FE UPTAKE INDUCING PEPTIDE1 maintains Fe translocation by controlling Fe deficiency response genes in the vascular tissue of Arabidopsis

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Abstract

FE UPTAKE INDUCING PEPTIDE1 (FEP1), also named IRON MAN3 (IMA3) is a short peptide involved in the iron deficiency response in *Arabidopsis thaliana*. Recent studies uncovered its molecular function, but its physiological function in the systemic Fe response is not fully understood. To explore the physiological function of FEP1 in iron homeostasis, we performed a transcriptome analysis using the *FEP1* loss-of-function mutant *fep1-1* and a transgenic line with estrogen-inducible expression of *FEP1*. We determined that FEP1 specifically regulates several iron deficiency-responsive genes, indicating that FEP1 participates in iron translocation rather than iron uptake in roots. The iron concentration in xylem sap under iron-deficient conditions was lower in the *fep1-1* mutant and higher in *FEP1*-induced transgenic plants compared to the wild type. Perls staining revealed a greater accumulation of iron in the cortex of *fep1-1* roots than in the wild-type root cortex, although total iron levels in roots were comparable in the two genotypes. Moreover, the *fep1-1* mutation partially suppressed the iron overaccumulation phenotype in the leaves of the *oligopeptide transporter3-2* (*opt3-2*) mutant. These data suggest that FEP1 plays a pivotal role in iron movement and in maintaining the iron quota in vascular tissues in Arabidopsis.

1 Introduction

Iron (Fe) is an essential element for plant growth and development and is required for the activity of many enzymes and sensor proteins intrinsic to all organisms (Marschner, 2012). Although Fe is abundant in the soil, most of it exists as poorly bioavailable ferric Fe (Fe^{3+}), especially in aerobic and neutral- or higher-pH environments. Fe deficiency is a major problem in many crops, especially those cultivated in alkaline soils, causing chlorosis, lower growth rates, poor yields, and inferior quality. Plants have developed two elaborate mechanisms to acquire Fe from the soil and maintain proper endogenous Fe levels (strategy I and II), which are used by different species (Kobayashi and Nishizawa, 2012; Kobayashi, 2019; Grillet and Schmidt, 2019). Plants adapt to limited Fe bioavailability in the soil by activating Fe deficiency-responsive genes (Rodríguez-Celma et al., 2013; Gao et al., 2019).

During the strategy I Fe deficiency response in Arabidopsis (*Arabidopsis thaliana*), the expression of several genes encoding basic helix-loop-helix (bHLH)-type transcription factors of the Ib subfamily is induced, such as *bHLH38* and *bHLH39* (Wang et al., 2007; Yuan et al., 2008; Sivitz et al., 2012). These Ib bHLH transcription factors, along with the bHLH protein FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT), cooperatively activate *FERRIC REDUCTION OXIDASE2* (*FRO2*) and *IRON-REGULATED TRANSPORTER1* (*IRT1*) transcription to facilitate Fe uptake from the soil (Vert et al., 2002; Connolly et al., 2003). Ib *bHLH* gene expression is itself regulated by bHLH transcription factors belonging to the IVc subfamily, such as bHLH105 (also named IAA-LEUCINE RESISTANT3 [ILR3]) and bHLH115 (Li et al., 2016; Liang et al., 2017; Tissot et al., 2019), which function negatively or positively by forming heterodimers with the bHLH protein POPEYE (PYE) (Long et al., 2010; Selote et al., 2015; Kobayashi, 2019) or UPSTREAM REGULATOR OF IRT1 (URI)/bHLH121 (Kim et al., 2019; Gao et al., 2020), respectively. The E3 ligase BRUTUS (BTS) downregulates the activity of these IVc bHLH transcription factors by promoting their ubiquitin-dependent degradation (Long et al., 2010; Selote et al., 2015). The hemerythrin motif-like domain of BTS has Fe-binding activity and modulates its E3-ligase activity as a function of Fe status (Kobayashi et al., 2013; Selote et al., 2015; Rodríguez-Celma et al., 2019). Based on these findings, a cellular regulatory system for Fe deficiency–responsive genes has been proposed (Brumbarova et al., 2015; Kobayashi, 2019; Kroh and Pilon, 2019; Gao and Dubos, 2021).

FE UPTAKE INDUCING PEPTIDEs (FEPs)/IRON MANs (IMAs) are short peptides (~50 amino acids [aa]) that play pivotal roles in the Fe deficiency response in Arabidopsis (Hirayama et al., 2018; Grillet et al., 2018). The genes encoding these peptides are expressed mainly in the vascular tissues under Fe deficiency conditions. The Arabidopsis genome harbors at least six *FEP/IMA* genes. FEP1 has a unique amino acid sequence, and loss of FEP1 function has a clear chlorosis phenotype (Hirayama et al., 2018), suggesting

that FEP1 holds a specific function among FEP/IMA peptides. *FEP/IMA* overexpression activates the transcription of Ib *bHLH* genes and induces Fe accumulation in the roots and shoots. Fe accumulates to lower levels specifically in the leaves of the *fep1* loss-of-function mutant and not in the roots, suggesting that FEP1/IMA3 is involved in the systemic Fe-deficiency response (Hirayama et al., 2018). This hypothesis is supported by grafting experiments between wild-type (WT), *IMA1*-overexpressing, and *ima8x* mutant plants (Grillet et al., 2018). Many vascular plants have FEP/IMA peptides (Hirayama et al., 2018; Grillet et al., 2018). *FEP/IMA* homologs in rice (*Oryza sativa*) play roles similar to their Arabidopsis counterparts (Kobayashi et al., 2021). A recent study proposed a model for FEP/IMA-mediated activation of Fe deficiency-responsive genes (Li et al., 2021). According to this model, FEP/IMA peptides stabilize IVc bHLH transcription factors by competing for physical association with BTS (which degrades both FEP/IMA peptides and IVc bHLH transcription factors). One of the IVc bHLH transcription factors, bHLH115, induces *FEP1/IMA3* and *BTS* transcription, thus constituting a positive and a negative feedback loop to maintain Fe homeostasis (Li et al., 2021). In addition, FEP3/IMA1 interferes with the interaction between bHLH IVc TFs and BTS LIKE1 or BTS LIKE2, two BTS homologs that negatively regulate Fe uptake in roots (Hindt et al., 2017; Lichtblau et al., 2022). According to these studies, it is likely that Fe deficiency in shoots induces FEP/IMA peptides that move to roots and inhibit BTS/BTSLs, activating Fe deficiency–responsive genes in roots. These studies offered clues to understanding the molecular function of these peptides at the cellular level. However, the physiological roles of the FEP/IMA peptides at the tissue or organ levels, and in the systemic Fe deficiency response are still obscure. The systemic regulation of Fe homeostasis involves communication between roots and leaves, to control Fe uptake from the soil by roots and its incorporation into photosynthetic or respiratory enzymes in leaves. Fe transporters such as FERRIC REDUCTASE DEFECTIVE3 (FRD3), YELLOW STRIPE-LIKE (YSL) family members, IRON REGULATED 1 (IREG1)/FERROPORTIN1 (FPN1) are involved in Fe translocation between organs and tissues (Green and Rogers, 2004; Waters et al., 2006; Morrissey et al., 2009; Gayomba et al., 2015; Zhang et al., 2019). OLIGOPEPTIDE TRANSPORTER3 (OPT3), another putative Fe transporter, plays a pivotal role in Fe movement from the xylem to the phloem as well as in the systemic Fe deficiency response (Zhai et al., 2014; Mendoza-Cózatl et al., 2014; Khan et al., 2018). The knockdown mutant *opt3-2* in Arabidopsis is characterized by increased Fe accumulation in its leaves and reduced Fe movement from sink to source tissues (Mendoza-Cózatl et al., 2014; Zhai et al., 2014). Transcriptome analysis revealed the distinct effects of the *opt3-2* mutation on gene expression patterns between shoots and roots. Despite its higher Fe contents in shoots, *opt3-2* causes the constitutive activation of Fe uptake gene expression, such as the Ib *bHLH* genes, *IRT1*, and *FRO2*, in the roots but not in the shoots (Khan et al., 2018). Recently, Ngueyn et

