The Effect of the Upregulated Expression of Fe Regulators IMA1 and bHLH104 in Arabidopsis on the Root Length and Fe Homeostasis Under Pi Starvation

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Research Article

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Abstract

Phosphate (Pi) and iron (Fe) are two essential mineral nutrients for plant growth and development. Pi starvation triggers the Fe local redistribution and over-accumulation, resulting in the reduction of the primary root, while represses the expression of Fe uptake genes. Nevertheless, the antagonistic mechanism between P and Fe nutrition in plant remain not addressed. Here, the effect of the upregulated expression of Fe regulators *IMA1* and *bHLH104* driven by the different-type promoters (*proCaMV 35S*, the promoters of Pi-starvation responsive genes *proIPS1* and *proPHT1;4*) in response to Pi starvation was investigated in Arabidopsis. The results showed that the expression of Fe uptake genes *IRT1* and *FRO2* was successfully upregulated in *proIPS1::IMA1*, *proPHT1;4::IMA1* and *proIPS1::bHLH104* under Pi starvation while decreased in *pro35S::IMA1*, *pro35S::bHLH104* and *proPHT1;4::bHLH104*, compared with that in the corresponding plants under Pi sufficiency. Although the length and Fe distribution in roots of them didn't have significant difference with wild type under Pi starvation, the Fe distribution and total Fe contents were significantly increased in shoots of *proIPS1::IMA1*, *proPHT1;4::IMA1* and *proIPS1::bHLH104* while were decreased in *proPHT1;4::bHLH104*. The higher Fe concentrations in the Pi-starved transgenic plants also conferred the obviously tolerance to Fe deficiency. Their biomasses and total P concentrations showed no difference with wild type, regardless of Pi sufficiency or deficiency. Therefore, this approach would be a novel manipulation to modify Fe nutrient via coupling with Pi starvation in plants.

Key Messages

Under Pi starvation, upregulating Fe regulators induces the different expression of Fe uptake genes and Fe homeostasis disorder in *Arabidopsis*, but cannot rescue the reduction of primary root.

Introduction

Phosphorus (P) is an essential mineral element for plant growth and development, involving in a myriad of important biological processes as a structural component in nucleic acids and phospholipids, in energy metabolism, in the regulation of enzymatic activities, and in signal transduction cascades (Hinsinger, 2001, Raghothama and Karthikeyan, 2005, Rausch and Bucher, 2002). However, most of soil P is firmly fixed in minerals (e.g. Ca-P, Al-P and Fe-P) and cannot be directly used by plants, resulting in that the readily bioavailability (inorganic phosphate) represents a constraint for plant productivity in natural and agricultural ecosystems (Lynch, 1995). When suffering phosphate (Pi) starvation, plants modulate an array of morphological, biochemical and genetic modifications, which include remodeling the root system architecture; enhancing the excretion of organic acids and phosphatases, nucleases and ribonucleases; activating Pi transporters to increase Pi uptake and utilization (Bates and Lynch, 2001, Chevalier, et al., 2003, Li, et al., 2002, Lopez-Bucio, et al., 2002, Lynch, 2011, Lynch and Brown, 2001, Plaxton and Tran, 2011). Meanwhile, plant alters the homeostasis of other mineral nutrients to increase the acclimation of Pi-deficiency stress, which play crucial roles in regulating the Pi uptake and utilization and even affecting the growth and development (Baxter, 2009, Gniazdowska, et al., 1999, Hu and Chu, 2020, Linkohr, et al.,

2002, Rouached, et al., 2010, Rufty, et al., 1993, Wang, et al., 2019). Of these, the antagonistic interaction between Pi and Fe is thought as a proactive strategy in the remodification of the root architecture to cope with Pi shortage (Abel, 2017). Fe redistribution and accumulation as well as Fe-mediated ROS signals triggered by Pi starvation led to the inhibition of the primary root elongation, which could be rescued by decreasing ambient Fe status (Balzergue, et al., 2017, Gutierrez-Alanis, et al., 2017, Huang, et al., 2018, Mora-Macias, et al., 2017, Ward, et al., 2008, Zheng, et al., 2019). Similarly, Fe plaques on rice root surface is obviously enriched under Pi starvation (Ding, et al., 2018). In addition, Hirsch et al. found that Pi deficiency promoted the modification of Fe storage from the vacuole to the chloroplasts (Hirsch, et al., 2006). Above all, alteration of Fe homeostasis plays crucial roles in adaptive response to Pi starvation.

At molecular level, the signaling pathways of Pi-starvation response are dissected into systemic and local responses depending upon the internal Pi status in plant and the external Pi availability, respectively (Gruber, et al., 2013, Lopez-Bucio, et al., 2002, Thibaud, et al., 2010). When Pi starvation is sensed, a large set of Pi-starvation responsive genes are induced to optimize Pi uptake and internal Pi utilization efficiency under the control of the master transcriptional factors *PHR1* and *PHR1-like 1 (PHL1)* directly or indirectly (Bustos, et al., 2010, Puga, et al., 2017, Rubio, 2001). In addition, many Fe-deficiency responsive genes were also involved in the response to Pi starvation. The metal uptake and transport genes, such as *IRT1*, *FRO2*, *NAS1* and *NAS2* are downregulated in systemic responses to Pi deficiency while the repression could be relieved in mutant *phr1phl1* (Bustos, et al., 2010, Thibaud, et al., 2010), indicating that the alteration of Fe homeostasis caused by Pi starvation is regulated by the central transcriptional factor PHR1. The expression of *FERs*, which are responsible for metal storage and detoxification, is locally induced under the control of *PHR1* dependent on Pi homeostasis (Bournier, et al., 2013, Hirsch, et al., 2006, Ravet, et al., 2009, Thibaud, et al., 2010). An integrative analysis of multiple Pi-starvation and Fe-deficiency transcriptomic data exhibits that 579 different-expression genes are overlapped at both stresses. Of these, 90 genes are upregulated under Fe deficiency while downregulated under phosphate starvation in *Arabidopsis* root and 12 genes contain the P1BS cis-element in the promoter regions, which interacts with the transcription factor PHR1/PHL1 (Bustos, et al., 2010, Li and Lan, 2015). Recent research showed that the core regulatory factor OsPHR1 and the possible Fe sensors OsHRZs form a reciprocal inhibition module to coordinate Pi and iron signaling and homeostasis in rice (Guo, et al., 2021). In brief, Fe-deficiency responsive genes is clearly pointed to be involved in the systemic response to Pi deficiency at transcriptional level.

