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The Putative Peptide Gene *FEP1* Regulates Iron Deficiency Response in Arabidopsis

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Iron is an essential element for all organisms, and plants have developed sophisticated systems to acquire iron and maintain iron homeostasis. We found that an *Arabidopsis thaliana* ABA-hypersensitive mutant, *aba hypersensitive germination2-1* (*ahg2-1*), that is known to be defective in mitochondrial mRNA regulation, had increased expression of iron deficiency response genes. The *ahg2-1* mutant had lower heme levels than the wild type. Transcriptome data further revealed that novel genes encoding short polypeptides were highly expressed in this mutant. The expression of one of these genes, which we named *FE-UPTAKE-INDUCING PEPTIDE 1* (*FEP1*), was induced under iron-deficient conditions and was observed in the vascular tissues of the leaves and roots, as well as in leaf mesophyll cells. Notably, deletion or insertion mutations of *FEP1* exhibited impaired iron accumulation in shoots but normal iron levels in roots. Artificially induced expression of *FEP1* was sufficient to induce iron deficiency response genes, such as *basic HELIX–LOOP–HELIX 38* (*bHLH38*), *bHLH39*, *IRON-REGULATED TRANSPORTER1* (*IRT1*) and *FERRIC REDUCTION OXIDASE2* (*FRO2*), and led to iron accumulation in planta. Further analysis confirmed that the encoded peptide, but not the *FEP1* RNA, was responsible for this activity. Remarkably, the activation of *bHLH39* by FEP1 was independent of FER-LIKE IRON DEFICIENCY INDUCED (FIT), a key transcription factor in the iron deficiency response. Taken together, our results indicate that FEP1 functions in iron homeostasis through a previously undescribed regulatory mechanism for iron acquisition in Arabidopsis.

Keywords: Arabidopsis • Iron deficiency response • Short peptide • Systemic response.

Abbreviations: bHLH, basic helix–loop–helix; CRISPR/Cas9, clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9; ER, estradiol; FEP, FE-UPTAKE-INDUCING PEPTIDE; FIT, FER-LIKE IRON DEFICIENCY INDUCED; GFP, green fluorescent protein, GUS, β-glucuronidase; ICP-MS, inductively coupled plasma mass spectrometry; LUC, luciferase; MS, Murashige and Skoog; qRT–PCR, quantitative reverse transcription–PCR.

Introduction

Iron is an essential element for many enzymes and sensory components intrinsic to all organisms, with heterotrophs including humans acquiring iron via autotrophs such as plants. Plants have developed an elaborate system to maintain sufficient iron levels, using Strategy I or II, depending on the species. When plants experience low iron in the soil, the expression of a set of iron deficiency response genes is altered (Hindt and Guerinot 2012, Kobayashi and Nishizawa 2012, Thomine and Vert 2013, Brumbarova et al. 2015). During Strategy I responses in Arabidopsis, several genes encoding basic helix–loop–helix (bHLH)-type transcription factors, such as *bHLH38* and *bHLH39*, are activated (Wang et al. 2007). These Ib subfamily bHLH transcription factors along with another bHLH protein, FER-LIKE IRON DEFICIENCY INDUCED (FIT), co-operatively activate *FRO2* and *IRT1*, which encode a ferric reduction-oxidase and an iron transporter, respectively (Vert et al. 2002, Connolly et al. 2003, Yuan et al. 2008). Another bHLH protein, POPYE (PYE), is a negative regulatory factor for iron deficiency responses. *PYE* is induced slowly under iron-deficient conditions and down-regulates several genes involved in metal translocation (Long et al. 2010). Despite this progress in understanding iron biology, the regulatory mechanism upstream of these key components remains to be fully elucidated (Aksoy et al. 2013). In addition, the molecular mechanisms by which plants sense iron availability and regulate iron homeostasis systemically, presumably through long-distance signaling, are unknown. Iron-transporting factors such as FRD3, OPT3, YSL family members and IREG1/FPN1 were shown to be involved in iron translocation among tissues (Green and Rogers 2004, Waters et al. 2006, Morrissey et al. 2009, Mendoza-Cózatl et al. 2014, Zhai et al. 2014), yet the related regulatory mechanisms are largely unknown.

Absorbed iron is incorporated into organometallic clusters, including iron–sulfur clusters and heme, mainly in mitochondria and chloroplasts (Couturier et al. 2013, Vigani et al. 2013, Balk and Schaedler 2014). The iron is subsequently utilized in many proteins, including factors functioning in respiration and photosynthesis. Accordingly, iron acquisition is regulated by

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various developmental and physiological cues in plants. Plant hormones including auxin, cytokinin, ethylene and jasmonic acid have been implicated in the regulation of iron acquisition (see Hindt and Guerinot 2012). For example, an abiotic stress-related plant hormone, ABA, was also shown to take part in mineral uptake regulation (Fan et al. 2014). Several ABA-hypersensitive mutants such as *ahg2-1* and *ahg11* have defects in mitochondrial functions, implicating mitochondria in abiotic stress and ABA responses in plants (Liu et al. 2010, He et al. 2012, Murayama et al. 2012, Yuan and Liu 2012, Zhu et al. 2012, Hirayama et al. 2013, Sechet et al. 2015). Reactive oxygen and nitric oxide have been proposed as signals mediating this process as both are produced concomitantly with defective mitochondrial function and accumulate during ABA responses, although this idea remains controversial (Gupta et al. 2011, Hancock et al. 2011, Schwarzländer and Finkemeier 2013). Recently heme has also been reported to connect mitochondrial functions and ABA responses in plants (Vanhee et al. 2011).