al. showed that modulations of Fe levels in the vasculature by expressing *ZINC/IRON REGULATED TRANSPRTER-LIKE PROTEIN5* (*ZIP5*) in the phloem or by a deficiency in the synthesis of nicotianamine (NA, an Fe chelator) disturbed the Fe deficiency response in leaves and roots (Nguyen et al., 2022). The vasculature of the *nas* quadruple mutant accumulated more Fe but had increased expression of Fe deficiency–responsive genes in roots compared to that in the wild type under Fe-sufficient conditions (Nguyen et al., 2022). These observations suggest that the Fe status of vascular tissue is critical for the systemic Fe response. Moreover, long-distance signaling molecules have been postulated to play a pivotal role in the systemic Fe response. FEP/IMA peptides were identified as candidate long-distance signaling molecules by grafting experiments with wild-type and FEP/IMA defective plants (Grillet et al., 2018; Tabata et al, 2020). Taken together, it is assumed that the FEP/IMA peptides produced in Fe-starved shoots move to root cells and activate the Fe deficiency response through stabilizing IVc bHLH TFs by attenuating their interactions with BTS/BTSLs. However, it is difficult to discriminate shoot-to-root signaling by peptides from signaling by Fe in grafting experiments. Importantly, no study has been able to directly detect the movement of FEP/IMA peptides between cells, tissues, or organs yet. Therefore, the molecular mechanisms underlying Fe translocation and homeostasis need further exploration.

In this study, we conducted a comprehensive transcriptome analysis of *FEP1* loss- and gain-of-function lines to examine FEP1 function in the systemic Fe deficiency response by analyzing shoots and roots separately. Transcriptome data demonstrated that altered FEP1 function affects a distinct set of Fe homeostasis-related genes expressed in companion cells in shoots and roots, indicating that FEP1 has a pivotal role in Fe translocation. However, the effect of ectopically expressed *FEP1* differed between shoots and roots, suggesting that FEP1 has different physiological roles in these two tissues. We also determined that, under Fe-deficient conditions, Fe concentration in the xylem sap is lower in *fep1-1* plants but increases in a transgenic line containing an estrogen-inducible *FEP1* gene, and Fe accumulation increases in the cortex of *fep1-1* roots. We propose a negative relationship between *fep1-1* and *opt3-2* that perturbs Fe movement from the xylem to the phloem. These findings suggest that FEP1 has a pivotal role in Fe translocation in the vascular tissues of Arabidopsis.

2 Materials and Methods

2.1 Plant materials and growth conditions

Arabidopsis thaliana (L.) accession Columbia-0 (Col-0) was used in this study. All plants were grown on Murashige and Skoog (MS) plates (1× MS salt mix, 2% [w/v] sucrose, 2.5 mM MES, pH 5.8, and 0.8% [w/v] agar) or hydroponically in 1/10 Hoagland solution, pH 5.8, at

23°C under a 14-h light/10-h dark cycle. The *fep1-1* mutant and the *ER-FEP1* transgenic line were described previously (#1 line, Hirayama et al., 2018). The *fep1-1* and wild-type (WT) plants were grown hydroponically for 4 weeks in 1/10 Hoagland solution and then transferred to fresh 1/10 Hoagland solution with or without Fe (20 μM FeEDTA, sodium salt) and grown for an additional 5 d. The *ER-FEP1* transgenic plants were grown hydroponically for 4 weeks in 1/10 Hoagland solution and then for a further 5 d in Hoagland solution containing estradiol (final concentration 0.5 μM). Entire roots and shoots of *fep1-1*, WT, and *ER-FEP1* transgenic plants were sampled and immediately frozen before RNA extraction, with three biological replicates per sample. For all experiments, the liquid medium was changed every 2 d. The *opt3-2* mutant (SALK_021168) was obtained from the Arabidopsis Biological Resource Center (ABRC).

2.2 Transcriptome deep sequencing (RNA-seq)

Total RNA was isolated from all plants using Sepasol reagent (Nacalai Tesque, Kyoto, Japan) and purified using a Qiagen Plant Mini Kit (Qiagen Japan). Total RNA quality and integrity were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Japan Ltd., Tokyo). Libraries for RNA sequencing were constructed using a TruSeq Stranded mRNA Sample Preparation Kit (Illumina K.K. Tokyo, Japan) according to the manufacturer's instructions and assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Library clusters were generated using a cBot with a TruSeq PE Cluster Kit (Illumina) and sequenced on a HiSeq 4000 instrument (Illumina) using a TruSeq SBS Kit (Illumina). The single-end sequencing method was used to obtain 50-bp sequences. The RNA-seq data are archived in the DNA Data Bank of Japan under accession number PSUB012213.

2.3 RNA-seq analysis

The RNA-seq reads were quality-checked and trimmed using Trimmomatic (v0.36) (Bolger et al., 2014) with the LEADING:20, TRAILING:20, and MI MINLEN:36 parameters. The cleaned RNA-seq reads were mapped to the reference *Arabidopsis thaliana* genome sequence (TAIR10) using HISAT 2-2.10.1 (Kim et al., 2015) with default parameters (Supplemental Table S1). The mapping results were summarized with SAMtools 1.9 (Li et al., 2009) and used to obtain read counts using Subread 1.5.2 (Liao et al., 2013) with a dataset of annotated *Arabidopsis thaliana* genes (TAIR10.42). Differentially expressed genes (DEGs) between samples were identified using edgeR 3.26 (Robinson et al., 2010) in R 3.6 using a threshold of |fold-change| > 2; the false-discovery rate (FDR) for each comparison was calculated by adjusting the *p*-value using the Benjamini–Hochberg procedure (FDR < 0.01). Co-expressed gene networks were constructed based on Pearson's correlation coefficients using Z-score normalized gene expression levels and visualized in Cytoscape

3.7 (Shannon et al., 2003). The RNA-seq data for *opt3-2* published by Khan *et al.* (Khan et al., 2018) were downloaded from the National Center for Biotechnology Information (GSE79275) and processed as described above. Transcripts per million (TPM) were calculated with StringTie v2.2.1 (Pertea et al., 2015).

2.4 RT-qPCR

Reverse transcription quantitative PCR (RT-qPCR) was performed on a LightCycler (Roche Diagnostics, Basel, Switzerland) in a total volume of 20 µL containing 10 µL TB Green Primer Ex Taq II (Takara Bio Inc., Otsu, Japan), 8 pmol of each primer, and a cDNA mixture synthesized using 50 ng total RNA. The amplification program consisted of 40 or 50 cycles at 95°C for 10 s and 60°C for 1 min. Relative transcript levels were calculated using the comparative Ct method, taking the expression of *ACT2* as an internal control. The primers used for qPCR are listed in Supplemental Table S2.

2.5 Perls staining

Seedlings grown hydroponically for 2 weeks in 1/10 Hoagland solution were used for Perls staining. Fresh roots, petioles, and basal stems were embedded in 2% (w/v) agar with 3% (w/v) gelatin and 100-µm thick cross-sections were prepared using a microslicer (Linear Slicer PRO10; Dosaka EM, Kyoto, Japan). For Perls staining, sections were exposed to a staining solution (4% [v/v] HCl and 4% [w/v] potassium ferrocyanide mixed in equal amounts) for 1 min. After rinsing with water, the signal was observed under an optical microscope.

