	0.66	0.83
MD11G1287000	*MdALMT13*	0.60	0.70				
MD03G1266500	*MdALMT13 L1*	0.54	0.54				

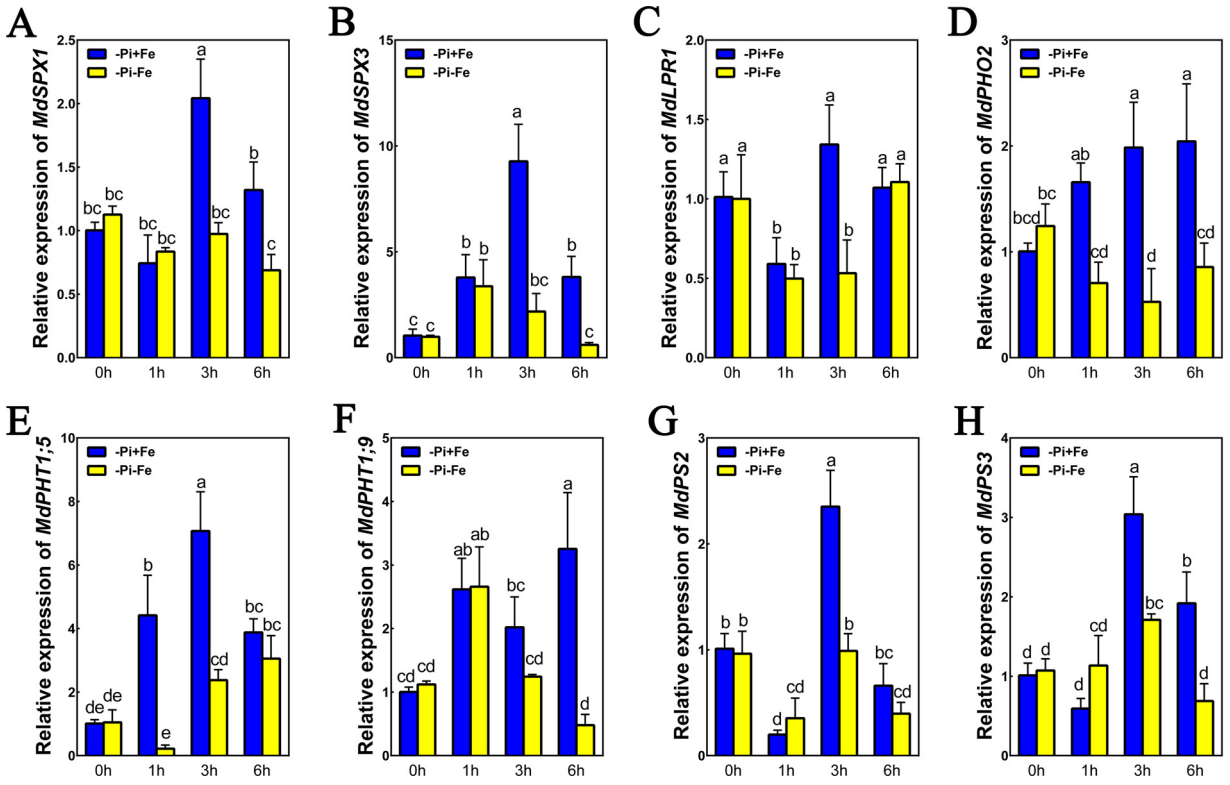

Fig. 11. Expression of low Pi response related genes under different Fe conditions. Apple roots treated with –Pi + Fe or –Pi–Fe in growth medium for 0–6 h. Relative expression of (A) *MdSPX1*, (B) *MdSPX3*, (C) *MdLPR1*, (D) *MdPHO2*, (E) *MdPHT1;5*, (F) *MdPHT1;9*, (G) *MdPS2*, (H) *MdPS3*. Changes in expression are expressed as the mean and SD. Error bars represent standard deviations (n = 3 seedlings). Different letters represent significantly different values at $P < 0.05$.