To address the physiological role of mitochondrial function in plant stress responses, we analyzed the *ahg2-1* mutant as a mitochondrial-defective model in Arabidopsis. *AHG2* encodes a poly(A)-specific RNase that is postulated to regulate mitochondrial mRNA stability (Nishimura et al. 2005, Hirayama et al. 2013). We found that the mutant was up-regulated in many genes related to iron metabolism and in several novel genes with the potential to encode short polypeptides. One of these novel genes, which we named *FE-UPTAKE-INDUCING PEPTIDE 1* (*FEP1*), strongly induced the expression of iron deficiency response genes in a FIT-independent manner. In addition, *FEP1*-deficient mutants showed impaired iron re-distribution. Since *FEP1*-like genes are found in several plant genomes, this previously uncharacterized polypeptide may be an important factor in iron deficiency responses in plants.

Results

Genes encoding novel short polypeptides are up-regulated in the *ahg2-1* mutant

To address the physiological status of the *ahg2-1* mutant, we conducted RNA sequencing (RNA-seq) analysis of the wild type and *ahg2-1* plants. Many genes were up- or down-regulated in the *ahg2-1* mutant compared with the wild type (Supplementary Table S1), consistent with our previous microarray data (Nishimura et al. 2009). Several genes responsive to iron deficiency, such as *IRT1*, *FRO2*, *bHLH38* and *bHLH39*, were up-regulated in the mutant, while an iron overload response gene, *FER1*, was down-regulated (**Fig. 1A**). The expression patterns of these genes implied that iron homeostasis was disrupted in the *ahg2-1* mutant. Indeed, we found that *ahg2-1* was more sensitive to low iron conditions (**Fig. 1B**) and that the heme content was significantly reduced in the mutant under iron-deficient conditions (**Fig. 1D**). However, *FIT*, which encodes a key transcriptional regulator in iron homeostasis, was not activated in the *ahg2-1* mutant (**Fig. 1A**). Notably, quantitative reverse transcription–PCR (qRT–PCR) analysis showed that transcripts for *At1g47395*, *At1g47400* and *At2g30766*, which encode short polypeptides (<100 amino acids) with similar amino acid sequences, were accumulated in the mutant (**Fig. 1C, E;** Supplementary Table S1). As shown below, the products of these three genes were involved in regulation of the iron deficiency response genes in Arabidopsis. Therefore, we named *At2g30766*, *At1g47395* and *At1g47400* as *FEP1*, *FEP2* and *FEP3*, respectively, after FE-UPTAKE-INDUCING PEPTIDE.

Expression pattern of *FEP* genes

To explore the physiological function of FEPs, we compared the expression patterns of *FEP1*, *FEP2* and *FEP3* under mineral-starved conditions with those of other iron-responsive genes. RNA was isolated from wild-type Arabidopsis hydroponically grown in medium lacking Fe, Mn, Cu or Zn for 5 d. The iron deficiency response genes were up-regulated under iron-deficient conditions as reported previously (**Fig. 2**). Under normal growth conditions, all *FEP* genes were highly expressed in aerial parts. *FEP2* and *FEP3* were also expressed at the same levels in roots as shoots, while *FEP1* showed much lower expression in roots. Interestingly, all *FEP* genes were strongly induced in roots and shoots under iron-deficient conditions after 1 d, and their expression peaked after 3 d. In contrast, the expression of the *FEP* genes was not affected strongly in the media deficient in Mn, Cu or Zn. These expression patterns of the *FEP* genes were very similar to those of *bHLH38* and *bHLH39*, suggesting that the *FEP* genes could be iron deficiency response genes. Among three *FEP* genes, the induction of *FEP1* was 100-fold higher than that of the other two *FEP* genes in roots. Accordingly, we focused on *FEP1* for further analysis.

The expression patterns of *FEP1* and *bHLH39* were analyzed using transgenic Arabidopsis lines expressing promoter–*GUS* (β-glucuronidase) reporter constructs. In the *proFEP1:GUS* lines, GUS staining was observed mainly in leaves under iron-sufficient conditions. When plants were subjected to Fe deficiency, the GUS staining in leaves was further enhanced (Supplementary Fig. S1A), consistent with the qRT–PCR data. In roots, although GUS staining was undetectable under iron-sufficient conditions, it was easily observed under iron-deficient conditions. Under iron-sufficient conditions, the *bHLH39* promoter-driven GUS activity was detected mainly in roots (Supplementary Fig. S1A). Upon transfer to iron-deficient conditions, GUS staining was further enhanced in roots and was observed weakly in the vasculature of leaves.

To address the distribution of *FEP1* and *bHLH39* expression in more detail, we performed immunostaining using an antibody against GUS on root and leaf cross-sections of these transgenic plants, which allowed us to detect the tissue distribution of *FEP1* expression more precisely. As shown in **Fig. 3A**, in *proFEP1:GUS* lines, GUS signal in the roots was detected strongly in a few cells located near the phloem under iron-deficient conditions. In the *probHLH39:GUS* lines, GUS signal in the roots was strongly detected in similar cells to the *proFEP1:GUS* lines and moderately detected in endodermis cells. In leaf sections, GUS was detected in several cells near the phloem in the vasculature in the *proFEP1:GUS* and