In the local response of Pi starvation, Fe also plays crucial roles in the modification of root system architecture (RSA), such as inhibiting the elongation of primary root (PR), increasing the density and length of lateral root and root hairs, ultimately to increase the scavenging ability of Pi in soil, especially topsoil (Bates and Lynch, 1996, Gonzalez, et al., 2005, Gruber, et al., 2013, Ward, et al., 2008). In several long-root mutants (*lpr1*, *lpr2*, *stop1*, *almt1*), Fe accumulation in the root apoplast and cell wall is significantly less than that in wild type under Pi deficiency. On the contrary, the mutants (*pdr2* and *als3*), whose roots are sensitive to Pi deficiency, always accumulate more Fe in the root (Balzergue, et al., 2017, Dong, et al., 2017, Mora-Macias, et al., 2017, Ticconi, 2004). More recently, the *STOP1-ALMT1* pathway, which previously was responsible for the tolerance to low pH and Al toxicity, has been documented to be

involved in Fe accumulation and redistribution, resulting in the reduction of primary root (Balzergue, et al., 2017, Hoekenga, et al., 2006, Luchi, et al., 2007, Mora-Macias, et al., 2017). The zinc finger-type transcription factor STOP1 activated the malate transporter gene *ALMT1* to exudate malate, which contributes to Fe retention into root apoplast (Godon, et al., 2019, Hirsch, et al., 2006, Hoekenga, et al., 2006, Luchi, et al., 2007). The Fe accumulation triggers the peroxidase-dependent cell wall stiffening and Fe remobilization process across the different zones of the root (Balzergue, et al., 2017, Grillet, et al., 2014, Mora-Macias, et al., 2017). In addition, sufficient Fe accumulation in nucleus promotes substantially accumulation of STOP1, which is repressed by another Al-toxicity responsive genes *ASL3/STAR1* (Dong, et al., 2017, Godon, et al., 2019, Huang, et al., 2010, Larsen, et al., 2005, Wang, et al., 2019). In root tip, the Fe-redox cycle mediated by the LPR1/2 initiates ROS, further contributing to Fe redistribution and callose deposition (Muller, et al., 2015, Svistoonoff, et al., 2007). The locally sequestration and redistribution of Fe^{3+} in root tip induced the expression of *CLE14* (Gutierrez-Alanis, et al., 2017). The secretion of peptide CLE4 is perceive by CLV2/PEPR2 to downregulate of the SHR/SCR and PIN/AUXIN pathway, which are essential for stem cell specification and maintenance in RAM (Benfey, et al., 1993, Bennett and Scheres, 2010, Di Laurenzio, et al., 1996, Gutierrez-Alanis, et al., 2017, Meng and Feldman, 2010). Concomitantly, the interaction between LPR1 and PDR2, which encodes a single P5-type ATPase, facilitates cell-specific apoplastic Fe and callose deposition, further interfering the proper expression of *SCR* and the symplastic communication of SHR (Muller, et al., 2015). Taken together, Fe over-accumulation and redistribution in root tip as well as the Fe-induced oxidative stress in the apoplast of root tips cause the arrest of root cell elongation and the exhaustion of the root apical meristem, finally leading to the inhibition of the primary root. However, the root phenotype of mutant *pdr2* under Pi starvation is independent on external Fe level (Ticconi, et al., 2009). Therefore, it remains to be explored that the alteration of Fe homeostasis causes the inhibition of the primary root triggered by Pi deprivation.

Considering the important roles of Fe nutrition in response to Pi starvation, therefore, we raised a hypothesis whether the enhancement of Fe uptake could relieve the inhibition of primary root triggered by Pi deficiency. In this work, we selected two functionally independent regulators *IMA1* and *bHLH104* in response to Fe deficiency and constructed the constitutive and inducible expression of them driven by different-type promoters of CaMV 35S as well as Pi-starvation responsive genes *IPS1* and *PHT1;4*, respectively. The effect of the upregulated expression of Fe regulators on the expression of Fe uptake genes, root length and Fe homeostasis in response to Pi starvation was investigated. The study further developed the understanding of the antagonism between P and Fe. Moreover, this approach would be a novel manipulation to modify Fe nutrient via coupling with Pi nutrient in plants.

Materials And Methods

2.1 Growth Condition

The *Arabidopsis thaliana* (L.) Heynh, ecotype Columbia (Col-0) was used in this study. The seeds were surface-sterilized using 75% alcohol for 3 min, 0.5% bleach and Tween for 10 min, washed 3-5 times with sterile ultrapure water, and stratified for 2-3 days at 4°C in the dark. Then they were sown on the standard

medium, containing 5 mM KNO₃, 2 mM MgSO₄, 2 mM Ca(NO₃)₂, 2.5 mM KH₂PO₄, 70 µM H₃BO₃, 14 µM MnCl₂, 10 µM NaCl, 1 µM ZnSO₄, 0.5 µM CuSO₄, 0.2 µM Na₂MoO₄, 40 µM Fe-EDTA, 1.0% sucrose and 0.918 g/L MES, 0.8% agar, pH 5.5 (Estelle and Somerville, 1987). And they were cultured in a growth chamber at 22°C with 16 h light / 8 h darkness photoperiod at a light intensity of 60 µmol m⁻² s⁻¹. For Pi-deficiency treatment, the KH₂PO₄ was replaced with equal quantity of KCl in standard medium. The Fe-deficiency medium was prepared by omitting the Fe-EDTA and adding 100 µM Ferrozine (Sigma, USA).

2.2 Plasmid construction and Plant Transformation

To construct the plant expression vectors, the sequence information of two promoters of P-deficiency responsive genes and two Fe-deficiency responsive genes was acquired from TAIR (The *Arabidopsis* Information Resource www.arabidopsis.org). The full-length cDNA of *IMA1* (AT1G47400) and *bHLH104* (AT4G14410) was amplified from the Fe-deficiency root RNA and cloned into SmaI and SalI sites between the CaMV 35S promoter and the NOS terminator of the binary vector pCambia2301, resulting in the constructs *pro35S::IMA1* cDNA and *pro35S::bHLH104* cDNA, respectively. With respect to the inducible expression of *IMA1* and *bHLH104*, the promoters of *IPS1* (AT3G09922) and *PHT1;4* (AT2G38940) were used to replace the CaMV 35S promoter in *pro35S::IMA1* and *pro35S::bHLH104* cDNA, respectively. The promoters of *PHT1;4* and *IPS1* were amplified in the genomic DNA that 2987bp and 1068bp upstream sequences from both genes' translation start sites, severally. All the primers used were listed in Table S1.