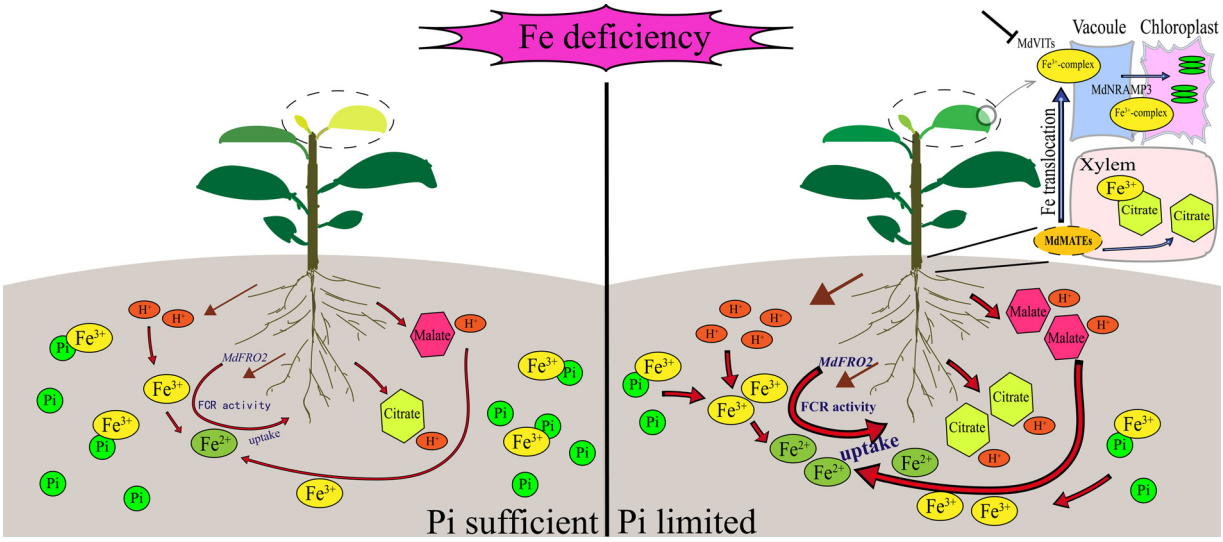

Fig. 12. The model of how low Pi treatment alleviates Fe deficiency in apple. Under the conditions of Fe deficiency, low Pi treatments promote the expression of *MdFRO2* and increase the FCR activity. Simultaneously, malate and citrate are synthesized and effluxed in apple roots, thus releasing H^+ and acidifying the rhizosphere environment. Additionally, the low Pi concentration weakened the antagonistic effects of Fe and Pi in the soil, releasing more Fe^{3+} and then reducing it to Fe^{2+} for root absorption, and *MdMATEs* mediated the involvement of citrate in the transport of the citrate-Fe^{3+} complex in the xylem. In the leaves, the transport of MdVITs to the vacuole Fe pool is limited, and transported to other organelles, such as chloroplasts, by the action of *MdNRAMP3*. The bold font and line segments represent the enhancement of the process, the arrows with line segments represent the promotion of the process, and the bounded line segments represent suppression. A gene depicted by a dotted line indicates that the gene might be involved in a related reaction.

4.3. Low Pi regulates Fe uptake and transport by releasing malate and citrate

Malate and citrate efflux is an effective way for plants to cope with Al and P stress [40,41,53,56,80]. In higher plants, citrate is involved in the transport of xylem citrate-Fe^{3+} mediated by FRD3 [41,63,79,81]. In the present study, low Pi treatments allowed malate and citrate to efflux from the apple roots (Fig. 4). In the transcriptome results, the expression of *MdALMT2*, *MdALMT10*, *MdALMT13*, and *MdMATE43* was down-regulated (Table 2). However, the efflux of organic acids was increased (Fig. 4), which might be due to the feedback regulation mechanism in plants causing their expression to be down-regulated, just as

BTS prevents excessive absorption of Fe in *Arabidopsis*. In addition, another MATE family gene, *MdDTX51*, was up-regulated 13.5 times, which might serve to mediate Fe transport in the xylem of apple plants. In order to verify the efficacy of the malate and citrate efflux, malate and citrate were applied to –Fe +5 mM Pi-treated and –Fe –Pi-treated apple plants. The results showed that all these plants demonstrated a reversal of the symptoms of Fe and P deficiency (Figs. 5 and 6). This suggested that malate and citrate might be simultaneously involved in two pathways related to Fe and P stress: one involves the formation of an Fe^{3+} complex *in vivo*, and the other involves malate and citrate efflux acidifying the rhizosphere environment to promote Fe uptake. Additionally, our results further illustrated the importance of Fe for helping apple plants cope with P deficiency. Although the Fe content in the -Fe +5 mM Pi +0.5 mM malate and citrate-treated *Arabidopsis* roots did not change significantly compared to the -Fe +5 mM Pi treatment at 14 days (Supplemental Fig S4), malate and citrate are likely to be absorbed and transported simultaneously, similar to what occurs in apples. Perhaps *Arabidopsis* is more efficient at Fe transport or Fe reutilization than apple plants. As such, it will be interesting to study the differences in nutrient uptake and utilization between herbaceous and woody plants.

Combining our data and RNA-seq analyses, we have proposed a model illustrating how apple plants cope with Fe deficiency under low Pi conditions (Fig. 12). Under the condition of Fe deficiency, low Pi treatments promote the expression of *MdFRO2* (Table 2) and increase the FCR activity (Fig. 2). Simultaneously, malate and citrate are synthesized and effluxed in apple roots (Fig. 4A), thus releasing H^+ and acidifying the rhizosphere environment (Fig. 2 and 4B, C). Additionally, the low Pi concentration weakens the antagonistic effects of Fe and Pi in the soil, releasing more Fe^{3+} and then reducing it to Fe^{2+} for root absorption, and *MdMATEs* mediates the involvement of citrate in the transport of the citrate-Fe^{3+} complex in the xylem (Table 2) [59]. In the leaves, the transport of MdVITs to the vacuole Fe pool is limited, and transported to other organelles such as chloroplasts by the action of *MdNRAMP3* (Table 2).