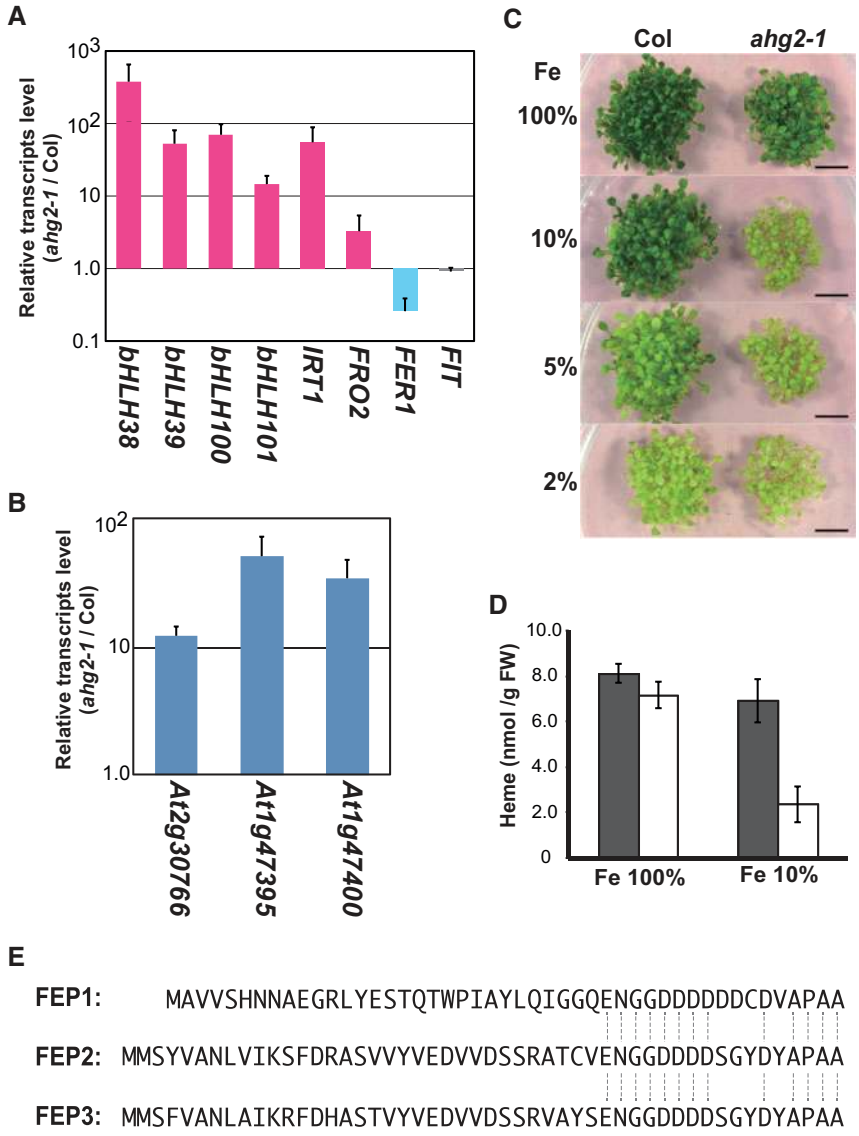

Fig. 1 The iron deficiency-related phenotypes and the *FEP* expression levels of the *ahg2-1* mutant. (A) qRT–PCR experiments of iron deficiency response genes. Total RNA obtained from 3-week-old plants (Col-0 wild type and the *ahg2-1* mutant) were used for cDNA synthesis. qRT–PCR data of each gene were normalized with the *ACT2* gene and shown as relative expression levels (*ahg2-1*/Col-0). Error bars indicate the SD (n = 4). (B) Relative expression levels of *FEP* genes deduced as in (A). (C) Plant phenotype under iron-deficient conditions. The wild type (Col-0) and the *ahg2-1* mutant were grown on agar medium containing standard Murashige and Skoog salts for 12 d. The medium with 0.1 mM $FeSO_4$ was named 'Fe 100%'. For the Fe-deficient plates, the amount of $FeSO_4$ was reduced to 10% (10 μM) or 2% (2 μM) (named Fe 10% and Fe 2%). Scale bar = 1 cm. (D) Effects of Fe deficiency on the amounts of heme. Error bars indicate the SE (n = 3 or 4). The P-values were calculated using R software (one-way ANOVA followed by Tukey's test). (E) Deduced amino acid sequence of the FEP peptides. The conserved amino acid residues are indicated with dotted lines.

probHLH39:GUS transgenic lines under iron-deficient conditions (**Fig. 3B**). GUS was also detected in mesophyll cells in the *proFEP1:GUS* lines under iron-deficient conditions (**Fig. 3B;** Supplementary Fig. S1B). These data indicate that the expression patterns of *FEP1* and *bHLH39* are distinct but overlap in the cells near the phloem under iron-deficient conditions.

FEP1-defective mutants show impaired root to shoot translocation of iron

To see whether FEP1 has physiological roles in the iron deficiency response, *FEP1*-defective mutants were generated using the CRISPR/Cas9 (clustered regulatory interspaced short palindromic repeat/CRISPR-associated protein 9) genome editing technology. We generated two mutant lines, *fep1-1* and *fep1-2*, homozygous for a 10 bp deletion and 1 bp insertion in the coding sequence of *FEP1*, respectively (Supplementary Fig. S2A, B). The *fep1* mutants were fertile and appeared to have normal morphology, but the leaf color was slightly brighter and the growth rates were slightly slower than in the wild type (**Fig. 4A–C;** Supplementary Fig. S2C, D). Inductively coupled plasma mass spectrometry (ICP-MS) analysis showed that, in the *fep1-1* mutant, the shoot iron concentration was notably lower while those of roots were slightly higher than those of the