2.6 Measurement of Fe concentrations in xylem sap and different organs

Various Arabidopsis genotypes (Col-0, *fep1-1*, *opt3-2*, and *ER-FEP1*) were grown in 1/10 Hoagland solution, pH 5.8, for approximately 4 weeks. The nutrient solution was changed every 2 d. The *ER-FEP1* transgenic plants were treated with DMSO only (as mock control) or 1 µM estrogen for 7 d before collecting xylem sap. For the Fe deficiency treatment, plants grown hydroponically as described above were transferred to a solution containing no Fe or 20 µM FeEDTA for 3 d and then exposed to a solution containing 20 µM FeEDTA for 6 h or 2 µM of the stable isotope ^{57}Fe(II) for 24 h. The xylem sap was collected with a pipette from the stem cut just under the rosette. Whole leaves and roots were harvested and the roots were washed four times with cold 1 mM $CaCl_2$ solution. Dried samples were digested with 60% (w/v) HNO_3 at 135°C. The concentrations of all elements in the digest solution and xylem sap were determined by inductively coupled plasma mass spectrometry (ICP-MS) with isotope mode.

2.7 Constructing transgenic plants

The *FEP1pro:FEP1-GFP* gene, containing the green fluorescent protein (*GFP*) gene at the *Msc*I restriction site of the *FEP1* gene with the *FEP1* promoter, was inserted into a binary vector, pBI101. Construction of the *FEP1-GFP* fusion gene and cloning of the *FEP1* promoter region were described previously (Hirayama et al., 2018). The resulting construct was introduced into the *fep1-1* mutant via Agrobacterium (*Agrobacterium tumefaciens*)-mediated transformation (Clough and Bent, 1998).

2.8 Immunostaining

After being grown on MS plates for 2 weeks, seedlings harboring the *FEP1pro:FEP1-GFP* transgene were transferred to a 1/10 Hoagland solution, pH 5.8, in an environmentally controlled growth room with a 14-h light/10-h dark cycle at 23°C. After 2 weeks, plants were exposed to a 1/10 Hoagland solution, pH 5.8, without Fe for 7 d. Different tissues were harvested for immunostaining using an anti-GFP antibody (Invitrogen anti-GFP, A11122, 1:1,000 dilution) as described previously (Yamaji and Ma, 2007).

2.9 Determination of root ferric-chelate reductase activity

The ferric-chelate reductase activity of the roots was determined as described previously with some modifications (Romera et al., 1996). Briefly, seedlings (4 weeks old) grown in 1/10 Hoagland solution were exposed to 18 mL solution containing 0.2 mM $CaSO_4$, 5 mM MES, 0.1 mM Fe(III)-EDTA, and 0.2 mM BPDS (pH 5.5) in a 20-mL beaker. After 1 h, an aliquot of the assay solution was sampled and A535 was determined. The Fe(II)-BPDS concentration was calculated using the extinction coefficient of 22.14 mM^{-1} cm^{-1}, and the reductase activity was normalized by root fresh weight.

3 Results

3.1 Transcriptomes of loss- and gain-of-function *FEP1* lines

We performed RNA-seq on the roots and shoots of the wild-type (WT) Col-0, a loss-of-function *fep1* mutant (*fep1-1*) and a transgenic line carrying an estrogen-inducible *FEP1* expression cassette (*ER-FEP1*) (Supplemental Figure S1A) (Hirayama et al., 2018). We monitored the expression patterns of several Fe deficiency-responsive genes in *fep1-1* and the WT under Fe-sufficient and Fe-deficient conditions and confirmed their upregulation in response to Fe deficiency in both roots and shoots (Supplemental Figure S1B). These results were in agreement with our previous observations based on RT-qPCR (Hirayama et al., 2018). Principal component analysis of the data indicated that the results obtained for each biological replicate are highly reproducible (Supplemental Figure S1C). These results showed that our RNA-seq datasets provide a collective transcriptome resource for studying FEP1-dependent transcriptional regulation underlying Fe homeostasis in plants.

By comparing the transcriptomes from the roots and shoots of the WT, *fep1-1*, and *ER-FEP1* plants under Fe-sufficient and Fe-deficient conditions, we identified differentially expressed genes (DEGs) using a threshold of |fold-change| > 2 and a false discovery rate (FDR) < 0.01 across the samples (Supplemental Figure S2A, Supplemental Tables S3, S4). Specifically, we obtained 1,462 genes responsive to Fe deficiency in shoots and 2,013 genes in roots. We also determined that these DEGs include genes reported to function in a regulatory network involved in Fe homeostasis (Kim et al., 2019; Gao et al., 2020; Schwarz and Bauer, 2020). Importantly, we observed that many genes in the network are differentially regulated between *fep1-1* and the WT under Fe-sufficient conditions: 23 genes were upregulated and seven genes were downregulated in mutant shoots and 26 upregulated and 12 downregulated in mutant roots relative to the WT (Figure 1A, 1B, Supplemental Figure S2B). Under Fe-deficient conditions, numerous genes were differentially regulated in *fep1-1* compared to the WT in both shoots (868 genes) and roots (227 genes) (Supplemental Tables S3, S4), in which most of the genes in the gene regulatory network showed similar trends of upregulation and downregulation in *fep1-1* roots and shoots. These data are consistent with the notion that FEP1 is involved in Fe homeostasis.

3.2 Loss of FEP1 function activates genes involved in Fe uptake under Fe-sufficient conditions

To identify genes that are potential regulatory targets of FEP1, we constructed a gene co-expression network using our transcriptome dataset. Genes expressed in at least one sample were included in this network, and the correlation between genes was determined based on their Pearson's correlation coefficient values (a value > 0.9 indicates a co-expressed gene pair). We thus generated a co-expression network comprising approximately 5,000 nodes (genes) and more than 75,000 edges (links) (Supplemental Figure S3A, S3C). A small side branch of the network was prominent in the co-expression network and contained *FEP1* and several Fe deficiency-responsive genes (hereafter referred to as the FEP1 subnetwork): *FEP1*-related short peptide genes (*FEP2/IMA2*, *FEP3/IMA1*, *IMA4*, and *IMA6*), four Ib *bHLH* genes (*bHLH38*, *bHLH39*, *bHLH100*, and *bHLH101*), *BTS*, *PYE*, *OPT3*, *FRO3*, *NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN4* (*NRAMP4*), *OBP3-RESPONSIVE GENE 1* (*ORG1*), and *ZINC INDUCED FACILITATOR 1* (*ZIF1*), and two defensin-like genes (At1g13608 and At1g13609) (Supplemental Figure S3B, S3D). We superimposed the expression changes of the genes in *fep1-1* and in the WT under Fe-sufficient conditions and established that the genes upregulated in the mutant are tightly linked to the FEP1 subnetwork in shoots and roots (Figure 2A, 2B, Supplemental Figure S3E, S3F). The FEP1 subnetwork genes included several components of the recently updated gene regulatory network of Fe homeostasis (Kim et al., 2019; Gao et al., 2020) and largely

overlapped with the genes belonging to cluster1 in the co-expression network defined by Schwarz and Bauer (Schwarz and Bauer, 2020). The top 20 genes with the highest co-expression (highest Pearson's correlation coefficients) with *FEP1* among shoot or root DEGs are listed in Supplemental Table S5.