Then the modified binary vectors were transformed into *Agrobacterium tumefaciens* GV3101 to generate transgenic *Arabidopsis*. The T-DNA constructs were introduced into Col-0 plants via the floral dipping method as described previously (Clough and Bent, 1998).

2.3 RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufactures' instructions. 1 µg of the total RNA was used as the template to synthesize cDNA following manufactures' instructions of PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, with gDNA Eraser, Cat#RP047A). Afterwards, SYBR® Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, Cat# RR420A) was used for qRT-PCR on the Thermo PIKOREAL 96 Real-Time PCR System, with the following program: 95°C for 5 min, 95°C for 5 s, 60°C for 30 s, 40 cycles. The gene *TUA3* (*tubulin alpha-3*, At5g19770) was employed as endogenous control to normalize the expression levels of target genes. The qRT-PCR primers used in this work were listed in Table S1.

2.4 Fe Histochemical staining assay

Fe(II) distribution in plants was observed using the Perls staining as the previous described method (Roschzttardtz, et al., 2009). In brief, the seedlings were vacuum infiltrated with Perls staining solution (containing 2% (v/v) HCl and 2% (w/v) K-ferrocyanide) for 15 min and incubated for another 30 min at room temperature. Then the seedlings were rinsed with ultrapure water for 3-4 times and imaged using a Zeiss Stemi 305 microscope.

2.5 Measurement of nutrient element contents

Shoots were harvested and washed with ultrapure water for 3-4 times. The samples were dried for 2 days in a conventional oven at 70°C after being blotted with tissue papers. The dry samples were digested completely in 65% ~ 68% HNO₃ at 120°C. The measurement of mineral (Fe, Mn and Zn) nutrients was determined by inductively coupled plasma spectroscopy (ICP-MS). The total P was measured based on the method of molybdate blue-colorimetric (Murphy and Riley, 1962).

2.6 Chlorophyll content analysis

The total chlorophyll content was measured according to the method described by Arnon (Arnon, 1949). In brief, the shoots of *Arabidopsis* were collected and extracted in 80% acetone in dark until the pigments were decolorized completely. The absorbance values were recorded at the wavelength of 645 nm and 663 nm using a spectrophotometer (SpectraMax Plus 384 Absorbance Microplate Reader). Total chlorophyll content was calculated using the following formula: Chlorophyll content = $(20.2 \times A_{645} + 8.02 \times A_{663}) \times V / FW$.

Results

3.1 Fe uptake genes were differentially induced in the transgenic plants of the upregulated expression of *bHLH104* under Pi starvation

Pi deficiency disturbs Fe homeostasis in plants, such as increasing Fe accumulation, modifying Fe distribution but repressing the expression of Fe uptake-associated genes (Hirsch, et al., 2006, Thibaud, et al., 2010, Wang, et al., 2019). Recently studies revealed Fe accumulation and redistribution in root tip play important roles in remodeling the root system architecture, especially the reduced primary root under Pi deficiency in *Arabidopsis* (Balzergue, et al., 2017, Gutierrez-Alanis, et al., 2017, Mora-Macias, et al., 2017). Hence, we raised a hypothesis that whether upregulating the expression of Fe-deficiency regulatory factors could reduce Fe accumulation on the root tip via enhancing Fe uptake, further relieving the inhibition of primary root triggered by Pi deficiency. Accordingly, we selected the upstream transcriptional factor *bHLH104* in the Fe-deficiency regulatory network. And the inducible expression was constructed by combining with the promoters of Pi-starvation responsive genes *PHT1;4* and *IPS1*, which play crucial roles in Pi absorption and sensing signal of Pi homeostasis in plants, respectively. Meanwhile, we also constructed the constitutive expression by CaMV 35S. The transgenic plants were generated and three independent lines for each transgene were determined by qRT-PCR (Figure S1). Under Pi sufficiency, the expression of *bHLH104* was significantly higher in the transgenic plants of *pro35S::bHLH104*. Moreover, its expression was subtly upregulated in *proPHT1;4::bHLH104* and *proIPS1::bHLH104* compared with that in wild type. Correspondingly, the Fe uptake genes *IRT1* and *FRO2* were more highly expressed in *pro35S::bHLH104*. And the transcripts of *IRT1* were slight higher in *proIPS1::bHLH104* and *proPHT1;4::bHLH104* in comparison with that in wild type whereas the expression of *FRO2* was comparable (Table S2). The Fe regulator *IMA1* was highly induced in *pro35S::bHLH104* and

proIPS1::bHLH104 while the expression of *FIT* was only higher in *pro35S::bHLH104* than in wild type (Table S2). When 10-day-old seedlings were grown on Pi-deprived medium for 3 days, the expression of *bHLH104* was more highly upregulated in transgenic plants than in wild type (Figure S1 A-C). The expression of *IRT1*, *FRO2* and *IMA1* were higher in *pro35S::bHLH104* and *proIPS1::bHLH104* while showed no significantly in *proPHT1;4::bHLH104* compared with that in wild type under Pi starvation (Table S2). In contrast, the expression of *FIT* was only higher in *proIPS1::bHLH104* than that in wild type under Pi starvation (Table S2). Compared with that in the corresponding plants on the standard medium, in wild type, the expression of *IMA1*, *IRT1* and *FRO2* were inhibited under Pi starvation while the expression of *FIT* and *bHLH104* showed little affected by Pi deprivation (Fig. 1A). In *pro35S::bHLH104* and *proPHT1;4::bHLH104*, the expression of *IRT1*, *FRO2* and *IMA1* were inhibited under Pi starvation than that under Pi sufficiency (Fig. 1A). However, the inducible expression of *IRT1*, *FRO2* and *FIT* were successful in *proIPS1::bHLH104* compared with that under Pi sufficiency (Fig. 1A). Above all, the results indicated that the Fe-deficiency responsive genes were successfully induced by the upregulated expression of *bHLH104* via the promoter of *IPS1* while their expression was failed when *bHLH104* was driven by the promoters of *PHT1;4* under Pi starvation.