4.4. Pi might regulate hormone and coumarins-mediated Fe deficiency response

Hormone regulation has an important influence on Fe absorption and utilization. In *Arabidopsis*, auxin acts upstream of nitric oxide (NO) to promote the expression of *FRO2*, thereby increasing FCR activity and facilitating Fe uptake [82]. Gibberellin (GA) removes the restriction on the root length imposed by the DELLA protein, positively regulates *FRO2* and *IRT1*, and promotes Fe absorption [83,84]. Abscisic acid (ABA) promotes the expression of *YSL2*, *NAS1*, and *NRAMP3* to promote Fe transport and reutilization [23]. The ethylene response factors ETHYLENE INSENSITIVE 3 (EIN3) and ETHYLENE INSENSITIVE 3–LIKE (EIL1) physically interact with FIT and affect FIT abundance, triggering iron deficiency response signals at both the transcriptional and post-transcriptional levels [85]. In addition, under the condition of Pi deficiency, auxin and ethylene participate in the development of root hairs and root elongation in *Arabidopsis*, thereby increasing Pi uptake [86,87]. In this current study, based on our transcriptome analysis, the auxin-responsive protein (SAUR and IAA) families in the auxin-activated signaling pathway and the ethylene-responsive transcription factor (ERF) family in the ethylene-activated signaling pathway were largely differentially expressed in the roots and shoots (Supplemental Table S1 and S2). In addition, ABA synthesis and metabolism-related genes were also differentially expressed (Supplemental Table S1 and S2). However, Trull et al. indicated that *aba1* and *abi2* mutants were not significantly different from wild type in regard to Pi stress [88]. As such, there might exist another or multiple regulatory networks of Pi-mediated hormones that respond to Fe stress.

Coumarins are closely related to the response to Fe deficiency. In *Arabidopsis*, β-glucosidase (BGLU42) acts downstream of MYB72 to mediate the removal of glycones from Fe-mobilizing coumarins, which are excretes to the rhizosphere via PDR9 to promote Fe uptake [89]. In addition, the P450 dependent monooxygenase CYP82C4 is induced by Fe deficiency and participates in the conversion of fraxetin to sideretin [11,48,90]. According to previous studies, the combined deficiency of Fe and Pi caused down-regulation of CYP82C4 mRNA expression [91,92]. Coumarins also exists in apples, and about 13 coumarin derivatives can inhibit the browning of fruits [93], but no research on Fe deficiency has been reported. In this current study, *MdCYP82C4* expression was up-regulated in apple roots treated with -Fe +0.5 mM Pi compared to -Fe +5 mM Pi treatments. However, only *MdBGLT* (a homologous gene of beta-glucosidase 17 in *Arabidopsis*) was up-regulated in coumarin synthesis-related genes and other genes, such as *MdBIPSs* and *MdBGLs*, were down-regulated (Supplemental Table S3). Perhaps, under the condition of -Fe +0.5 mM Pi, *MdCYP82C4* is more involved in the Fe deficiency response of apple plants than coumarin biosynthesis. In addition, whether *MdCYP82C4* participates in coumarin biosynthesis like *AtCYP82C4* needs further research. According to the transcriptome, coumarin synthesis-related genes *MdBIPSs* and scopolin beta-glucosidase activity genes *MdBGLs* were down-regulated in apple roots (Supplemental Table S3), indicating that coumarins might also play a role in coping with Fe and Pi stress in apples. Similar to coumarins in *Arabidopsis* [44,46,51], malate and citrate are synthesized in apple roots in the absence of Fe or Pi (Fig. 4; Supplemental Fig. S2). However, the difference is that compared with –Fe +1.25 mM Pi treatment, malate and citrate increased exudation under the conditions of –Fe–Pi, –Fe +0.125 mM Pi, and –Fe +0.5 mM Pi (Fig. 4). In addition to these species differences, there are also large differences in the subset of coumarins (such as scopolin, esculin, fraxin, and sideritin glucoside) involved in the response to Fe or Pi stress [50–52,94,95]. Perhaps, like coumarins, the isomers of malic acid and citric acid, such as L-malic acid with D-malic acid or citric acid with isocitric acid, could be synthesized differently in response to environmental stress.

In summary, we verified in apple plants that Pi responds effectively to Fe deficiency in a dose-dependent manner. In addition, low Pi responds to Fe deficiency in two ways: one is to promote Fe activation directly, and the other is to promote the efflux of malate and citrate, thus promoting Fe uptake and transport. The interaction of Fe and Pi has a crucial role in the absorption and utilization of Fe and Pi nutrition. Although Fe and Pi antagonize each other, an appropriate amount of Pi is still necessary to cope with Fe deficiency, and an appropriate amount of Fe is also necessary during Pi deficiency.

Contribution

Y.J.H., C.X.Y. and J.C.Z. helped to modify the manuscript. J. C.Z., X. S. T. and X.F.W. provided experimental materials. X.N.W. and W.S. helped determine the physiological indicators related to Fe. X.L.J., J.P.A. and Q.Z. helped with primer design.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2020.110526.