We previously observed that the *fep1-1* mutation causes Fe deficiency phenotypes in shoots, even under Fe-sufficient conditions (Hirayama et al., 2018). We reasoned that Fe deficiency activates Fe deficiency-responsive genes, at least in the *fep1-1* shoots. To test this idea, we explored gene expression levels in shoots and roots in *fep1-1* and the WT upon Fe deficiency. Accordingly, we compared the reads per million mapped reads (RPM) values of each DEG between *fep1-1* and the WT under Fe-sufficient or Fe-deficient conditions. Under Fe-sufficient conditions, all the FEP1 subnetwork genes were expressed at higher levels in *fep1-1* in shoots and roots when compared to the WT, while the expression of most other genes was unchanged (Figure 2C, left panels). FEP1 subnetwork genes were upregulated in *fep1-1* roots, even though their Fe concentration is comparable to that of WT roots, as we showed previously (Hirayama et al., 2018). The expression levels of FEP1 subnetwork genes in the shoots and roots of Fe-sufficient *fep1-1* plants were almost the same as those in Fe-deficient WT (Figure 2C, center panels). FEP1 subnetwork genes were also upregulated in *fep1-1* tissues under Fe-deficient conditions (Figure 2C, right panels). These observations indicated that the *fep1-1* mutation activates a distinct set of Fe deficiency-responsive genes, including FEP1 subnetwork genes, regardless of the Fe supply, with expression changes similar to those in the WT under Fe-deficient conditions.

3.3 FEP1 differentially regulates Fe deficiency-responsive genes in roots and shoots

We previously demonstrated that the induced ectopic expression of *FEP1*, via the *ER-FEP1* transgenic line, is sufficient to activate Fe deficiency-responsive genes and Fe accumulation in roots and shoots (Hirayama et al., 2018). Here, we used the same *ER-FEP1* line to assess *FEP1*-induced global gene expression changes in roots and shoots. *ER-FEP1* expression rose within a day into estrogen treatment and remained high and constant for the following 6 d in both shoots and roots (Figure 3A). We observed the concomitant upregulation of *bHLH39* and *IRT1* expression in roots but not in shoots, highlighting the distinct responses of roots and shoots to *FEP1* induction.

We identified 522 upregulated genes and 641 downregulated genes in ER-FEP1 roots treated with estrogen relative to the mock control, including Fe deficiency-responsive genes, suggesting that induced *FEP1* expression affects the Fe-deficiency response in roots (Figure 3B, Supplemental Figure S4C, Supplemental Table S3). Superimposing these upregulated genes in *FEP1*-induced roots on our gene co-expression network illustrated the upregulation of FEP1 subnetwork genes (Figure 3C, Supplemental Figure S4A). By contrast, among the

genes upregulated in *FEP1*-induced shoots, only seven genes, including *BTSL1* and *FEP1*, overlapped with the genes upregulated in Fe-deficient WT shoots (Figure 3B, Supplemental Table S3), and we observed limited changes in the expression of FEP1 subnetwork genes upon *FEP1* induction (Figure 3C, Supplemental Figure S4B). These findings indicate a substantial gap in the response to *FEP1* induction between roots and shoots, as ectopic *FEP1* induction activated the Fe deficiency-responsive genes in roots but not in shoots.

The genes directly regulated by FEP1 should be affected reciprocally by loss and gain of FEP1 function. We thus turned to a comparison of *fep1-1* and *ER-FEP1* RNA-seq data to identify such genes. We selected all upregulated or downregulated genes in *fep1-1* but with the opposite expression pattern upon *FEP1* induction (|fold change| > 0.75 and FDR < 0.1) in shoots and roots (Supplemental Table S6). Notably, no gene fulfilled these criteria in the shoots. In the roots, genes participating in the biosynthesis of iron chelators or coumarins were upregulated in *fep1-1* and downregulated in the estrogen-treated *ER-FEP1* line (Supplemental Table S6). These genes were considered to be high-confidence candidate targets of FEP1. The expression of these genes was also downregulated in WT roots under Fe-deficient conditions, indicating that these genes are responsive to Fe levels.

3.4 FEP1 is involved in Fe translocation

Our transcriptome data indicated that FEP1 differentially regulates a distinct set of Fe deficiency-responsive genes, including FEP1 subnetwork genes, between shoots and roots. We confirmed the differential expression of these genes by RT-qPCR (Supplemental Figure S5). The distinct effects of modulating *FEP1* levels in shoots and roots were consistent with previous studies, indicating that FEP/IMA peptides are involved in shoot-to-root communication during the Fe deficiency response (Hirayama et al., 2018; Grillet et al., 2018).

Among the FEP1 subnetwork genes, *OPT3* encodes a putative transporter implicated in Fe translocation in vascular tissues (Zhai et al., 2014; Mendoza-Cózatl et al., 2014; Khan et al., 2018). In the gene-co-expression network based on the RNA-seq data, *FEP1* only connected to *OPT3* with a high correlation value (Figure 2B). *OPT3* was upregulated in *fep1-1* shoots and roots and in *FEP1* induced roots (Figures 2B, 3C, Supplemental Figure S3F). To test the effect of the *opt3-2* mutation on the expression of FEP1 subnetwork genes, we reanalyzed published RNA-seq data from roots and shoots of WT and the *opt3-2* mutant grown under Fe-sufficient conditions. We determined that the effect of the *opt3-2* mutation on the expression of FEP1 subnetwork genes in shoots is different from that of the *fep1-1* mutation. For example, several genes were downregulated in *opt3-2* when they were upregulated in *fep1-1* (Supplemental Figure S6A) (Khan et al., 2018). In addition, a considerable members of genes downregulated in *fep1-1* shoots were upregulated in *opt3-2*, while substantial members of genes upregulated in estrogen-induced *ER-FEP1* shoots

were similarly upregulated in *opt3-2* shoots (Supplemental Figure S6B). The *opt3-2* mutant has higher Fe contents in leaves than the WT (Mendoza-Cózatl et al., 2014; Zhai et al., 2014), whereas *fep1-1* accumulates relatively less Fe in the shoots than the WT (Hirayama et al., 2018). This relation between *fep1-1* and *opt3-2* indicated that FEP1 might also have a pivotal role in Fe translocation, as does OPT3.

3.5 FEP1 promotes Fe accumulation in xylem sap

To examine the effect of modulated FEP1 function on Fe translocation, we measured Fe concentrations in the xylem sap of *fep1-1*, *opt3-2*, and WT plants. Under Fe-sufficient conditions, the Fe concentration in the xylem sap was significantly higher in *opt3-2* than in the WT (P<0.01, Figure 4A), which was consistent with a previous report (Zhai et al., 2014). The Fe concentration in the xylem sap of Fe-sufficient *fep1-1* plants was almost the same as that in the WT. When plants were pretreated under Fe-deficient conditions for 3 d and then exposed to Fe for 6 h, the Fe concentration in the xylem sap was similarly high in Fe-deficient WT and *opt3-2* plants (Figure 4A), but much lower in *fep1-1*. We also observed that the Fe concentration of the xylem sap of estrogen-treated *ER-FEP1* plants is higher than that of the controls (WT or DMSO-treated *ER-FEP1* plants) (Figure 4B). We performed a short-term (24-h) labeling experiment with a stable heavy Fe isotope (^{57}Fe) using plants exposed to Fe deficiency for 3 d or maintained in Fe-sufficient conditions. The ^{57}Fe concentration in the xylem sap was significantly lower in Fe-deficient *fep1-1* than in the WT and *opt3-2* under the same conditions (P<0.01, Figure 4C). However, in Fe-sufficient plants, the ^{57}Fe concentration in the xylem sap was comparable among the three lines. These results are consistent with the notion that FEP1 has a pivotal role in Fe translocation.