3.2 Upregulated expression of *IMA1* driven by different-types promoters universally induced the expression of Fe uptake genes

IRON MAN1 (*IMA1*), a novel member of small peptides harboring a short C-terminal amino-acid sequence consensus motif, is critical for the acquisition and cellular homeostasis of Fe as the potential hubs in the regulatory network of Fe-deficiency response (Grillet, et al., 2018, Kobayashi, 2019). Therefore, we sequentially constructed the constitutive and inducible expression of *IMA1* to further explore the effect of Pi starvation on the expression of Fe uptake genes. Consistently, the expression of Fe uptake genes *FIT*, *IRT1* and *FRO2* was significantly higher in *pro35S::IMA1* than in wild type, regardless of Pi supply, although their expression of them was decreased under Pi starvation compared with that in the corresponding plants under Pi sufficiency (Figure 1B, Table S2). In the transgenic plants of the inducible expression of *IMA1*, the expression of *IRT1* and *FRO2* was no significant difference under Pi sufficiency while significantly upregulated under Pi starvation, compared with that in wild type. The expression of *FIT* was successfully induced in *proPHT1;4::IMA1* while was slight decreased in *proIPS1::IMA1* under Pi starvation in comparison with that in the corresponding plants under Pi sufficiency (Figure 1B). Combined with the results above, we found that the patterns of the expression of Fe uptake-associated genes were various in the inducible expression of *IMA1* and *bHLH104*. As shown in Figure 1C, the expression of Fe uptake genes also showed significant difference among the transgenic plants of the upregulated expression of Fe regulators *bHLH104* and *IMA1*. The Fe uptake genes were successfully induced in *proIPS1::bHLH104* and *proPHT1;4::IMA1* while their expression were inhibited in *proPHT1;4:: bHLH104*. Nevertheless, the expression of these genes also showed dramatically difference among these transgenic plants. Of these, the relative expression (-P treatment / +P treatment) of *FIT* and *FRO2* was highest in *proIPS1::bHLH104* while the *IRT1* only showed the strongest induction in *proPHT1;4::IMA1*. In contrast, the expression of them was severely inhibited in *proPHT1;4:: bHLH104*. With respect to the different Fe regulators, the expression of *FIT* was more significantly induced in the transgenic plants of the

upregulated expression of *bHLH104* than that in the inducible expression of *IMA1*. On the contrary, the expression of *IRT1* was more highly induced in *proPHT1;4::IMA1* and *proIPS1::IMA1* than *proIPS1::bHLH104*. The expression of *FRO2* was comparable in *proPHT1;4::IMA1* and *proIPS1::bHLH104*. The analysis of two-way ANOVA also revealed that the expression of *FIT* in *proIPS1::bHLH104* showed significant higher than that in *proPHT1;4::bHLH104* and *proIPS1::IMA1* while no difference with that in *proPHT1;4::IMA1*. Nevertheless, its expression were comparable in the transgenic plants of the inducible expression of *IMA1*. In contrast, the expression of *IRT1* displayed obvious dependence on the promoters. Its expression was significantly different between *proPHT1;4::bHLH104* and *proPHT1;4::IMA1* while the effect was little when they were induced under the control of *proIPS1*. The expression of *FRO2* dramatically affected by the different promoters in the transgenic plants of the upregulated expression of *bHLH104*. Taken together, the expression of Fe uptake genes was differentially induced in the transgenic plants of the inducible expression of *IMA1* and *bHLH104* driven by the different promoters of Pi-starvation responsive genes (Figure 1D).

3.3 Effect of upregulated expression of *IMA1* and *bHLH104* on the root length and Fe distribution under Pi starvation

In view of the significantly differential expression of the genes associated with Fe uptake in transgenic plants under Pi starvation, therefore, we evaluated the effect of Pi starvation on the root elongation and Fe distribution in transgenic plants. When 7-day-old seedlings were grown on Pi-starvation medium for 7 days, the root length and biomass were significantly reduced compared with that on Pi-sufficiency medium. However, the root length in transgenic plants showed no significant difference with that in wild type, regardless of Pi supply (Figure 2A-B, S2 A-D, S3, S4). Sequentially, we further observed the distribution of Fe in root via Fe histochemical staining based on Perls staining under Pi starvation. Although more blue Fe signals were observed in root tips of plants under Pi deficiency than under Pi sufficiency, there was no significant difference between wild type and transgenic plants (Figure 2C-D, S2 E-H). Above all, the results indicated that changing the expression of Fe uptake-associated genes was failed to rescue the inhibition of the primary root triggered by Pi starvation.

3.4 Upregulated expression of *bHLH104* and *IMA1* via Pi-starvation responsive promoters affects Fe distribution in shoots

To further investigate the effect of Pi deprivation on Fe homeostasis, we sequentially analyzed the Fe distribution in shoots of the upregulated expression of *bHLH104* and *IMA1*. Under Pi sufficiency, the results of histochemical staining of Fe (Fe²⁺) displayed that visibly blue signals were observed in the overexpression of *bHLH104* and *IMA1* under Pi sufficiency while not observed in the shoots of transgenic plant of the inducible expression as well as wild type. When grown on the Pi-deficiency medium, a few blue Fe signals were observed at the edge of shoots in wild type (Figure 3A-D and S6 A-B). In contrast, the obviously more blue Fe plaques were aggregated in the shoots of *pro35S::bHLH104*, *pro35S::IMA1*, *proIPS1::bHLH104*, *proPHT1;4::IMA1* and *proIPS1::IMA1* than that in wild type while scarcely any blue Fe signals were observed in *proPHT1;4::bHLH104* (Figure 3A-D and S6 A-B). Nevertheless, the blue Fe