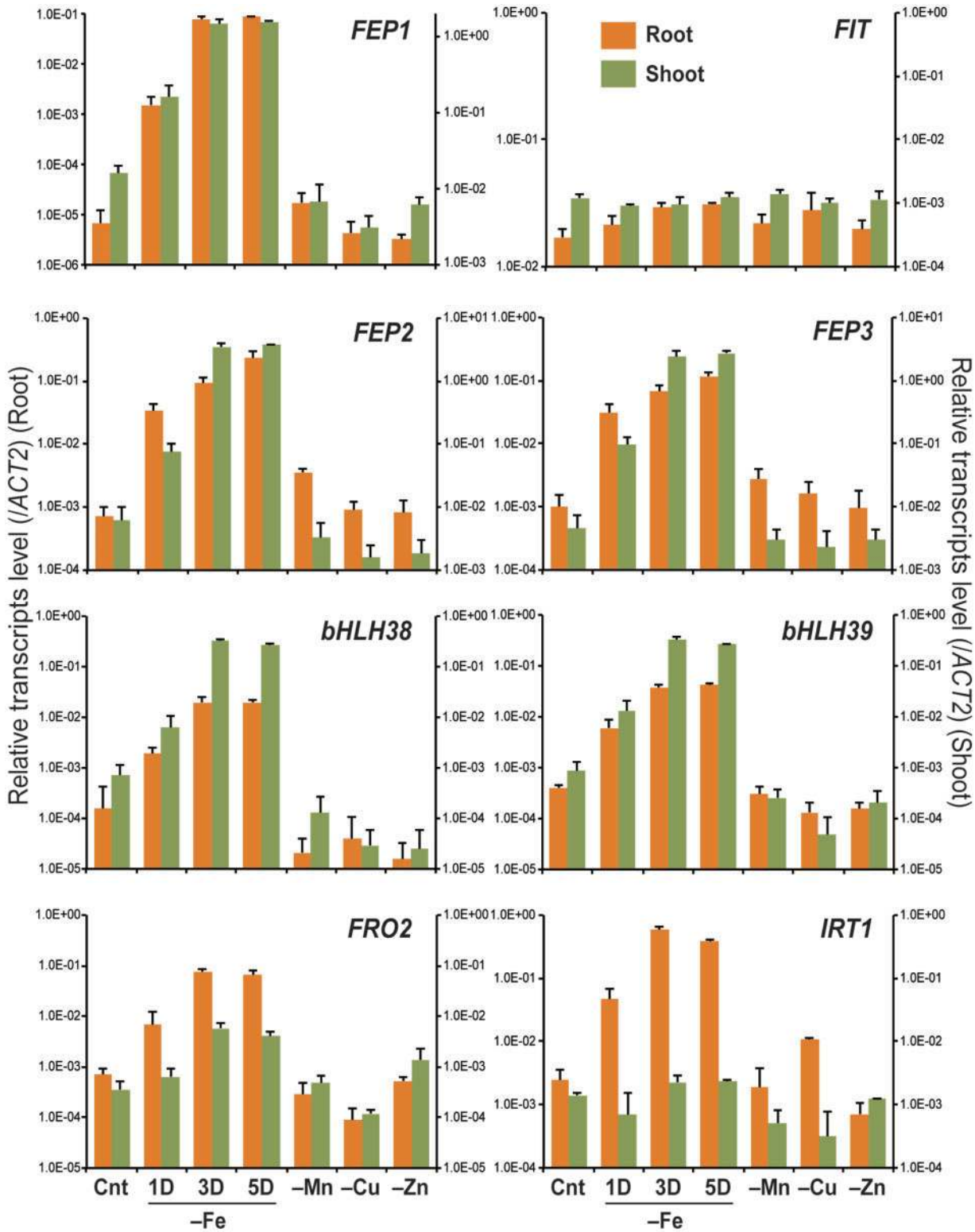

Fig. 2 Responses of the expression of *FEP1*, *FIT*, *FEP2*, *FEP3*, *bHLH38*, *bHLH39*, *FRO2* and *IRT1* to metal deficiency stresses. Total RNA was obtained from the roots or shoots of wild-type Arabidopsis grown hydroponically and treated with metal-sufficient or -deficient conditions (1, 3 or 5 d for iron and 5 d for other metals). The transcript levels were determined by qRT–PCR and normalized with the *ACT2* transcript level (orange, roots; green, shoots). The error bar indicates the SD (n = 3).

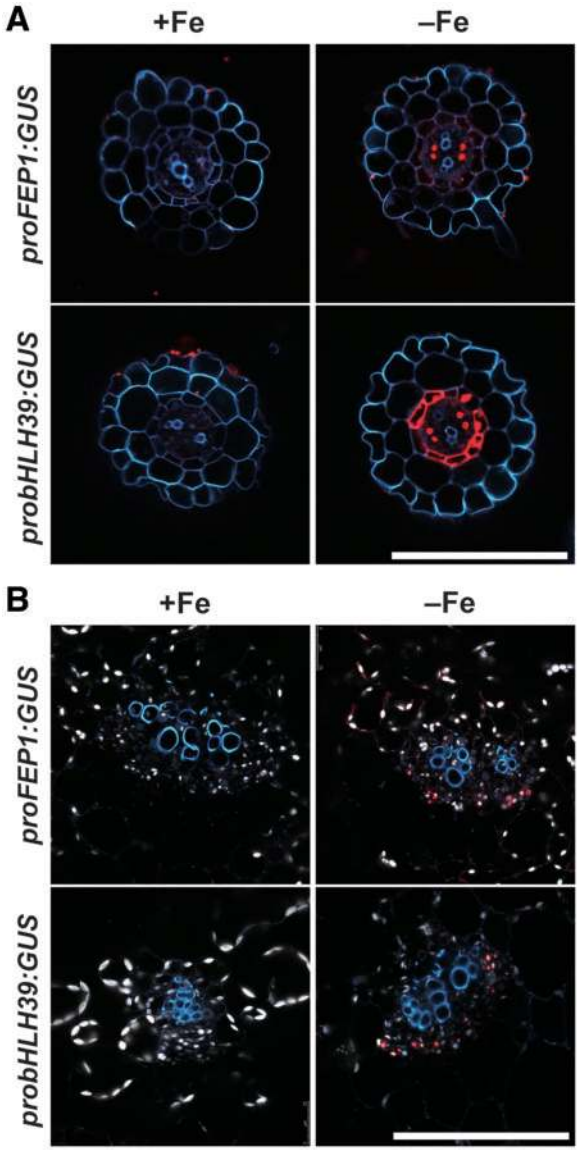

Fig. 3 Tissue-specific expression of *FEP1*. (A, B) Immunostaining for GUS in the *proFEP1:GUS* or *probHLH39:GUS* transgenic plants (A, root section; B, leaf section). Two-week-old plants were treated with iron-sufficient or iron-deficient medium for 5 d. Cross-sections of roots were subjected to immunostaining using anti-GUS antibody and Alex-Fluor 555-conjugated secondary antibody. The Alex-Fluor 555 was visualized with a laser confocal microscope. Scale bar = 100 μm.