3.6 The *fep1-1* root cortex accumulates more Fe

To assess ferric Fe deposition *in situ*, we conducted Perls staining in petioles, stems, and roots using the *frd3-7* mutant, which exhibits a constitutive Fe deficiency response and accumulates Fe in its vasculature, as a positive control (Figure 5). In agreement with a previous report, we detected strong Perls staining in the *frd3-7* root stele (Scheepers et al., 2020) and enhanced staining near the phloem in *opt3-2* petioles (Zhai et al., 2014). In *fep1-1,* we observed Perls staining in the cortex but not in the epidermis or stele of the roots, suggesting that Fe movement from the cortex into the stele is compromised in *fep1-1*. We observed the same strong Perls staining in the root cortex of the *fep1-1 opt3-2* double mutant, although the shoot phenotype typically seen with the *fep1-1* mutation was suppressed (see below). This result suggested that the high Fe content in the cortex is not responsible for the *fep1-1* chlorotic shoot phenotype.

3.7 Tissue specificity of FEP1 localization

To determine where the *FEP1* gene is expressed and where the FEP1 peptide accumulates within cells, we produced a transgenic line in the *fep1-1* background harboring a *FEP1-GFP* transgene driven by the *FEP1* promoter (*FEP1pro:FEP1-GFP*). We previously confirmed the transcriptional activation induced by FEP1-GFP in protoplast experiments (Hirayama et al., 2018). We rarely detected GFP fluorescence in roots or shoots under Fe-sufficient conditions, but fluorescence became visible under Fe-deficient conditions (Supplemental Figure S7). To localize FEP1-GFP in tissues more precisely, we conducted immunostaining experiments using sections of various tissues with an anti-GFP antibody. After a 7-d Fe deficiency treatment, we detected GFP mainly in root and shoot vascular tissues (Figure 6). In root sections, we detected strong immunohistochemical signal in a few cells near the phloem, consistent with a previous study using the *FEP1pro:GUS* (*β-glucuronidase*) transgenic lines (Hirayama et al., 2018), and a moderate but clear signal in pericycle cells (Figure 6E). In leaf and shoot sections, the anti-GFP antibody detected GFP in many cells in vascular tissues, which differed from the results of our previous study using the *FEP1pro:GUS* line (Hirayama et al., 2018).

3.8 *opt3-2* suppresses the *fep1-1* shoot phenotype

To further investigate the role of FEP1 in Fe translocation, we generated the *fep1-1 opt3-2* double mutant (hereafter, *fep1 opt3*) by genetic crossing. We previously reported that *fep1-1* shoots have an Fe-deficient phenotype, such as lower Fe concentration in their shoots, bright green leaves, and upregulation of Fe deficiency-responsive genes in shoots, whereas *fep1-1* roots display subtle phenotypes (Hirayama et al., 2018). The leaves of the *fep1 opt3* double mutant were greener than those of *fep1-1* and similar to those of *opt3-2* and the WT. The SPAD values of *fep1 opt3* true leaves were higher than those of *fep1-1* and similar to those of the WT and *opt3-2* (Figure 7A), indicating that the *opt3-2* mutation suppresses the *fep1-1* phenotype. The shoot Fe concentration was lower in *fep1-1* and much higher in *opt3-2* than in the WT, but was higher in the *fep1 opt3* double mutant relative to the WT and *fep1-1* (Figure 7B), again indicating that the *opt3-2* and *fep1-1* mutations genetically interact. The upregulation of Fe deficiency-responsive genes in *fep1-1* shoots was suppressed by the *opt3-2* mutation, although the observed effects differed between genes (Figure 7C). Specifically, *NRAMP4* and *FRO2* expression was the same in *fep1 opt3* and *opt3-2*, whereas the expression levels of other genes in the double mutant were intermediate between those of *fep1-1* and *opt3-2.*

The Fe concentration in *opt3-2* roots was twice as high as that in *fep1-1* and the WT (Supplemental Figure S8A), which was consistent with a previous report (Mendoza-Cózatl et al., 2014). The Fe concentration in *fep1 opt3* roots was the same as in the WT and *fep1-1*.

The ferric-chelate reductase activity in the roots, which is one of the biochemical makers for Fe uptake, was significantly higher in *opt3-2* roots compared with in WT roots, as reported previously (Khan et al., 2017). By contrast, the reductase activities of *fep1-1* and *fep1 opt3* roots were not different from those of WT roots (Supplemental Figure S8B). These data indicates that the *fep1-1* mutation suppresses the *opt3-2* root phenotype. However, the expression levels of Fe deficiency-responsive genes in roots were higher in *fep1 opt3* than in *fep1-1*, *opt3-2*, or the WT (Supplemental Figure S8C). The expression of *FERRITIN1* (*FER1*), which encodes an Fe storage protein and whose levels reflect the Fe levels of the tissue, was lowest in *fep1 opt3* roots, even though the Fe concentration in *fep1 opt3* roots was the same as that of the WT and half that of *opt3-2* roots. These results indicated that the effects exerted by FEP1 and OPT3 on Fe homeostasis differ between roots and shoots.

4 Discussion

To understand the physiological role of FEP1*,* we analyzed the transcriptome of FEP1 loss- and gain-of-function lines, which revealed that the *fep1-1* mutation activates a distinct set of genes, referred to here as the FEP1 subnetwork genes, in shoots even under Fe-sufficient conditions (Figures 1, 2). When comparing the FEP1 subnetwork genes to the gene co-expression network reported by Schwarz and Bauer (Schwarz and Bauer, 2020), we determined that the FEP1 subnetwork genes largely overlap with members of a cluster which was supposed to be composed of FIT-independent genes, including those encoding Ib bHLHs. This observation suggests that the upregulation of other Fe deficiency-responsive genes is mainly regulated by FIT and Ib bHLHs. However, *IRT1* and *FRO2* expression levels, which are regarded as FIT-dependent genes, were unchanged or downregulated in *fep1-1* relative to the WT. These effects caused by modulation of FEP1 output demonstrated that FEP1 has a unique role in the Fe deficiency response. FEP/IMA peptides regulate the degradation of IVc bHLH transcription factors via the E3 ligase BTS and BTSLs (Li et al., 2021, Lichtblau et al., 2022)). Since the genes encoding these bHLHs are located at the top of the gene regulatory network of Fe deficiency-responsive genes, their BTS/BTSL-mediated degradation through physical interaction is a critical step in Fe homeostasis. In the proposed model, FEP/IMA peptides compete with BTS/BTSL for binding to these bHLHs and consequently stabilize them. At least two of these IVc bHLHs, bHLH105 and bHLH115, activate *FEP1* and *BTS* expression, thus forming positive and negative feedback loops. Therefore, the balance between intracellular Fe levels, BTS levels, FEP/IMA peptide levels, and IVc bHLH levels, determine the Fe deficiency response (Li et al., 2021). This model is largely consistent with the results of *in vitro* or *in vivo* assays using transient expression systems, loss-of-function lines, and gain-of-function lines of genes involved in the regulation of Fe deficiency-responsive genes.

In this model, six FEP/IMA peptides in Arabidopsis inhibit the interaction between IVc bHLHs and BTS/BTSL by competing for the BTS binding site on the IVc bHLHs. In this study, *FEP1* expression in the shoots was higher than that of *FEP2* or *FEP3,* while in the roots, *FEP1* expression was low compared to that of *FEP2* and *FEP3* (Supplemental Table. S7). Therefore, the loss of one FEP/IMA peptide may be compensated by other FEP/IMA peptides, at least in roots. However, the FEP1 subnetwork genes are activated in *fep1-1* roots and shoots (Figure 1, 2). It is possible that the unique function of FEP1, being the direct target of two IVc bHLHs, causes this *fep1-1* phenotype. According to the model, a defect in FEP1 function would downregulate Fe deficiency-responsive genes by destabilizing IVc bHLHs. However, we demonstrated here that these genes are upregulated in the shoots and roots of the *fep1-1* mutant.