plaques displayed significant differences between the upregulated expression of *bHLH104* and *IMA1*. For instance, in the shoots of transgenic plants related to *bHLH104*, the blue Fe plaques mainly enriched at the edge of leaves while the blue Fe signals were filled in the leaf vein of the whole shoots in the upregulated expression of *IMA1* (Figure 3A and C, S6 A). Therefore, we further measured the Fe contents in shoots (Fig. 3A-D and S6 C-D). Under Pi sufficiency, the Fe contents were 24.9%~83.9% and 23.2% ~ 82.2% greater in *pro35S::bHLH104* and *pro35S::IMA1* than that in wild type, respectively, while no significant difference in transgenic plants of the inducible expression of them (Figure S6 C-D). As shown in Fig. 3 and S6, Pi starvation significantly triggers the increase of the Fe contents in *Arabidopsis*. In comparison with that in wild type under Pi starvation, nevertheless, the Fe contents were increased by 20.2% ~ 58.4%, 118.0% ~ 134.3% and 45.7% ~ 106.1% in *proIPS1::bHLH104*, *proPHT1;4::IMA1* and *proIPS1::IMA1*, respectively, while Fe content was decreased 16.0% ~ 45.1% in *proPHT1;4::bHLH104* (Figure 3E-H). Despite the different accumulation of Fe between them, the biomass of shoots showed no significant difference between transgenic plants and wild type whether under Pi sufficiency or Pi deficiency (Figure S5 B, D and F). Considering the significant different Fe accumulation in transgenic plants under Pi starvation. Thus, we sequentially evaluated the chlorophyll contents when 5-day-old seedlings were grown on the medium with Pi (+P) or without Pi (-P) for 3 days and then they were transferred to the medium with Fe (+Fe) or without Fe (-Fe) for another 7 days (Fig. 4). Under +P+Fe and -P+Fe treatments, no significant difference was observed between transgenic plants and wild type. Under +P-Fe, in contrast, the chlorophyll content was 40.0% ~ 83.8%, 13.3% ~ 32.8%, 24.3% ~ 91.8% and 30.0% ~ 52.9% greater in *pro35S::bHLH104*, *pro35S::bHLH104*, *pro35S::IMA1* and *proPHT1;4::IMA1*, respectively, while showed no significant difference in *proPHT1;4::bHLH104* and *proIPS1::IMA1*, compared with that in wild type. When the Pi-starvation seedlings were transferred to Fe-deficiency medium, the chlorophyll content was 34.8% greater in wild type than that under +P-Fe conditions. Nevertheless, the chlorophyll contents in *pro35S::bHLH104*, *pro35S::bHLH104*, *pro35S::IMA1* and *proPHT1;4::IMA1* was still higher than that in wild type under -P-Fe conditions. The chlorophyll contents were subtly increased in *proPHT1;4::IMA1* while showed reduced tendency in *proPHT1;4::bHLH104* in comparison with that in wild type under -P-Fe conditions (Fig. 4). Nevertheless, the Pi contents were no significant difference between wild type and the transgenic plants (Fig S7). Above all, the upregulated expression of *IMA1* and *bHLH104* driven by the promoters of Pi-starvation responsive genes *PHT1;4* and *IPS1* exhibited the different Fe distribution and Fe contents in shoots of *Arabidopsis*. The results also showed that these manipulation approaches would be novel to regulate the Fe nutrient in plants based on different purposes.

3.5 Effect of upregulated expression of *IMA1* and *bHLH104* on the contents of Zn and Mn

Given that Fe contents showed significant differences among the transgenic plants of the constitutive and inducible expression of *IMA1* and *bHLH104*, we further assessed the effect on the content of other micronutrients, such as Mn and Zn. As shown in Table 1, the contents of Mn and Zn were significantly increased in the transgenic plants of the overexpression of *IMA1* under Pi sufficiency while there was no significant difference between the inducible expression of *IMA1* and the wide type. Phosphate starvation triggered the Zn accumulation while decreased Mn content in wild type. Moreover, Zn contents were increased by 19.5% ~ 32.6% and 7.7% ~ 22.3% and 20.1% ~ 33.1% in *pro35S::IMA1*, *proIPS1::IMA1* and

proPHT1;4::IMA1 under Pi starvation, respectively. However, Zn contents were universally decreased in the transgenic plants of the upregulated expression of *bHLH104*, especially in *proIPS1::bHLH104*, where Zn contents were 18.5% ~24.9% less than wild type under Pi starvation. Mn contents showed the similar tendency with Fe contents in transgenic plants. The Mn contents were subtly increased in the transgenic plants in the upregulated expression of *IMA1* while decreased in that of *bHLH104*. Above all, the upregulated expression of *IMA1* obviously increased the content Zn and Mn while the upregulated expression of *bHLH104* didn't. Combined with the Fe distribution and accumulation, therefore, the upregulated expression of *IMA1* displayed more extensive function in regulation of micronutrients (Fe, Mn, Zn) while *bHLH104* just had tissue-specificity expression in the regulation of Fe homeostasis.

TABLE 1. The effect of Pi starvation on the Mn and Zn contents in the transgenic plants of the upregulated expression of Fe regulators *IMA1* and *bHLH104*.

		+P		-P	
		Zn (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	Mn (mg/kg)
Col-0		52.09±1.49	95.58±8.75	56.64±2.16	66.96±1.63
<i>pro35S::IMA1</i>	#9-2	58.54±2.63 *	105.46±19.43	68.14±5.32 *	78.71±8.19
	#13-8	60.97±1.88 **	153.84±30.03	67.67±2.44 **	80.23±18.07
	#19-1	59.1±1.96 *	153.47±31.7	75.11±3.96 **	91.09±12.77
<i>proPHT1;4::IMA1</i>	#2-10	47.95±2.1	104.99±11.83	75.39±8.48 *	75.33±4.53
	#3-2	56.23±1.82	123.35±20.11	71.38±3.00 **	74.93±7.35
	#5-2	50.85±1.52	104.99±8.58	68.03±3.73 **	72.85±11.17
<i>proIPS1::IMA1</i>	#20-3	55.34±2.5	102.13±8.27	64.27±5.72	74.99±4.25
	#27-2	52.11±1.75	117.86±5.78 *	69.28±1.97 **	80.32±2.85 *
	#31-2	51.33±3.92	103.21±7.81	60.99±6.53	76.86±5.32
<i>pro35S::bHLH104</i>	#16-5	67.35±7.69	103.6±11.07	62.18±3.6	67.23±9.49
	#18-8	56.22±5.83	102.18±2.49	53.29±1.67	61.14±9.76
	#20-5	51.01±5.33	89.96±7.95	47.51±3.88	60.34±11.48
<i>proPHT1;4::bHLH104</i>	#2-8	44.71±6.09	70.83±2.1 *	37.36±3.35 **	51.26±7.69
	#8-7	40.14±4.5 *	69.49±7.31 *	37.96±3.12 **	48.1±8.31 *
	#11-2	37.36±2.04 **	71.29±4.86 *	37.3±6.79 **	51.37±9.59 *
<i>proIPS1::bHLH104</i>	#12-5	40.91±3.64 *	77.91±5.14	52.09±3.89	62.62±10.11
	#19-6	42.46±2.88 *	80.65±3.75	46.82±3.41	57.41±10.84
	#22-6	39.12±0.90 **	73.48±2.92 *	44.82±1.81 *	54.91±5.44 *