wild type regardless of whether or not iron was supplied (**Fig. 4D, E**). The calculated iron translocation rates from roots to shoots were apparently reduced in the *fep1-1* mutant (**Fig. 4F**). The concentrations of other minerals, with the exception of Zn and Mn, were not affected significantly by the *fep1-1* mutation (Supplementary Fig. S3). Under iron-deficient conditions, the Zn concentration of the roots was increased and the Mn concentration of the roots was decreased in the mutant.qRT–PCR analysis revealed that, even under iron-sufficient conditions, *FER1* transcripts accumulated less in the *fep1-1* shoots (**Fig. 5**), consistent with its lower iron levels. In addition, the genes involved in regulation of the iron deficiency response, such as *bHLH38*, *bHLH39* and *FEP2*, were activated in the *fep1-1* shoots (13×, 9× and 18×, respectively) and moderately in the roots (6×, 4× and 5×, respectively) but their up-regulated levels were significantly lower than those under the iron deficiency conditions (**Figs. 2, 5**). These genes were further activated under iron deficiency conditions in *fep1-1*, suggesting that other genes are also necessary for the activation of these iron deficiency response genes. The transcript levels of iron uptake genes, *IRT1* and *FRO2*, in the roots were not different between the *fep1-1* mutant and the wild type. When grown on normal Murashige and Skoog (MS) agar medium, both *fep1-1* and *fep1-2* exhibited a similar up-regulation of *bHLH38*, *bHLH39* and *FEP2* only in the shoots, suggesting that the *fep1* defect disturbs the regulation of iron metabolism more strongly in shoots than in roots, and confirming that these phenotypes are caused by the *fep1* defect (Supplementary Fig. S4). Under the same conditions, stronger up-regulation of *bHLH38*, *bHLH39* and *FEP2* was observed in the *frd3-7* mutant, which is defective in a citrate efflux transporter required for iron translocation from roots to shoots and exhibits a constitutive iron deficiency response (Durrett et al. 2007, Roschzttardtz et al. 2011). In *frd3-7*, *FRO2* and *IRT1* were also activated. The expressions of *bHLH38*, *bHLH39* and *FEP2* in shoots were similar between the *fep1* and *frd3-7* mutants. However, the expressions of those genes in roots and of *IRT1* and *FRO2* in roots and shoots were quite different between the *fep1* and *frd3-7* mutants. These results suggest that *fep1* disturbs the iron translocation among plant tissues differently from *frd3-7*. The expression of the genes shown to be involved in iron translocation, such as *FRD3*, *OPT3*, *YLS1*, *YLS2*, *YLS3* and *FPN1*, were not significantly altered in *fep1* and *frd3-7* under the experimental conditions tested (Supplementary Fig. S4).

Transient expression of FEP1 induces an iron deficiency response in planta

To address the physiological function of FEP1, we generated transgenic plants in which the expression of *FEP1* could be transiently induced by exposure to estradiol (ER) (Zuo et al. 2000). When roots of these transgenic plants were treated with ER, *FEP1* was up-regulated approximately 6,000-fold within a day (**Table 1**). In the same root samples, the expression of *bHLH39* was elevated 10–100×. Upon induction of *FEP1* by the treatment with ER, *bHLH38*, *IRT1* and *FRO2* were up-regulated, while *FER1* was down-regulated.

Using this ER–FEP1 inducible expression system, we examined whether FEP1 modulates the iron levels in planta by ICP-MS. Under iron-sufficient conditions, an ER-induced *FEP1* caused an elevation of endogenous iron content, as was expected (**Fig. 6**). This FEP1-inducible iron accumulation was dependent on iron supply in the media, suggesting that this iron accumulation relied on the iron uptake ability of plants. Comprehensive analysis of minerals revealed that the *FEP1* induction by ER did not affect the endogenous levels of other minerals examined (Cu, Zn, Mg, Mn, K, P or Ca) (Supplementary Fig. S5). Therefore, it is more plausible that the high accumulation of endogenous iron by FEP1 is the