This discrepancy might be explained by the secondary effect of the Fe deficiency response; lower levels of IVc bHLHs induce Fe deficiency in cells and/or tissues, which in turn activates the Fe deficiency-responsive genes. While Fe levels in *fep1-1* shoots were lower than in WT shoots (Figure 7B), Fe levels in *fep1-1* roots were not changed (Hirayama et al., 2018), although the expression of Fe deficiency-responsive genes was activated. In addition, compared to the WT, *bHLH39* expression was significantly higher in *fep1 opt3* shoots, which accumulated more Fe (Figure 7C). To explain our transcriptome results, we may need to consider additional factors involved in the regulation of Fe deficiency-responsive genes. Importantly, experimental conditions affect the expression of Fe deficiency response genes. In our previous study, we showed that the *bHLH38* and *bHLH39* genes were activated in the roots of *fep1-1* plants grown hydroponically but not in the roots of *fep1-1* plants grown on MS media under Fe-sufficient conditions (Hirayama et al., 2018). This could explain why the expression levels of these genes were not changed in *fep1-1* roots in our current study, where plants were grown hydroponically. Additionally, Li et al. showed that the expression levels of these genes were not activated in roots of the *ima3/fep1* mutant grown on plate medium (Li et al., 2021).

Alternatively, FEP1 may function in a tissue- or cell lineage-specific manner. *FEP1* is highly expressed in phloem companion cells (Supplemental Table S8) (You et al., 2019). We showed that *FEP1* is preferentially expressed in vascular tissues in this study with the *FEP1pro:FEP1-GFP* line (Figure 6) and in our previous study using the *FEP1pro:GUS* line (Hirayama et al 2018). In addition, transcripts of FEP1-subnetwork genes, including *BTS*, were enriched in a companion cell transcriptome (Supplemental Table S8) (You et al., 2019). Interestingly, all four IVc *bHLH* genes and *bHLH121* were highly expressed in phloem companion cells (You et al., 2019). By contrast, the expression of *FIT* and Ib *bHLH* genes was not as high in these cells. The preferential activation of *FEP1* and *BTS* by IVc bHLH, shown by Li et al., also can be explained by the tissue-specific expression of *FEP1* and *BTS*

(Li et al., 2021). These data suggest that FEP1 preferentially regulates its target genes in vascular tissues, including companion cells, and that the *fep1-1* mutation results in a localized Fe deficiency under Fe-sufficient conditions. This idea is supported by the results of our physiological studies. The Fe concentration of the xylem sap was lower in Fe-deficient *fep1-1* than in the WT and *opt3-2* (Figure 4A), indicating that Fe translocation to the xylem is impaired in *fep1-1*. Consistent with this notion, Perls staining revealed that Fe translocation from the cortex to the stele is compromised in *fep1-1* roots (Figure 5). We hypothesize that the lower stability of IVc bHLH proteins in *fep1-1* would diminish the expression of Fe deficiency-responsive genes, including those involved in Fe translocation from the cortex to the stele. Low levels of Fe translocation from the cortex to the stele in *fep1-1* would impose Fe deficiency in vascular tissues, including the xylem, thus activating Fe deficiency-responsive genes in these tissues.

To examine the effect of gain of FEP1 function, we used an estrogen-inducible system because constitutive ectopic expression of *FEP1* caused severe growth retardation (Hirayama et al., 2018). In this estrogen-inducible system, the ectopic expression of *FEP1* is temporally controlled but is ubiquitous. As FEP1 functions in specific cells or tissues, the transcriptome data generated from this system require us to consider the possible secondary effects resulting from *FEP1* ectopic expression. However, the transcriptome data also offer useful clues about the distinct effects of *FEP1* induction in shoots and roots (Figure 3). According to the FEP/IMA working model, high levels of FEP1 stabilize IVc bHLHs, which upregulates Fe deficiency-responsive genes and Fe uptake (Li et al., 2012). Ectopic expression of *FEP1* increased Fe concentrations in the xylem sap (Figure 4B) and induced higher Fe accumulation in shoots and roots (Hirayama et al., 2018). However, *bHLH39* expression was not induced in shoots upon *FEP1* induction (Figure 3A). Presumably, sufficient or excess Fe modulates the extent of gene activation induced by ectopic expression of *FEP1* in shoots but not in roots.

Higher Fe levels destabilize BTS *in planta*, but the molecular mechanisms by which BTS suppresses the Fe deficiency response under Fe-sufficient conditions have not been fully elucidated (Kobayashi et al., 2013; Selote et al., 2015; Kobayashi, 2019). *bHLH105, bHLH115,* and *BTS* expression was higher in roots than in shoots in the WT under Fe-sufficient conditions, while *FEP1* expression was lower in roots than in shoots (Supplemental Table S4). According to the FEP/IMA working model proposed by Li et al., the relative amounts of FEP1, IVc bHLHs, and BTS determine the regulation of Fe deficiency-responsive genes (Li et al., 2021). Since FEP1, IVc bHLHs, and BTS are modified post-translationally, the transcript levels of their encoding genes do not reflect their protein levels; nonetheless, we observed significant differences in their transcript levels between roots and shoots. This result suggests that the equilibrium between FEP1, IVc bHLHs, and BTS differs between

shoots and roots, and that changes in Fe or FEP1 accumulation might differentially affect the Fe-deficiency response in shoots and roots. Further detailed analysis of the relationship between these factors is needed to understand the discrepancy in the responses in shoots and roots.

One of the most intriguing observations of this study is the strong Fe deposition in the *fep1-1* cortex (Figure 5). Fe is taken up through the symplastic pathway (Kim and Guerinot, 2007). It has been postulated that Fe taken up into the symplast moves freely to the endodermis through the plasmodesmata. Since the ferric-chelate reductase activity of *fep1-1* roots was similar to that of WT roots (Supplemental Figure S8B), the Fe deposition at the cortex of *fep1-1* could be the result of altered Fe loading to the xylem. In fact, Fe translocation into the xylem sap was significantly lower in Fe-deficient *fep1-1* plants than in Fe-deficient WT plants (Figure 4C). The higher Fe deposition observed in the *fep1-1* cortex was not suppressed in the *fep1-1 opt3-2* double mutant (Figure 5), suggesting that Fe accumulation in the cortex is unlikely to be a secondary effect of the *fep1-1* shoot phenotype. Therefore, we propose that FEP1 is required for the function of one or more Fe translocators in the cortex or endodermis, although the reason for higher Fe deposition at the cortex of *fep1-1* roots is currently unclear. A recent study showed that the *irt1-1* mutant exhibited compromised Fe translocation from roots to shoots and a higher Fe deposition at the cortex than the WT, which is reminiscent of the *fep1-1* phenotype (Quintana et al., 2022). Interestingly, using several point mutations of *IRT1*, the authors demonstrated that IRT1 has a role in root-to-shoot Fe partitioning besides the Fe uptake function, suggesting the presence of unknown regulatory systems for systemic Fe regulation (Quintana et al., 2022).