References

[1] T. Kobayashi, N.K. Nishizawa, Iron uptake, translocation, and regulation in higher plants, Annu. Rev. Plant Biol. 63 (2012) 131–152.
[2] M.L. Guerinot, Y. Yi, Iron: nutritious, noxious and not readily available, Plant Physiol. 104 (1994) 815–820.
[3] J. Jeong, M.L. Guerinot, Homing in on iron homeostasis in plants, Trends Plant Sci. 14 (2009) 280–285.
[4] W. Schmidt, Iron solutions: acquisition strategies and signaling pathways in plants, Trends Plant Sci. 8 (2003) 188–193.
[5] G. Vert, N. Grotz, F. Dédaldéchamp, F. Gaymard, M.L. Guerinot, J.F. Briat, C. Curie, IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth, Plant Cell 14 (2002) 1223–1233.
[6] C. Curie, G. Cassin, D. Couch, F. Divol, K. Higuchi, M.L. Jean, J. Misson, A. Schikora, P. Czernic, S. Mari, Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters, Ann Bot-London 103 (1) (2008) 1–11.
[7] H. Inoue, T. Kobayashi, T. Nozoye, M. Takahashi, Y. Kakei, K. Suzuki, M. Nakazono, H. Nakanishi, S. Mori, N.K. Nishizawa, Rice osysl15 is an iron-regulated iron(iii)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings, J. Biol. Chem. 284 (6) (2009) 3470–3479.
[8] Y. Ishimaru, M. Suzuki, T. Tsukamoto, K. Suzuki, M. Nakazono, T. Kobayashi, H. Nakanishi, Rice plants take up iron as an $Fe3^+$-phytosiderophore and as $Fe2^+$, Plant J. 45 (3) (2006) 335–346.
[9] E.L. Walker, E.L. Connolly, Time to pump iron: iron-deficiency-signaling mechanisms of higher plants, Curr. Opin. Plant Biol. 11 (5) (2008) 530–535.
[10] C. Liu, T. Gao, Y. Liu, J. Liu, F. Li, Z. Chen, W. Huang, Isotopic fingerprints indicate distinct strategies of Fe uptake in rice, Chem. Geol. 524 (2019) 323–328.
[11] F. Gao, K. Robe, F. Gaymard, E. Izquierdo, C. Dubos, The transcriptional control of iron homeostasis in plants: a tale of bHLH transcription factors? Front. Plant Sci. 10 (2019) 6.
[12] D. Selote, R. Samira, A. Matthiadis, J.W. Gillikin, T.A. Long, Iron-binding E3 ligase mediates iron response in plants by targeting basic helixloop-helix transcription factors, Plant Physiol. 167 (2015) 273–286.
[13] J. Rodríguez-Celma, J.M. Connorton, I. Kruse, R.T. Green, M. Franceschetti, Y.T. Chen, Y. Cuid, H.Q. Ling, K.C. Yeh, J. Balk, Arabidopsis BRUTUS-LIKE E3 ligases negatively regulate iron uptake by targeting transcription factor FIT for recycling, Proc. Natl. Acad. Sci. U. S. A. 116 (35) (2019) 17584–17591.
[14] N. Wang, Y. Cui, Y. Liu, H. Fan, J. Du, Z. Huang, Y. Yuan, H. Wu, H.Q. Ling, Requirement and functional redundancy of Ib subgroup bHLH proteins for iron deficiency responses and uptake in Arabidopsis thaliana, Mol. Plant 6 (2) (2013) 503–513.
[15] M. Klatte, M. Schuler, M. Wirtz, C. Fink-Straube, R. Hell, P. Bauer, The analysis of Arabidopsis nicotianamine synthase mutants reveals functions for nicotianamine in seed iron loading and iron deficiency responses, Plant Physiol. 150 (1) (2009) 257–271.
[16] T.A. Long, H. Tsukagoshi, W. Busch, B. Lahner, D.E. Salt, P.N. Benfey, The bHLH transcription factor POPEYE regulates response to iron defciency in *Arabidopsis* roots, Plant Cell 22 (2010) 2219–2236.
[17] R. Nechushtai, A.R. Conlan, Y. Harir, L. Song, O. Yogev, Y. Eisenberg-Domovich, O. Livnah, D. Michaeli, R. Rosen, V. Ma, Y. Luo, J.A. Zuris, M.L. Paddock, Z.I. Cabantchik, P.A. Jennings, R. Mittler, Characterization of Arabidopsis NEET reveals an ancient role for NEET proteins in iron metabolism, Plant Cell 24 (5) (2012) 2139–2154.
[18] N. Tissot, K. Robe, F. Gao, S. Grant-Grant, J. Boucherez, F. Bellegarde, A. Maghiaoui, R. Marcelin, E. Izquierdo, M. Benhamed, A. Martin, F. Vignols, H. Roschzttardtz, F. Gaymard, J.F. Briat, C. Dubos, Transcriptional integration of the responses to iron availability in Arabidopsis by the bHLH factor ILR3, New Phytol. (2019).
[19] L.J. Zhou, C.L. Zhang, R.F. Zhang, G.L. Wang, Y.Y. Li, Y.J. Hao, The SUMO E3 ligase MdSIZ1 targets MdbHLH104 to regulate plasma membrane H+-ATPase activity and iron homeostasis, Plant Physiol. 179 (1) (2019) 88–106.