Note: The micronutrient contents were measured when the 10-day-old seedlings were grown on Pi-starvation medium for 7 days. Values were means ± SD of 3 biologic replicates. “*” and “**” indicate the significant difference between transgenic plants and wild type at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Discussion

Phosphorus (P) and iron (Fe) are essential mineral elements for plant growth and development. Pi deficiency increased Fe homeostasis in plants while represses the expression of Fe uptake-associated genes (Abel, 2011, Hirsch, et al., 2006, Ward, et al., 2008). Recent reports indicated that Fe over-accumulation and redistribution on root surface result in the inhibition of the primary root in *Arabidopsis* under Pi starvation (Balzergue, et al., 2017, Gutierrez-Alanis, et al., 2017, Mora-Macias, et al., 2017, Wang,

et al., 2019). Moreover, the inhibition was alleviated by decreasing exogenous Fe concentration (Huang, et al., 2018, Ward, et al., 2008). In brief, Fe plays crucial roles in the systemic and local response to Pi starvation in plants. Hence, we wondered whether the upregulated expression of Fe uptake genes could reduce Fe accumulation on the root surface, further relieving the inhibition of primary root under Pi starvation. Sequentially, we constructed the inducible expression of *bHLH104* and *IMA1* driven by the promoters of Pi starvation responsive genes *IPS1* and *PHT1;4* as well as the constitutive expression of them via the *CaMV 35S* promoter. Of these, *bHLH104* is a key transcription factor in the regulatory network of Fe deficiency, while *IMA1* is characterized as a long-distance interorgan signal involved in the uptake, translocation and cellular homeostasis of Fe in a wide range of plants. And both of them positively activated the expression of Fe-uptake system of *FIT-FRO2/IRT1*. (Grillet, et al., 2018, Kobayashi, 2019, Li, et al., 2016, Zhang, et al., 2015). In this work, we found that the expression of *FRO2* and *IRT1* was significantly inhibited under Pi starvation in wild type, consistent with previous reports (Fig S1). Moreover, in *pro35S::bHLH104* and *pro35S::IMA1*, their expression showed significantly decreased under Pi starvation than that under Pi sufficiency although they were more highly expressed than that in the wild type, regardless of Pi supply (Figure 1A-B, S1 C and F). In the transgenic plants of the inducible expression of Fe regulators, the expression of *bHLH104* and *IMA1* was successfully induced under Pi starvation, respectively (Figure S1A-B, C-D). Nevertheless, Fe uptake genes *IRT1* and *FRO2* were differentially expressed in the transgenic plants. In *proIPS1::bHLH104*, the expression of *FIT*, *FRO2* and *IRT1* was highly induced under Pi starvation while failed in *proPHT1;4::bHLH104* (Figure 1A, Table S2), indicating that the regulatory expression of Fe uptake genes under the control of *bHLH104* significantly depended on the promoters under Pi starvation. Previous research displayed that the primary expression patterns of *bHLH104* were localized in the pericycle of the root maturation zone and veins of leaves maturation zone and in rosette leaves, cauline leaves, stems, and siliques but not in flowers (Zhang, et al., 2015). The expression of *IPS1* was mainly delimited by the endodermis of the root while *PHT1;4*, as a high affinity transporter, was predominantly induced in the epidermis, including the cortex and the root cap of root (Martin, et al., 2000, Misson, et al., 2004). Therefore, the higher induction of Fe uptake genes in *proIPS1::bHLH104* seems to benefit from the functional overlap of *IPS1* and *bHLH104*. However, the ectopic expression of *bHLH104* might impair its precise function to control the expression of Fe uptake genes driven by *proPHT1;4*. Moreover, the results also sustained by recent research that the inducible expression of *bHLH104* under control of *MYB72* promoter enhanced the tolerance to Fe deficiency while the sensitivity to Fe deficiency was elevated when *bHLH104* was driven by the promoter of *IRT1* (Wang, et al., 2017). Similarly, *MYB72* and *IRT1* were strongly expressed in the root stele and in the root epidermis, respectively (Vert et al., 2002; Palmer et al., 2013). Therefore, the regulation of *bHLH104* might exist tissue specificity in the Fe-deficiency regulatory network. In contrast, the expression of *FRO2* and *IRT1* was universally upregulated in the transgenic plants of the inducible expression of *IMA1* (Figure 1B, Table S2), indicating that the upregulated expression of *IMA1* displayed extensive regulation in Fe uptake. Recent research also displayed that *IMA1* have more broad effects functionally as the potential hubs in the regulatory network of Fe-deficiency response due to the extensive expression in companion cells and circulate in the sieve elements throughout the plant (Grillet, et al., 2018). Further investigation showed that the expression of Fe uptake genes were significant difference in the transgenic plants of the

inducible expression of *bHLH104* and *IMA1* driven by different promoters (Figure 1C-D). *IRT1* were more strongly expressed in the upregulated expression of *IMA1* while *FIT* and *FRO2* was more highly expressed in the transgenic plants of the upregulated expression of *bHLH104*, indicating that *IMA1* might predominantly function in the direct regulation of Fe uptake while *bHLH104* was mainly responsible for the regulation of *FIT* (Figure 2C). Recently researches also illustrated that the *IMAs* genes, as a potential hubs of Fe-deficiency response, can directly activate *bHLH39* independent on *FIT* while *bHLH104* is able to regulate the expression of *FIT* by directly combining with the promoter of *FIT* (Hirayama, et al., 2018, Kobayashi, 2019, Zhang, et al., 2015). The two-way ANOVA also revealed that the *IPS1* and *PHT1;4* promoters displayed significantly distinct regulation to the expression of Fe uptake-associated genes in the transgenic plants of the inducible expression of *IMA1* and *bHLH104* (Figure 2D).