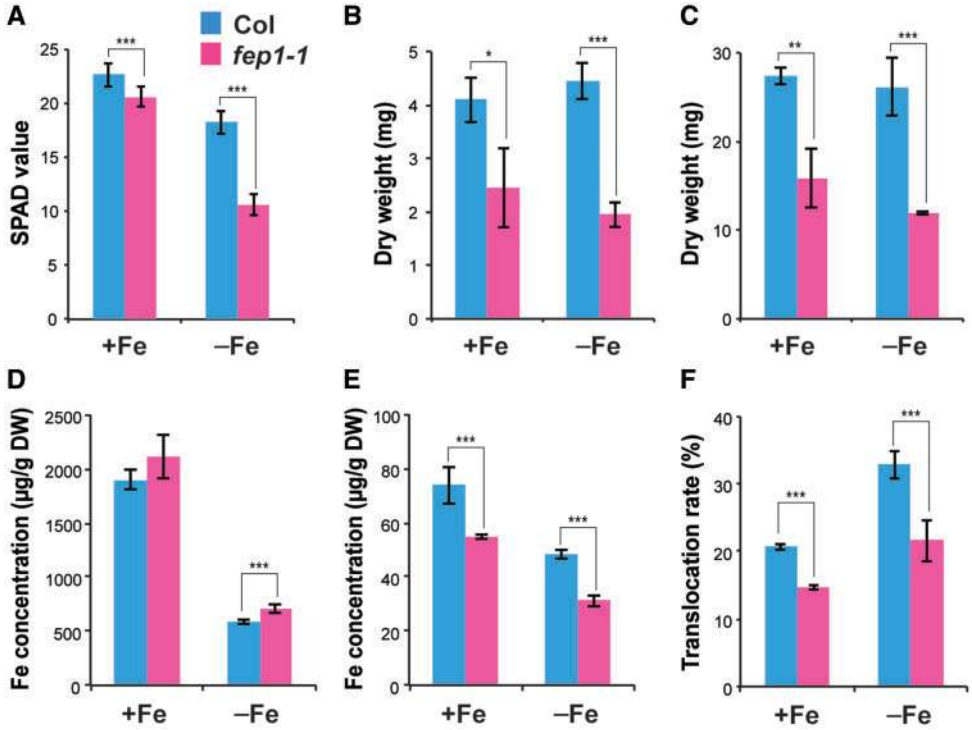

Fig. 4 Phenotype of the *fep1* mutant. (A) SPAD value, (B, C) dry weight of the roots (B) and shoots (C). (D–F) Concentration of Fe in the roots (D) and shoots (E), and calculated root to shoot translocation (F). Wild-type or *fep1-1* Arabidopsis grown hydroponically were subjected to a solution containing iron (+Fe) or without iron (−Fe) for 5 d. Error bars indicate the SD (n = 9 for A, n = 3 for B–F). ***$P < 0.01$, **$P < 0.05$ and *$P < 0.1$, by Student's t-test.

direct effect of the modulation of iron homeostasis rather than an indirect effect caused by other physiological perturbations.

A cascade of gene expression occurs upon FEP1 induction

To characterize the gene expression regulation by FEP1, a time-course assessment of gene induction was examined with the ER-inducible *FEP1* line. The transcript levels of *FEP1*, *bHLH39* and *IRT1* were examined at 1–24 h after the ER treatment. The *FEP1* transgene was activated in 1 h after the ER treatment, and its expression level reached its maximum at around 10 h (**Fig. 7**). The activation of *bHLH39* was detected around 2 h after the ER treatment. Although slightly increased expression of *bHLH39* was also observed at 2 h in the ER–green fluorescent protein (GFP) transgenic line, presumably due to physical stresses conferred by the ER treatment, the increased expression of this gene was apparent at 5 h in the ER-inducible *FEP1* transgenic plant. The up-regulation of *IRT1* was detected at 5 h after the ER treatment. These data indicate that the order of activation over time is ER–FEP1 > bHLH39 > IRT1, implying that FEP1 activates the regulatory system previously proposed for the iron deficiency response genes. Interestingly, endogenous *FEP1* was also induced upon ER treatment. The induction was apparent at 2 h after the treatment, peaked at 10 h and rapidly decreased at 24 h (**Fig. 7**). This result indicates that *FEP1* is activated by FEP1 itself, and was then negatively regulated after the induction.

Table 1 Expression levels in roots under FEP1 or GFP induction[a]

	FEP1[b]	GFP[b]
FEP1	6,625.4 ± 2,105.88	1.92 ± 0.72
bHLH38	40.7 ± 3.04	0.5 ± 0.11
bHLH39	15.79 ± 2.66	0.79 ± 0.17
IRT1	46.34 ± 12.14	0.14 ± 0.03
FRO2	61.18 ± 13.99	0.08 ± 0.03
FER1	0.25 ± 0.02	0.94 ± 0.06
FIT	1.72 ± 0.30	1.05 ± 0.23

[a]RNA samples obtained from three independent root samples were used for qRT–PCR experiments. The expression levels were normalized with *ACT2* levels, and the changes between ER treatment 0 h and 24 h were calculated. SD is shown with ± (n = 3).
[b]Induced gene with the ER induction system in transgenic plants.

Transient expression of *FEP1* in protoplasts induces an iron deficiency response gene

The data presented above suggested that FEP1 regulates iron deficiency response genes such as *bHLH39*. To understand the molecular mechanism by which FEP1 regulates *bHLH39*, we examined the effect of *FEP1* on the activation of a *probHLH39:LUC* (luciferase) reporter gene in detail in Arabidopsis leaf protoplasts. As summarized in **Fig. 8A**, LUC activity was significantly induced in the protoplasts transiently expressing *FEP1*. To address the characteristics of *FEP1* necessary for its gene-activating ability, we constructed several recombinant *FEP1* genes (**Fig. 8A;** Supplementary Fig. S6A).