[20] Q. Zhao, Y.R. Ren, Q.J. Wang, Y.X. Yao, C.X. You, Y.J. Hao, Overexpression of MdbHLH104 gene enhances the tolerance to iron deficiency in apple, Plant Biotechnol. J. 14 (2016) 1633–1645.
[21] S. Thomine, F. Lelièvre, E. Debarbieux, J.I. Schroeder, H. Barbier-Brygoo, AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency, Plant J. 34 (5) (2003) 685–695.
[22] S. Thomine, R. Wang, J.M. Ward, N.M. Crawford, J.I. Schroeder, Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to Nramp genes, P. Nati. Acad. Sci. USA. 97 (9) (2000) 4991–4996.
[23] G.J. Lei, X.F. Zhu, Z.W. Wang, F. Dong, S.J. Zheng, Abscisic acid alleviates iron deficiency by promoting root iron reutilization and transport from root to shoot in *Arabidopsis*, Plant Cell Environ. 37 (2014) 852–863.
[24] M. Zhang, M. Chen, Z. Wang, T. Wu, Y. Wang, X. Zhang, Z. Han, Synchrotron X-ray fluorescence microtomography profiling of Malus xiaojinensis provides insights into mechanisms of divalent metals transport subjected to Iron deficiency, HortScience 50 (6) (2015) 801–805.
[25] J.C. Zhang, X.F. Wang, X.N. Wang, F.P. Wang, X.L. Ji, J.P. An, K. Yang, Q. Zhao, C.X. You, Y.J. Hao, Abscisic acid alleviates iron deficiency by regulating iron distribution in roots and shoots of apple, Sci. Hortic. (2019) 109018.
[26] S.A. Kim, T. Punshon, A. Lanzirotti, L. Li, J.M. Alonso, J.R. Ecker, J. Kaplan, M.L. Guerinot, Localization of iron in Arabidopsis seed requires the vacuolar membrane transporter VIT1, Science 314 (5803) (2006) 1295–1298.
[27] J. Gollhofer, R. Timofeev, P. Lan, W. Schmidt, T.J. Buckhout, Vacuolar-iron-transporter1-like proteins mediate iron homeostasis in Arabidopsis, PLoS One 9 (10) (2014) e110468.
[28] H. Rouached, D. Secco, B.A. Arpat, Regulation of ion homeostasis in plants: current approaches and future challenges, Plant Signal. Behav. 5 (5) (2010) 501–502.
[29] V. Shanmugam, J.C. Lo, C.L. Wu, S.L. Wang, C.C. Lai, E.L. Connolly, K.C. Yeh, Differential expression and regulation of iron-regulated metal transporters in Arabidopsis halleri and Arabidopsis thaliana–the role in zinc tolerance, New Phytol. 190 (1) (2011) 125–137.
[30] O. Vedina, S. Toma, Forms of microelements in apple leaves under different conditions of iron and zinc nutrition, J. Plant Nutr. 23 (8) (2000) 1135–1143.
[31] M. Aktaş, F. Van Egmond, Effect of nitrate nutrition on iron utilization by an Fe-efficient and an Fe-inefficient soybean cultivar, Plant Soil 51 (2) (1979) 257–274.
[32] L. Sanchez-Calderon, J. Lopez-Bucio, A. Chacon-Lopez, A. Cruz-Ramirez, F. Nieto-Jacobo, J.G. Dubrovsky, L. Herrera-Estrella, Phosphate starvation induces a determinate developmental program in the roots of Arabidopsis thaliana, Plant Cell Physiol. 46 (2005) 174–184.
[33] L.F. Ruiz Herrera, M.W. Shane, J. Lo´pez-Bucio, Nutritional regulation of root development, Wiley Interdiscip. Rev. Dev. Biol. 4 (2015) 431–443.
[34] L.C. Williamson, S.P. Ribrioux, A.H. Fitter, H.M. Leyser, Phosphate availability regulates root system architecture in Arabidopsis, Plant Physiol. 126 (2001) 875–882.
[35] J.T. Ward, B. Lahner, E. Yakubova, D.E. Salt, K.G. Raghothama, The effect of iron on the primary root elongation of Arabidopsis during phosphate deficiency, Plant Physiol. 147 (3) (2008) 1181–1191.
[36] S. Svistoonoff, A. Creff, M. Reymond, C. Sigoillot-Claude, L. Ricaud, A. Blanchet, L. Nussaume, T. Desnos, Root tip contact with low-phosphate media reprograms plant root architecture, Nat. Genet. 39 (6) (2007) 792–796.
[37] A.P. Singh, Y. Fridman, N. Holland, M. Ackerman-Lavert, R. Zananiri, Y. Jaillais, H. Arnon, S. Savaldi-Goldstein, Interdependent nutrient availability and steroid hormone signals facilitate root growth plasticity, Dev. Cell 46 (1) (2018) 59–72.