GFP immunostaining in the root section of the *FEP1pro:FEP1-GFP* transgenic plants in the *fep1-1* background revealed that FEP1-GFP localizes in the endodermis, where we failed to detect GUS signal in the *FEP1pro:GUS* transgenic line in our previous study (Figure 6) (Hirayama et al., 2018). These observations indicate that FEP1 might move from cells near the phloem to the endodermis, like the Arabidopsis protein SHORT-ROOT that moves from the stele to the cortex (Nakajima et al., 2001). The candidate genes involved in Fe translocation at the endodermis can be identified from the transcriptome data of *fep1-1* roots. Exploring the list of genes differentially regulated in *fep1-1* roots indicated that *FRD3* is significantly downregulated under Fe-sufficient conditions and upregulated under Fe-deficient conditions (Supplemental Table S3). However, loss of FRD3 function caused Fe accumulation in the stele (Figure 5) (Scheepers et al., 2020), suggesting that this gene is less likely to be the candidate. YSL2 is another good candidate, because this transporter was reported to translocate Fe in roots (Schaaf et al., 2005), but *YSL2* expression was not significantly affected in *fep1-1* or *ER-FEP1*. Coumarins, a class of chemicals secreted from roots to facilitate Fe uptake, also accumulate in the cortex and are transported throughout

the whole plant body via the xylem sap (Robe et al., 2021). The expression of two FIT dependent genes, *CYTOCHROME P450 82C4* (*CYP82C4*) and *SCOPOLETIN 8-HYDROXYLASE* (*S8H*), involved in coumarin biosynthesis in Arabidopsis (Colangelo and Guerinot, 2004, Rajniak et al., 2018; Siwinska et al., 2018; Tsai et al., 2018), was lower in *fep1-1* roots under Fe-sufficient conditions compared to the WT (Figure 1B) and activated in roots upon *FEP1* induction (Supplemental Table S6). Consistently, a recent study showed that ectopic expression of *FEP2/IMA3* and *FEP3/IMA1* activated *CYP82C4* and *S8H* under Fe-sufficient conditions (Gautam et al., 2021)**.** Reduced or increased expression of these genes might contribute to Fe accumulation in the *fep1-1* root cortex or Fe accumulation in xylem of *FEP1*-induced root, respectively, but further studies are required to specify their contributions.

In conclusion, our transcriptome, physiological, and genetic studies demonstrate that FEP1 is involved in the Fe-deficiency response in vascular tissues, and is required for Fe homeostasis in Arabidopsis. Moreover, our data suggest that the regulatory system involving FEP1 may differ between roots and shoots. Taken together with the recent molecular study of FEP/IMA peptides, our results indicate that FEP1 regulates Fe deficiency-responsive genes in vascular tissues and plays a pivotal role in Fe movement across tissues. Our studies also suggests that FEP1 may move intercellularly to regulate Fe translocation. Additional studies of FEP1 and other FEP/IMA peptides will enable us to further elucidate the molecular mechanisms of Fe homeostasis in plants.

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Conflict of interest statement

None declared.

Author contributions

S.O. and T.H. designed the research; S.O., N.Y., G.J.L., S.H., J.F.M., and T.H. prepared the plant samples and conducted the physiological analysis; K.M. obtained the RNA sequence data; T.H., S.O., and K.M. performed the data analysis; S.O., J.F.M., K.M., and T.H. wrote the manuscript.

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Figure legends

Figure 1. DEGs between shoots and roots of Fe-sufficient *fep1-1.*

A, B, Heatmap representation of the expression levels of DEGs in *fep1-1* shoots (A) and roots (B) under Fe-sufficient conditions, shown as Log_2-transformed fold-change (left-most column of each figure). The differential expression of these genes between *fep1-1* and WT tissues under Fe-sufficient or Fe-deficient conditions are also shown. The colors (blue to orange) indicate the Log_2(fold change) in expression.

Figure 2. Co-expression network of the DEGs affected by the loss of FEP1 function.

A, Co-expression network drawn based on the correlation in expression levels of the expressed genes across all samples. The colors (blue to orange) of the nodes indicate the fold-change (Log_2-transformed) in expression levels between Fe-sufficient *fep1-1* and WT shoots. The dashed blue line indicates the FEP1 subnetwork. B, The FEP1 subnetwork. The co-expression network outlined by the dotted line in (A) was enlarged and reorganized for clarity. The colors (light blue to yellow) of the edges indicate the Pearson's correlation coefficient (0.9 to 1.0) between nodes. Each node is labeled with a gene name. C, Comparison of the expression levels (in RPM) of DEGs between *fep1-1* and the WT. Each DEG was plotted with the RPM of the indicated samples. Orange dots indicate FEP1 subnetwork genes.

Figure 3. *FEP1* induction differentially affects the expression of Fe deficiency–responsive genes in shoots and roots.

A, Time course of *FEP1*, *bHLH39*, and *IRT1* transcript levels in *ER-EFP1* transgenic plants following estrogen (ER) treatment. B, Venn diagrams showing the extent of overlap between the upregulated genes in ER-treated *ER-FEP1* roots and shoots and in Fe-deficient WT tissues. The numbers indicate the number of genes in each class. Known Fe deficiency-responsive genes are indicated. C, Expression levels of FEP1 subnetwork genes in ER-treated *ER-FEP1* roots (left panel) and shoots (right panel). The colors (blue to orange) of the nodes indicate the fold-change (Log_2-transformed) in expression levels between the estrogen- (+ER) and mock- (DMSO, −ER)- treated tissues. The colors (light blue to yellow) of the edges indicate the Pearson's correlation coefficient (0.9 to 1.0) between nodes. Each node is labeled with a gene name in the right panel.

Figure 4. FEP1 participates in the regulation of Fe translocation.

A, Fe concentrations in the xylem sap of hydroponically grown WT, *fep1-1*, and *opt3-2* plants. The plants were pretreated with (+Fe) or without Fe (−Fe) for 3 d and then exposed to 20 μM FeEDTA for 6 h before xylem sap collection. B, Fe concentrations in the xylem sap of

hydroponically grown *ER-FEP1* plants under Fe-sufficient conditions, pretreated with estrogen (+ER) or a mock solution of DMSO (−ER) for 5 d. C, Concentrations of ^{57}Fe in the xylem sap of WT, *fep1-1*, and *opt3-2* plants. The seedlings were pretreated with (+Fe) or without Fe (−Fe) for 3 d and then exposed to 2 μM ^{57}Fe. After 24 h, the xylem sap was collected for ^{57}Fe determination. In all panels, the mean values for xylem sap collected from four independent plants are shown (*n* = 4 or 5). Error bars indicate SD. *** indicates statistically significant differences (*P* < 0.01), as determined by Student's *t*-test.

Figure 5. Fe accumulates in the cortex of *fep1-1* roots.

Histochemical detection of ferric Fe deposition by Perls staining in cross-sections of petioles, stems, and roots of WT (Col-0), *fep1-1*, *frd3-7*, *opt3-2*, and the *fep1-1 opt3-2* double mutant (*fep1 opt3*). Plants were grown under Fe-sufficient conditions. Scale bars, 200 μm (petiole, stem), 50 μm (root).

Figure 6. Localization of FEP1-GFP driven by the *FEP1* promoter.

Immunostaining for GFP in the *fep1-1 FEP1pro:FEP1-GFP* transgenic line. A, Leaf section. B, Magnified image of the outlined region in (A). C, Shoot section. D, Magnified image of the outlined region in C. E, Root section. Two-week-old seedlings were grown on Fe-deficient medium for 7 d. Cross-sections of tissues were subjected to immunostaining using anti-GFP antibody and Alex-Fluor 555-conjugated secondary antibody. Alex-Fluor 555 fluorescence was visualized with a laser-scanning confocal microscope. Blue indicates the cell wall autofluorescence by 405-nm laser excitation. Scale bar: 100 μm.

Figure 7. The *opt3-2* mutation suppresses the *fep1-1* shoot phenotype.