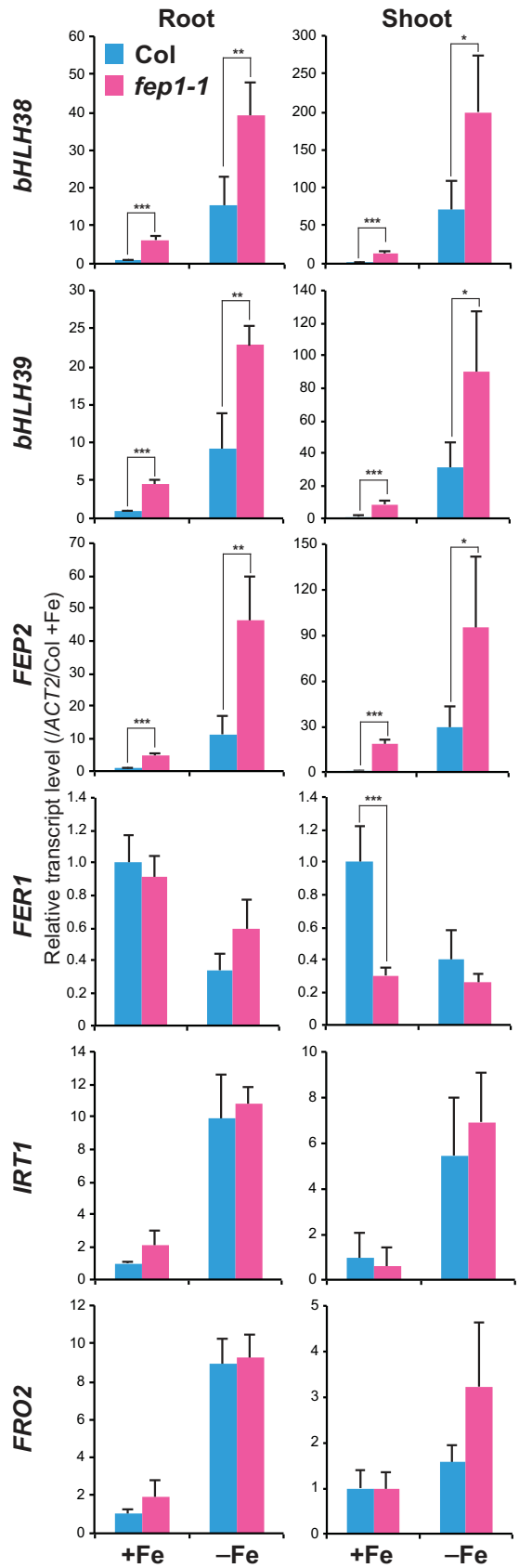

Fig. 5 Expression of iron deficiency response genes in the *fep1* mutant. Total RNA was extracted from hydroponically grown tissues used in **Fig.4** for qRT–PCR. The transcript levels were normalized with the *ACT2* transcript level and shown as the relative value compared with the wild type under iron-sufficient conditions. The error bar indicates the SD ($n = 3$). $^{***}P < 0.01$, $^{**}P < 0.05$ and $^{*}P < 0.1$, by Student's *t*-test.

A nonsense codon mutation at the start codon or fourth codon, or the insertion of a frameshift mutation completely abolished the FEP1 activity. However, synonymous substitutions, in which 37 bases out of 141 bases were substituted (Supplementary Fig. S6B), did not compromise the FEP1 activity. These results strongly suggest that the translated peptide, but not the transcribed RNA, is the functional molecule. This idea was consistent with the fact that *fep1-1* (10 bp deletion) and *fep1-2* (1 bp insertion) had an identical phenotype. Intriguingly, the expression of *FEP2* did not affect LUC activity in protoplasts, implying that FEP1 and FEP2 have different functions.

The amino acid sequence of the C-terminal half of FEP1, especially the C-terminal 17 amino acids, closely resembles those of FEP2 and FEP3. The insertion of two amino acid residues in the C-terminal half completely abolished the FEP1 activity, suggesting that the amino acid sequence of the C-terminal half is important for FEP1 function. We attempted to produce functional epitope- or fluorescent molecule-tagged FEP1; however, C-terminal fusions with any type of tag abolished the FEP1 activity. Therefore, this conserved C-terminal sequence, and the C-terminal structures seem to be important for the FEP activity. In addition, there are several open reading frames with potential to encode a FEP1-like short polypeptide in other plant genomes, implying that FEP-like genes are conserved among plants (Supplementary Fig. S6C).

The FEP1 recombinant protein with a two amino acid insertion in the N-terminal region possessed considerable activity, suggesting that insertions in the non-conserved region may not affect the FEP1 activity. Accordingly, we introduced a GFP tag sequence into the *Mfe*I and *Msc*I sites in the middle parts of *FEP1* in-frame, to produce recombinant FEP1–*Mfe*I–GFP and FEP1–*Msc*I–GFP genes, respectively (Supplementary Fig. S6A). These recombinant FEP1 proteins with GFP insertions were active in protoplast assays for *probHLH39* activity (**Fig. 8B**). The fluorescence of GFP-inserted FEP1 was found in the cytoplasm and nucleus (**Fig. 8C**), and immunoblotting with anti-GFP antibody detected a single band with higher molecular weight than GFP alone (**Fig. 8D**). These data imply that FEP1-fused GFP has not undergone proteolytic processing and functions in the cytoplasm or nucleus.

Activation of *bHLH39* by FEP1 is independent of FIT

A bHLH-type transcription factor, FIT, is a key regulator of the iron deficiency response genes in Arabidopsis (Colangelo and Guerinot 2004, Jakoby et al. 2004). To investigate whether gene activation by FEP1 is dependent on FIT, we used a T-DNA insertion mutant of *FIT* (Salk_126020), *fit-2*, grown in iron excess conditions, as the protoplast source. LUC activity was significantly higher in the cells expressing *FEP1* than in the cells expressing *GFP* (control) (**Fig. 8E**). This increased LUC activity was