[38] L. Zheng, F. Huang, R. Narsai, J. Wu, E. Giraud, F. He, L. Cheng, F. Wang, P. Wu, J. Whelan, H. Shou, Physiological and transcriptome analysis of iron and phosphorus interaction in rice seedlings, Plant Physiol. 151 (1) (2009) 262–274.
[39] W. Li, P. Lan, Genome-wide analysis of overlapping genes regulated by iron deficiency and phosphate starvation reveals new interactions in Arabidopsis roots, BMC Res. Notes 8 (1) (2015) 555.
[40] J. Mora-Macías, J.O. Ojeda-Rivera, D. Gutiérrez-Alanís, L. Yong-Villalobos, A. Oropeza-Aburto, J. Raya-González, L. Herrera-Estrella, Malate-dependent Fe accumulation is a critical checkpoint in the root developmental response to low phosphate, Proc. Natl. Acad. Sci. U. S. A. 114 (17) (2017) E3563–E3572.
[41] J. Müller, T. Toev, M. Heisters, J. Teller, K.L. Moore, G. Hause, S. Abel, Iron-dependent callose deposition adjusts root meristem maintenance to phosphate availability, Dev. Cell 33 (2) (2015) 216–230.
[42] A. Larbi, F. Morales, A. López-Millán, Y. Gogorcena, A. Abadía, P. Moog, J. Abadía, Technical Advance: Reduction of Fe (III)-Chelates by Mesophyll LeafDisks of Sugar Beet. Multi-Component Origin and Effects of Fe Deficiency, Plant Cell Physiol. 42 (1) (2001) 94–105.
[43] E.B. González-Vallejo, A. Abadía, J.A. González-Reyes, J. Abadía, A.F. López-Millán, F. Yunta, J.J. Lucena, Reduction of ferric chelates by leaf plasma membrane preparations from Fe-deficient and Fe-sufficient sugar beet, Funct. Plant Biol. 26 (6) (1999) 601–611.
[44] J. Hirsch, E. Marin, M. Floriani, S. Chiarenza, P. Richaud, L. Nussaume, M.C. Thibaud, Phosphate deficiency promotes modification of iron distribution in Arabidopsis plants, Biochimie 88 (11) (2006) 1767–1771.
[45] P. Fourcroy, P. Sisó-Terraza, D. Sudre, M. Savirón, G. Reyt, F. Gaymard, A. Abadía, J. Abadia, A. Álvarez-Fernández, J.F. Briat, Involvement of the ABCG 37 transporter in secretion of scopoletin and derivatives by Arabidopsis roots in response to iron deficiency, New Phytol. 201 (1) (2014) 155–167.
[46] N.B. Schmid, R.F. Giehl, S. Döll, H.P. Mock, N. Strehmel, D. Scheel, X. Kong, R.C. Hider, N. von Wirén, Feruloyl-CoA 6′-Hydroxylase1-dependent coumarins mediate iron acquisition from alkaline substrates in Arabidopsis, Plant Physiol. 164 (1) (2014) 160–172.
[47] J. Siwinska, K. Siatkowska, A. Olry, J. Grosjean, A. Hehn, F. Bourgaud, A.A. Meharg, M. Carey, E. Lojkowska, A. Ihnatowicz, Scopoletin 8-hydroxylase: a novel enzyme involved in coumarin biosynthesis and iron-deficiency responses in Arabidopsis, J. Exp. Bot. 69 (7) (2018) 1735–1748.
[48] H.H. Tsai, J. Rodriguez-Celma, P. Lan, Y.C. Wu, I.C. Vélez-Bermúdez, W. Schmidt, Scopoletin 8-hydroxylase-mediated fraxetin production is crucial for iron mobilization, Plant Physiol. 177 (1) (2018) 194–207.
[49] P. Fourcroy, N. Tissot, F. Gaymard, J.F. Briat, C. Dubos, Facilitated Fe nutrition by phenolic compounds excreted by the Arabidopsis ABCG37/PDR9 transporter requires the IRT1/FRO2 high-affinity root $Fe2^+$ transport system, Mol. Plant 9 (3) (2016) 485–488.
[50] H.H. Tsai, W. Schmidt, Mobilization of iron by plant-borne coumarins, Trends Plant Sci. 22 (6) (2017) 538–548.
[51] J. Ziegler, S. Schmidt, R. Chutia, J. Müller, C. Böttcher, N. Strehmel, D. Scheel, S. Abel, Non-targeted profiling of semi-polar metabolites in Arabidopsis root exudates uncovers a role for coumarin secretion and lignification during the local response to phosphate limitation, J. Exp. Bot. 67 (5) (2015) 1421–1432.
[52] R. Chutia, S. Abel, J. Ziegler, Iron and phosphate deficiency regulators concertedly control coumarin profiles in Arabidopsis thaliana roots during Iron, phosphate, and combined deficiencies, Front. Plant Sci. 10 (2019) 113.
[53] J.F. Ma, P.R. Ryan, E. Delhaize, Aluminium tolerance in plants and the complexing role of organic acids, Trends Plant Sci. 6 (6) (2001) 273–278.
[54] T. Sasaki, Y. Yamamoto, B. Ezaki, M. Katsuhara, S.J. Ahn, P.R. Ryan, H. Matsumoto, A wheat gene encoding an aluminum-activated malate transporter, Plant J. 37 (5)