Considering that the dramatically various expression of the genes responsible for Fe uptake in the inducible expression of *IMA1* and *bHLH104*, therefore, we further evaluated the effect on the upregulated expression of *IMA1* and *bHLH104* in response to Pi starvation. Unfortunately, the root length as well as the biomass was no difference between wild type and transgenic plants, regardless of Pi-sufficiency or -starvation conditions (Figure 3C and D, Figure S2 E-H, Figure S4 and Figure S5). The Fe distribution and accumulation in roots depending on perls staining also showed no significant difference between wild type and transgenic plants although obviously blue Fe signals on the surface of roots were observed under Pi starvation compared with that under Pi sufficiency (Figure 3A and B, Figure S2 A-D). Therefore, the results suggested that only enhancing the expression of Fe uptake genes was failure to reduce the Fe accumulation on the root surface and rescue the inhibition of primary root under Pi starvation. Fe accumulation and redistribution in root primarily result from the Fe efflux mediated by the module of *STOP1-ALMT1* (Balzergue, et al., 2017, Mora-Macias, et al., 2017). In this process, Fe-dependent redox reaction probably play crucial roles in the exhaustion of root apical meristem and cell wall stiffening, ultimately inhibiting the primary root triggered by Pi deficiency (Balzergue, et al., 2017, Muller, et al., 2015). More recent report showed that blue light-triggered malate-mediated photo-Fenton reaction also was critical in the inhibition of primary root under phosphate deficiency (Zheng, et al., 2019). Therefore, Fe-dependent ROS signaling triggered by low phosphate availability might play crucial roles in mediating the inhibition of primary root, resulting in Fe overaccumulation. The sufficient Fe in plants is essential for the accumulation of transcription factor STOP1 in the nucleus and further upregulated the expression of *ALMT1* under Pi starvation (Balzergue, et al., 2017, Godon, et al., 2019, Mora-Macias, et al., 2017). Therefore, our results indicated that this regulatory modulation of the reduced primary roots under Pi starvation might be independent on the regulatory network of Fe deficiency. Recent research exhibited that the central regulators of Pi starvation OsPHR2 and master regulators of Fe deficiency OsHRZs form a reciprocal inhibition module, further remolding Pi and iron signaling and homeostasis in rice (Guo, et al., 2021). The research indicated that the regulatory network of Fe deficiency also play critical roles in systemic response to Pi starvation.

Although the blue Fe plaques in roots showed no significant difference between wild type and transgenic plants, in this study, we observed that the obvious blue Fe plaques were largely enriched in the shoot of transgenic plants (except for *proPHT1;4::bHLH104*) compared with the wild type plants under Pi

starvation (Figure 3A-D and Figure S5 A-B). Consistently, the Fe concentrations in shoots of them displayed the similar tendency with Fe distribution in them (Figure 3E-H and Figure S5 C-D). Nevertheless, the distribution of blue Fe plaques in shoots displayed visibly difference between the transgenic plants of the upregulated expression of two Fe regulators. The blue Fe signals appeared in the whole leaf veins in the upregulated expression of *IMA1* while the blue plaques were mainly enriched at the edge of the leaf in *pro35S::bHLH104* and *proIPS1::bHLH104* (Figure 3B-D and Figure S5 A-B). However, no significant blue Fe signal was observed in the shoots of *proPHT1;4::bHLH104* (Figure 3A). Correspondingly, the Fe contents were decreased in *proPHT1;4::bHLH104* while increased in *proIPS1::bHLH104* compared with wild type, further sustaining that the ectopic expression of *bHLH104* impaired Fe homeostasis in Arabidopsis (Figure 3E and G). Unexpectedly, the Fe contents in shoot were greater in *pro35S::bHLH104* under Pi sufficiency while no significant difference under Pi sufficiency compared with that in wild type (Figure S5 C). In contrast, the Fe contents were significantly higher in the upregulated expression of *IMA1* than in wild type and in the transgenic plants related to *bHLH104* (Figure 4 ,S5). The results showed consistent with the expression of Fe uptake genes, suggesting that *IMA1* and *bHLH104* might had the different regulatory mechanism to the Fe-deficiency response (Figure 1). Previous researches reported that the expression of *bHLH104* was not affected by Fe deficiency while the activity of the transcription factor was controlled by BTS dependent on Fe homeostasis at post-transcriptional levels (Hindt, et al., 2017, Selote, et al., 2015). High Fe contents might contribute to the degradation of bHLH104. In contrast, *IMA1* is positively induced in the early stage of Fe deficiency in companion cells and circulate in the sieve elements throughout the plant although the regulatory mechanism is still unclear (Grillet, et al., 2018). Recently, a research reported that IMAs can compete with bHLH105 and bHLH115 for the interaction with the negative regulator BTS to elevate the Fe-deficiency response (Li, et al., 2021). Therefore, *IMAs* played more broad roles in Fe-deficiency regulation than bHLH104. Above all, the inducible expression of *bHLH104* and *IMA1* driven by different-type promoter significantly altered the Fe distribution and accumulation under Pi starvation. Furthermore, the chlorophyll concentrations also displayed the consistent tendency in them when Pi-starved seedlings were transferred to Fe-deficiency medium (Figure 4). The above results indicated that the approaches were succeed in manipulating Fe homeostasis and the tolerance to Fe deficiency.

In order to improve Fe nutrient, lots of efforts have been done through upregulating expression of Fe-associated genes. Masuda et al. induced the expression of the genes related to Fe storage protein ferritin (*FERs*) and Fe(II)-nicotianamine transporter (*OsYSL2*) via combining with endosperm-specific promoters (*OsGluB1*, *OsGlb1*) or sucrose transporter promoter (*OsSUT1*), which significantly increased the Fe accumulation in rice seeds (Masuda, et al., 2013). However, it was less reported that the improvement of Fe nutrient by coupling the interaction between different nutrients. P immobility and high sorption capacity often results in the formation of Pi depletion zone in the rhizosphere (Lewis and Quirk, 1967). Moreover, the previous reports also indicated that overexpression of *IMA1* and the inducible expression of *bHLH104* via *MYB72* promoter can dramatically increase the Fe content in seed of transgenic plants and the tolerance to Fe deficiency in alkaline soil (Grillet, et al., 2018, Wang, et al., 2017). In agroecosystem, Pi fluctuation often happens due to the easy sorption and insolubility of P, resulting in the crops have to