A, SPAD values for the true leaves of WT (Col-0), *fep1-1*, *opt3-2*, and *fep1-1 opt3-2* (*fep1 opt3*) seedlings grown in soil for 2 weeks. Error bars indicate SD (*n* = 5). Significant differences were determined by Student's *t*-tests compared to the WT; *** *P* < 0.01. B, Fe concentrations in the shoots of WT, *fep1-1*, *opt3-2*, and *fep1 opt3* plants grown hydroponically under Fe-sufficient conditions. Error bars indicate SD (*n* = 5). Significant differences between indicated genotypes were determined by Student's *t*-tests; *P* values are indicated with asterisks (*** *P* < 0.01). C, Relative expression levels of Fe deficiency-responsive genes in the shoots of WT, *fep1-1*, *opt3-2*, and *fep1 opt3* plants grown hydroponically under Fe-sufficient conditions, by RT-qPCR. Results were normalized to *ACT2* transcript levels and are shown as relative to the WT, which was set to 1. Error bars indicate SD (*n* = 3). Significant differences were determined by Student's *t*-test compared to *fep1 opt3*; **, *P* < 0.05; ***, *P* < 0.01.

Supplemental Table S1. Mapping results of RNA-seq reads obtained in this study.

Supplemental Table S2. Oligonucleotide primers used for RT-qPCR.

Supplemental Table S3. List of DEGs identified in this study.

Supplemental Table S4. Summary of the comparison of shoot and root transcriptomes.

Supplemental Table S5. Co-expressed genes with *FEP1* in shoots and roots.

Supplemental Table S6. List of genes showing opposite regulation patterns in *fep1-1* and *ER-FEP1*.

Supplemental Table S7. Expression levels of several Fe deficiency-responsive genes in the organs of wild-type plants.

Supplemental Table S8. Expression of FEP1-subnetwork genes and several Fe deficiency-responsive genes in phloem companion cells.

Supplemental Figure S1. Experimental design and overview of the transcriptome data.
A, Schematic diagram of the experimental design of this study. *fep1-1* and WT Arabidopsis plants were grown under Fe-sufficient and Fe-deficient conditions, and shoots and roots were separately harvested for total RNA extraction. *ER-FEP1* transgenic Arabidopsis plants were treated with estrogen or mock solution, and total RNA was extracted from shoots and roots separately. B, Comparison of previously published RT-qPCR data (Hirayama et al., 2018) with the RNA-seq data obtained in this study for several Fe deficiency-responsive genes. The transcript levels were normalized to *ACT2* transcript levels and are shown as relative values compared to the WT under Fe-sufficient conditions (n = 3, error bars indicate SD). C, Principal component analysis (PCA) of the RNA-seq data (upper panel, shoots; lower panel, roots) obtained in this study.

Supplemental Figure S2. DEGs in *fep1* and WT tissues under various conditions.
A, MA plots of the RNA-seq transcriptome data for *fep1-1* and WT shoots or roots under Fe-deficient or -sufficient conditions. The x-axis represents the average expression level (log_2-transformed counts per million [CPM]) between two indicated samples. The y-axis represents the difference in the expression level (log_2-transformed fold change) between two indicated samples. Red dots indicate DEGs with FDR < 0.01. B, DEGs in *fep1-1* versus WT tissues

under Fe-sufficient conditions, in WT tissues under Fe-sufficient versus Fe-deficient conditions, and in *fep1-1* tissues under Fe-sufficient versus Fe-deficient conditions. The data for roots and shoots are shown separately, as are the upregulated (up) and downregulated (dw) DEGs. The numbers indicate the number of genes in each category.

Supplemental Figure S3. Co-expression network of DEGs in *fep1* and the *ER-FEP1* line.
A, C, E, Co-expression networks based on differential expression data between the indicated samples. B, D, F, The FEP1 subnetwork corresponding to the dashed line in panels (A, C, E), is enlarged and reorganized. The colors (blue to orange) of the nodes indicate the fold-change (Log_2-transformed) between the indicated samples.

Supplemental Figure S4. *FEP1* induction differentially affects the expression of Fe deficiency-responsive genes in shoots and roots.
A, B, Co-expression networks based on differential expression data between the indicated samples. The colors (blue to orange) of the nodes indicate the fold-change (Log_2-transformed) between the indicated samples. C, Venn diagrams of the downregulated genes in ER-treated *ER-FEP1* and in Fe-deficient WT roots and shoots. The numbers indicate the number of genes in each class. Known Fe deficiency-responsive genes are indicated.

Supplemental Figure S5. RT-qPCR analysis of the transcript levels of several FEP1 subnetwork genes.
A, B, RT-qPCR analysis of *BTS*, endogenous *FEP1*, *NRAMP4*, *OPT3*, *bHLH39*, and *ZIF1* expression levels in WT and *fep1-1* (A) or *ER-FEP1* plants (B). The transcript levels were normalized to *ACT2* transcript levels with Fe-sufficient WT samples (A) or mock-treated samples (B) set to 1. The mean values of independent samples are shown ($n = 3$). Error bars indicate SD. Significant differences were determined by Student's *t*-test compared to WT; **, $P < 0.05$; ***, $P < 0.01$.

Supplemental Figure S6. Expression patterns of FEP1 subnetwork genes in *opt3-2* and *fep1-1* under Fe-sufficient conditions and comparison of transcriptome data from *opt3-2*, *fep1-1*, and *ER-FEP1* plants.
A, The FEP1 subnetwork with differential expression data between *opt3-2* and WT shoots (left panel) or *fep1-1* and WT shoots (right panel). The colors (blue to orange) of the nodes indicate the fold-change (Log_2-transformed) between the samples. The colors (light blue to yellow) of the edges indicate the Pearson's correlation coefficient (0.9 to 1.0) between nodes in the transcriptome data in this study. Transcriptome data of *opt3-2* were obtained from NCBI GSE79275 (Khan et al., Plant Cell Env., 41, 2263-2276, 2018). B, Venn diagrams of

upregulated genes in *opt3-2* tissues and downregulated genes in *fep1-1* tissues compared to Fe-deficient WT (upper panel), and Venn diagrams of upregulated genes in *opt3-2* tissues and genes induced by ER treatment in the *ER-FEP1* line (lower panel). The number of genes in each class is indicated. Known Fe homeostasis-related genes are indicated.

Supplemental Figure S7. GFP fluorescence of *FEP1pro:FEP1-GFP* transgenic plant. The *fep1-1 FEP1pro:FEP1-GFP* transgenic plants grown MS plates for 2 weeks and transferred to Fe deficient condition for 5 d. A, Shoots were observed under a binocular with florescent light. Roots were observed under a microscope with fluorescent light (upper panel) or visible light (lower panel).

Supplemental Figure S8. The *opt3-2* mutation suppresses the *fep1-1* phenotypes. A, Fe concentrations in the roots of WT, *fep1-1*, *opt3-2*, and *fep1 opt3* plants grown hydroponically under Fe-sufficient conditions. Error bars indicate SD (n = 5). The P values of Student's t-test compared to the WT are shown, unless they are < 0.01. B, Ferric-chelate reductase assay. The reductase activity in roots of WT, *fep1-1*, *opt3-2*, and *fep1 opt3* plants grown hydroponically under Fe-sufficient conditions was measured. Error bars for WT, *fep1-1*, and *fep1 opt3* indicate SD (n = 3). The *opt3-2* data are the average of two root samples. ND indicates no significant differences among the indicated samples (Student's t-test; P > 0.5). C, Relative expression levels of Fe deficiency-responsive genes in roots of WT, *fep1-1*, *opt3-2*, and *fep1 opt3* plants grown hydroponically under Fe-sufficient conditions. RT-qPCR data were normalized to *ACT2* transcript levels, with WT values set to 1. Error bars indicate SD (n = 3). Significant differences were determined by Student's t-test compared to *fep1 opt3*; **, P < 0.05; ***, P < 0.01.