(2004) 645–653.
[55] Y. Sawaki, S. Iuchi, Y. Kobayashi, Y. Kobayashi, T. Ikka, N. Sakurai, M. Fujita, K. Shinozaki, D. Shibata, M. Kobayashi, H. Koyama, STOP1 regulates multiple genes that protect Arabidopsis from proton and aluminum toxicities, Plant Physiol. 150 (1) (2009) 281–294.
[56] J. Liu, J.V. Magalhaes, J. Shaff, L.V. Kochian, Aluminum-activated citrate and malate transporters from the MATE and ALMT families function independently to confer Arabidopsis aluminum tolerance, Plant J. 57 (3) (2009) 389–399.
[57] C. Liang, M.A. Piñeros, J. Tian, Z. Yao, L. Sun, J. Liu, H. Liao, Low pH, aluminum, and phosphorus coordinately regulate malate exudation through GmALMT1 to improve soybean adaptation to acid soils, Plant Physiol. 161 (3) (2013) 1347–1361.
[58] H. Liao, H. Wan, J. Shaff, X. Wang, X. Yan, L.V. Kochian, Phosphorus and aluminum interactions in soybean in relation to aluminum tolerance. Exudation of specific organic acids from different regions of the intact root system, Plant Physiol. 141 (2) (2006) 674–684.
[59] G.J.D. Kirk, E.E. Santos, G.R. Findenegg, Phosphate solubilization by organic anion excretion from rice (Oryza sativa L.) growing in aerobic soil, Plant Soil 211 (1) (1999) 11–18.
[60] A. Gaume, F. Mächler, C. De León, L. Narro, E. Frossard, Low-P tolerance by maize (Zea mays L.) genotypes: significance of root growth, and organic acids and acid phosphatase root exudation, Plant Soil 228 (2) (2001) 253–264.
[61] J.C. Brown, R.L. Chaney, Effect of iron on the transport of citrate into the xylem of soybeans and tomatoes, Plant Physiol. 47 (6) (1971) 836–840.
[62] L.S. Green, E.E. Rogers, FRD3 controls iron localization in Arabidopsis, Plant Physiol. 136 (1) (2004) 2523–2531.
[63] T.P. Durrett, W. Gassmann, E.E. Rogers, The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation, Plant Physiol. 144 (1) (2007) 197–205.
[64] Y. Zhang, P. Li, L. Cheng, Developmental changes of carbohydrates, organic acids, amino acids, and phenolic compounds in 'Honeycrisp'apple flesh, Food Chem. 123 (4) (2010) 1013–1018.
[65] X.Y. Lin, Y.Q. Ye, S.K. Fan, C.W. Jin, S.J. Zheng, Increased sucrose accumulation regulates iron-deficiency responses by promoting auxin signaling in Arabidopsis plants, Plant Physiol. 170 (2) (2016) 907–920.
[66] T. Soga, M. Imaizumi, Capillary electrophoresis method for the analysis of inorganic anions, organic acids, amino acids, nucleotides, carbohydrates and other anionic compounds, Electrophoresis 22 (16) (2001) 3418–3425.
[67] J.P. An, F.J. Qu, J.F. Yao, X.N. Wang, C.X. You, X.F. Wang, Y.J. Hao, The bZIP transcription factor MdHY5 regulates anthocyanin accumulation and nitrate assimilation in Apple, Hortic. Res. 4 (2017) 17023.
[68] X. Wang, Z. Wang, Z. Zheng, J. Dong, L. Song, L. Sui, L. Nussaume, T. Desnos, D. Liu, Genetic dissection of Fe-dependent signaling in root developmental responses to phosphate deficiency, Plant Physiol. 179 (1) (2019) 300–316.
[69] C. Godon, C. Mercier, X. Wang, P. David, P. Richaud, L. Nussaume, D. Liu, T. Desnos, Under phosphate starvation conditions, Fe and Al trigger accumulation of the transcription factor STOP1 in the nucleus of Arabidopsis root cells, Plant J. 99 (5) (2019) 937–949.
[70] A.J. Stewart, W. Chapman, G.I. Jenkins, I. Graham, T. Martin, A. Crozier, The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissues, Plant Cell Environ. 24 (11) (2001) 1189–1197.
[71] P. Dörmann, C. Benning, Galactolipids rule in seed plants, Trends Plant Sci. 7 (3) (2002) 112–118.
[72] C. Balzergue, T. Dartevelle, C. Godon, E. Laugier, C. Meisrimler, J.M. Teulon, A. Creff, M. Bissler, C. Brouchoud, A. Hagège, J. Müller, S. Chiarenza, H. Javot, N. Becuwe-Linka, P. David, B. Péret, E. Delannoy, M.C. Thibaud, J. Armengaud, S. Abel, J.L. Pellequer, L. Nussaume, J. Müller, Low phosphate activates STOP1-ALMT1 to rapidly inhibit root cell elongation, Nat. Commun. 8 (2017) 15300.
[73] J. Dong, M.A. Piñeros, X. Li, H. Yang, Y. Liu, A.S. Murphy, L.V. Kochian, D. Liu, An Arabidopsis ABC transporter mediates phosphate deficiency-induced remodeling of root architecture by modulating iron homeostasis in roots, Mol. Plant 10 (2) (2017) 244–259.
[74] C.C. Dalton, K. Iqbal, D.A. Turner, Iron phosphate precipitation in Murashige and Skoog media, Physiol. Plantarum 57 (4) (1983) 472–476.