constantly forage Pi by the regulation of Pi uptake system. To acclimate to Pi deficiency, moreover, plants often increase the Fe retention on root surface while repress the expression of the genes responsible for Fe uptake (Ding, et al., 2018, Gutierrez-Alanis, et al., 2018, Hirsch, et al., 2006). Therefore, it would be an effective strategy to increase Fe nutrient by coupling with the regulation of Pi homeostasis. In this work, the inducible expression of *IMA1* and *bHLH104* significantly increased the Fe accumulation in shoots. In addition, the biomass and total P contents had no significant difference between wild type and transgenic plants under Pi starvation (Figure S5 and S7). However, the expression of Fe uptake-associated genes in root and the Fe contents in shoot were decreased significantly in *proPHT1;4::bHLH104* (Figure 1A, 3A and E). Altogether, induced expression of Fe-deficiency responsive genes can change the Fe distribution and accumulation via coupling with Pi starvation. Therefore, it also provided a potential strategy to manipulate Fe nutrient via the tissue-specific expression of Fe-deficiency responsive genes.

Conclusion

The present study indicated that the upregulated expression of Fe-deficiency regulatory factors *IMA1* and *bHLH104* via the promoters of Pi-starvation responsive genes *PHT1;4* and *IPS1* successfully induced the expression of Fe uptake associated genes *IRT1/FRO2* under Pi starvation, except for *proPHT1;4::bHLH104*, in which, they were repressed. The alteration of the expression of Fe uptake genes in Arabidopsis showed little effect on the root length and the Fe distribution in roots. However, the upregulated expression of Fe regulators obviously affected the Fe homeostasis in shoots of the transgenic plants. And the chlorophyll contents showed the similar tendency when Pi-starved seedlings were transferred to Fe-deprivation medium. The present study provided a novel strategy to manipulate Fe nutrient via specifically induced Fe-deficiency regulatory factors.

Abbreviations

ALMT1	ALUMINUM-ACTIVATED MALATE TRANS-PORTER1
ASL3	ALUMINUM SENSI-TIVE3
BTS	BRUTUS
CLE14	CLAVATA3/ENDOSPERM SURROUNDING REGION
FIT	FER-LIKE REGULATOR OF IRON UPTAKE
FRO2	FERRIC REDUCTION OXIDASE 2
HRZ	Hemerythrin motif-containing Really Interesting New Gene- and Zinc-finger proteins
IMA	IRON MAN
IRT1	IRON TRANSPORTER 1
MCOs	Multicopper oxidases
NAS	NAS NICOTIANAMINE SYNTHASE
PEPR2	CLV2/PEP1 RECEPTOR 2
PHR1	PHOSPHATE STARVATION RESPONSE 1
SCR	SCARE-CROW
SHR	SHORT ROOT
STAR1	SENSITIVE TO ALUMINUM RHIZOTOXICITY1
STOP1	SENSITIVE TO PROTON RHIZOTOXICITY1
FER1	Ferritin1
RAM	Root Apical Meristem

Declarations

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Author Contributions

Ping Lan conceived and supervised the study; Xiangxiang Meng performed the experiments, analyzed the data and drafted the manuscript; Wenfeng Li and Renfang Shen provided infrastructure and polished the writing.

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Figures

Figure 1

The effect of Pi starvation on the expression of the Fe uptake genes in the upregulated expression of *bHLH104* and *IMA1*. (A) The heatmap of the relative expression of the Fe uptake genes in the transgenic plants of *bHLH104* driven by different-type promoters. (B) The heatmap of the relative expression of the Fe uptake genes in the transgenic plants of *IMA1* driven by different-type promoters. (C) The different expression of Fe uptake genes in the transgenic plants of the inducible expression of *bHLH104* and *IMA1*. (D) The two-way ANOVA analysis of the expression of Fe uptake genes in the transgenic plants of the inducible expression of *bHLH104* and *IMA1*. The RNA was extracted in the root when 10-day-old seedlings were grown Pi-sufficiency or -deficiency medium for 3 days. (A-B) The data (Log2FC) in the heatmaps were calculated from 3 biological replicates. Expression fold change (-P/+P) FC = 2Log2FC.

The asterisks indicate the significant difference between the transgenic plants and wild type by Student's t-test (* $p < 0.05$, ** $p < 0.01$). The expression fold change (FC) were analyzed by two-way ANOVA analysis by Duncan's multiple range. (C) The data was showed mean $\pm$ SE calculated from 3 biological replicates. (D) The different letters indicate the significantly difference among different transgenic plants ($P < 0.05$); an asterisk indicates there was a significant interaction between promoter and transgene in the effect on the expression of a gene ($P < 0.05$).

Figure 2

The effect of Pi starvation on the length and Fe (Fe) distribution of roots in wild type and transgenic plants. (A-B) The root length. (C-D) The perls staining in roots, bar = 1 mm. The root length and Perls staining were analyzed when 7-day-old seedling were grown on the Pi-starvation medium for 7 days. Bars in the graph showed mean $\pm$ SE calculated from 3 biological replicates. The asterisks indicate the significant difference between the transgenic plants and wild type by Student's t-test (* $p < 0.05$, ** $p < 0.01$).

Figure 3

The effect of Pi starvation on the distribution of Fe (⊠) and total Fe contents in shoots of the transgenic plants of the inducible expression of bHLH104 and IMA1. (A-D) The perls staining in shoots, bar = 1 mm. (E-H) The total Fe content. (A-D) Perls staining were performed when 7-day-old seedling were grown on the Pi-starvation medium for 7 days. (E-H) Fe contents were measured when 10-day-old seedling were grown on the Pi-starvation medium for 7 days. Bars in the graph show mean ± SD calculated from 3 biological replicates. The asterisks above the bars indicate statistically significant difference between transgenic lines and wild type by Student's t-test (* p < 0.05, ** p < 0.01).

Figure 4

The effect of Pi starvation on chlorophyll concentrations in the transgenic plants of the upregulated expression of bHLH104 and IMA1. The chlorophyll contents were analyzed when the 5-day-old seedlings were first grown on the medium with / without Pi for 3 days and then transferred to the medium with / without Fe for 7 days, respectively (+P+Fe, +P-Fe, -P+Fe, -P-Fe). Bars in the graph show mean ± SD calculated from 3 biological replicates. The asterisks above the bars indicate statistically significantly difference between transgenic lines and wild type by Student's t-test (* p < 0.05, ** p < 0.01).

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