[75] J.H. Rediske, O. Biddulph, The absorption and translocation of iron, Plant Physiol. 28 (4) (1953) 576.
[76] M. Bournier, N. Tissot, S. Mari, J. Boucherez, E. Lacombe, J.F. Briat, F. Gaymard, Arabidopsis ferritin 1 (AtFer1) gene regulation by the phosphate starvation response 1 (AtPHR1) transcription factor reveals a direct molecular link between iron and phosphate homeostasis, J. Biol. Chem. 288 (31) (2013) 22670–22680.
[77] C. Curie, J.F. Briat, Iron transport and signaling in plants, Annu. Rev. Plant Biol. 54 (2003) 183–206.
[78] S. Santi, W. Schmidt, Dissecting iron deficiency-induced proton extrusion in Arabidopsis roots, New Phytol. 183 (4) (2009) 1072–1084.
[79] E.E. Rogers, M.L. Guerinot, FRD3, a member of the multidrug and toxin efflux family, controls iron deficiency responses in Arabidopsis, Plant Cell 14 (8) (2002) 1787–1799.
[80] D.S. Lipton, R.W. Blanchar, D.G. Blevins, Citrate, malate, and succinate concentration in exudates from P-sufficient and P-stressed Medicago sativa L. seedlings, Plant Physiol. 85 (2) (1987) 315–317.
[81] R. Rellán-Álvarez, J. Giner-Martínez-Sierra, J. Orduna, I. Orera, J.Á. Rodríguez-Castrillón, J.I. García-Alonso, J. Abadía, A. Álvarez-Fernández, Identification of a tri-iron (III), tri-citrate complex in the xylem sap of iron-deficient tomato resupplied with iron: new insights into plant iron long-distance transport, Plant Cell Physiol. 51 (1) (2010) 91–102.
[82] W.W. Chen, J.L. Yang, C. Qin, C.W. Jin, J.H. Mo, T. Ye, S.J. Zheng, Nitric oxide acts downstream of auxin to trigger root ferric–chelate reductase activity in response to iron deficiency in *Arabidopsis thaliana*, Plant Physiol. 154 (2) (2010) 810–819.
[83] K. Matsuoka, J. Furukawa, H. Bidadi, M. Asahina, S. Yamaguchi, S. Satoh, Gibberellin-induced expression of Fe uptake-related genes in *Arabidopsis*, Plant Cell Physiol. 55 (2014) 87–98.
[84] M. Wild, J.M. Davière, T. Regnault, Tissue-specific is regulation of gibberellin signaling fine-tunes *Arabidopsis* iron-deficiency responses, Dev. Cell 37 (2) (2016) 190–200.
[85] S. Lingam, J. Mohrbacher, T. Brumbarova, T. Potuschak, C. Fink-Straube, E. Blondet, P. Genschik, P. Bauer, Interaction between the bHLH transcription factor FIT and ETHYLENE INSENSITIVE3/ETHYLENE INSENSITIVE3-LIKE1 reveals molecular linkage between the regulation of iron acquisition and ethylene signaling in Arabidopsis, Plant Cell 23 (5) (2011) 1815–1829.
[86] T.R. Bates, J.P. Lynch, Stimulation of root hair elongation in Arabidopsis thaliana by low phosphorus availability, Plant Cell Environ. 19 (5) (1996) 529–538.
[87] Z. Ma, T.I. Baskin, K.M. Brown, J.P. Lynch, Regulation of root elongation under phosphorus stress involves changes in ethylene responsiveness, Plant Physiol. 131 (3) (2003) 1381–1390.
[88] M.C. Trull, M.J. Guiltinan, J.P. Lynch, J. Deikman, The responses of wild-type and ABA mutant Arabidopsis thaliana plants to phosphorus starvation, Plant Cell Environ. 20 (1) (1997) 85–92.
[89] C. Zamioudis, J. Hanson, C.M. Pieterse, β-Glucosidase BGLU 42 is a MYB 72-dependent key regulator of rhizobacteria-induced systemic resistance and modulates iron deficiency responses in A rabidopsis roots, New Phytol. 204 (2) (2014) 368–379.
[90] J. Rajniak, R.F. Giehl, E. Chang, I. Murgia, N. von Wirén, E.S. Sattely, Biosynthesis of redox-active metabolites in response to iron deficiency in plants, Nature Chem. Biol. 14 (5) (2018) 442.
[91] R. Bustos, G. Castrillo, F. Linhares, M.I. Puga, V. Rubio, J. Pérez-Pérez, J. Paz-Ares, A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in Arabidopsis, PLoS Genet. 6 (9) (2010) e1001102.
[92] W. Li, P. Lan, Genome-wide analysis of overlapping genes regulated by iron deficiency and phosphate starvation reveals new interactions in Arabidopsis roots, BMC Res. Notes 8 (1) (2015) 555.
[93] I. Strelec, P. Burić, I. Janković, T. Kovač, M. Molnar, Inhibitory effect of coumarin derivatives on apple (cv. Idared) polyphenol oxidase, Croat. J. Food Sci. Technol. 9 (1) (2017) 57–65.
[94] K. Kai, M. Mizutani, N. Kawamura, R. Yamamoto, M. Tamai, H. Yamaguchi, K. Sakata, B.I. Shimizu, Scopoletin is biosynthesized via ortho-hydroxylation of feruloyl CoA by a 2-oxoglutarate-dependent dioxygenase in Arabidopsis thaliana, Plant J. 55 (6) (2008) 989–999.
[95] B.I. Shimizu, 2-Oxoglutarate-dependent dioxygenases in the biosynthesis of simple coumarins, Front. Plant Sci. 5 (2014) 549.