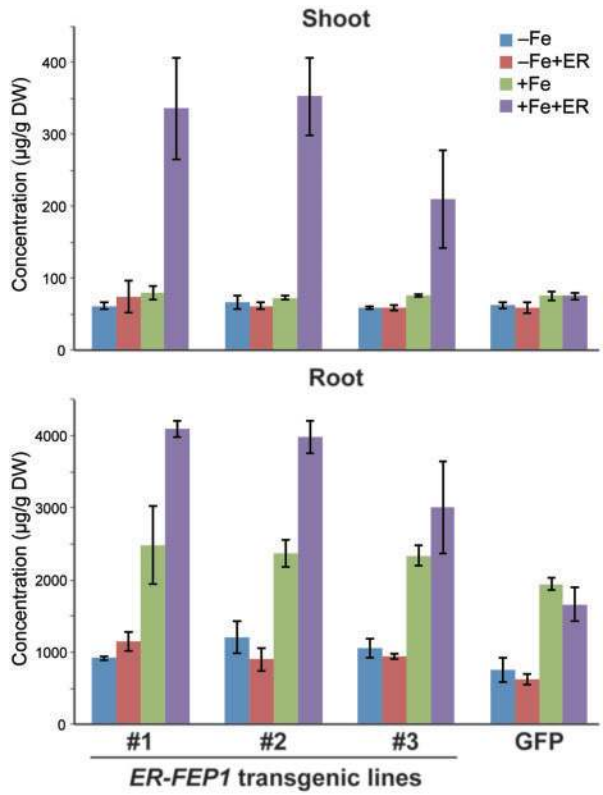

Fig. 6 Iron levels in the *FEP1*-induced plants. Three independent transgenic lines (#1–#3) possessing the estradiol (ER)-inducible *FEP1* were used for iron measurement by ICP-MS analysis. Plants grown hydroponically on 1/10 Hoagland's solution (pH 5.8) for 3 weeks were transferred to 1/10 Hoagland's solution containing iron (+Fe) or without iron (−Fe) in the absence or presence of 1.0 μM ER for 1 week. The shoots (upper panel) and roots (lower panel) were used for ICP-MS analysis. Error bars indicate the SD (n = 3).

not further enhanced by co-transfection with *pro35S:FIT*. These data suggest that FIT is dispensable for the activation of *bHLH39* by FEP1. FIT functions as a heterodimer with other bHLH-type transcription factors including bHLH39 (Yuan et al. 2008). Therefore, the effect of co-expression of FIT with bHLH39 was also assessed in wild-type protoplasts. When FIT and bHLH39 were co-expressed, the activation of the *bHLH39* promoter by FEP1 was significantly reduced (**Fig. 8F**). These data suggest that FIT and bHLH39 are not required for the up-regulation of *bHLH39* by FEP1, and imply that high levels of FIT and bHLH39 interfere with FEP1 activity. It is noteworthy that *FEP1* was expressed in *fit-2* at a level comparable with that of the wild-type plants under iron deficiency conditions shown in **Fig. 2**, like *bHLH39*, suggesting that the expression of *FEP1* is also independent of FIT (Supplementary Fig. S6D).

Ectopic expression of *FEP* genes activates iron deficiency response genes

To address the role in the iron distribution of *FEP1*, we generated transgenic Arabidopsis plants overexpressing *FEP1*, or another two *FEP* genes (*FEP2* or *FEP3*) from the 35S promoter. Interestingly, the *pro35S:FEP1* lines showed severe growth retardation (Supplementary Fig. S7A), with several T_1 plants becoming pale green and dying before flowering. Iron deficiency response genes, *bHLH38*, *bHLH39*, *FRO2* and *IRT1*, were significantly up-regulated in the roots of *pro35S:FEP1* (Supplementary Fig. S7B) Although they showed no visible differences in phenotype compared with the wild type, *pro35S:FEP2* and *pro35S:FEP3* were also up-regulated in these genes, albeit to lower levels than those in *pro35S:FEP1*. These data support the idea that these short polypeptides are involved in the iron deficiency response in Arabidopsis. In contrast, the expression of these genes was not affected or was slightly decreased in shoots in any of the overexpression lines (Supplementary Fig. S7B), implying that ectopic expression of *FEP* genes affect the systemic iron response.

Discussion

In this study, we described a gene, *FEP1*, encoding a novel peptide that has a pivotal role in the regulation of iron metabolism in Arabidopsis. This conclusion is supported by our data showing: (i) *FEP1* is activated under iron-deficient conditions in both roots and shoots (**Figs. 2, 3**); (ii) the *fep1* mutants have an iron deficiency phenotype in shoots (**Figs. 4, 5**); (iii) induced expression of *FEP1* alone caused up-regulation of several iron deficiency response genes and resulted in iron accumulation in the roots and shoots of the transgenic plants, which was dependent on iron supply in the medium (**Figs. 6–8**). A BLAST search showed that *Brassica*, *Camelina*, *Raphanus* and rice (*Oryza sativa*) may possess FEP1-like genes (Supplementary Fig. S6C), indicating that the iron deficiency regulation by FEP1 is conserved among plants. Taken together, this study reveals that polypeptides of <50 amino acids play pivotal roles in the regulation of iron metabolism in plants (Supplementary Fig. S8).

The known iron deficiency response regulator FIT activates genes involved in iron uptake through forming a heterodimer with the Ib subfamily bHLH transcription factors such as bHLH38 and bHLH39. However, the mechanisms that regulate these genes are still unknown. Our data showed that FEP1 is involved in the activation of *bHLH38* and *bHLH39*. Using an ER-inducible system, we demonstrated that *bHLH39* was activated within 2 h after *FEP1* was induced (**Fig. 7**). Given such a quick response, it is not likely that additional gene activation or drastic physiological changes intervene between the expression of *FEP1* and *bHLH39*. Our data further indicated that the activation of *bHLH39* by FEP1 is independent of FIT (**Table 1**; **Figs. 7, 8**). This result is consistent with a previous report showing that the bHLH genes were activated under iron deficiency conditions in the *fit* mutant (Wang et al. 2007). FEP1 might regulate the gene expression directly, through a previously undescribed molecular mechanism.

The *FEP1* transcripts in roots were increased 10,000× under iron-deficient conditions (**Fig. 2**), suggesting that *FEP1* is highly responsive to iron deficiency in wild-type Arabidopsis. According to our data, *FEP1* is activated by FEP1 itself (**Fig. 7**). Presumably, FEP1 amplifies the iron deficiency signal through self-activation. Our transient assays using protoplasts showed that the simultaneous expression of *FIT* and *bHLH39*

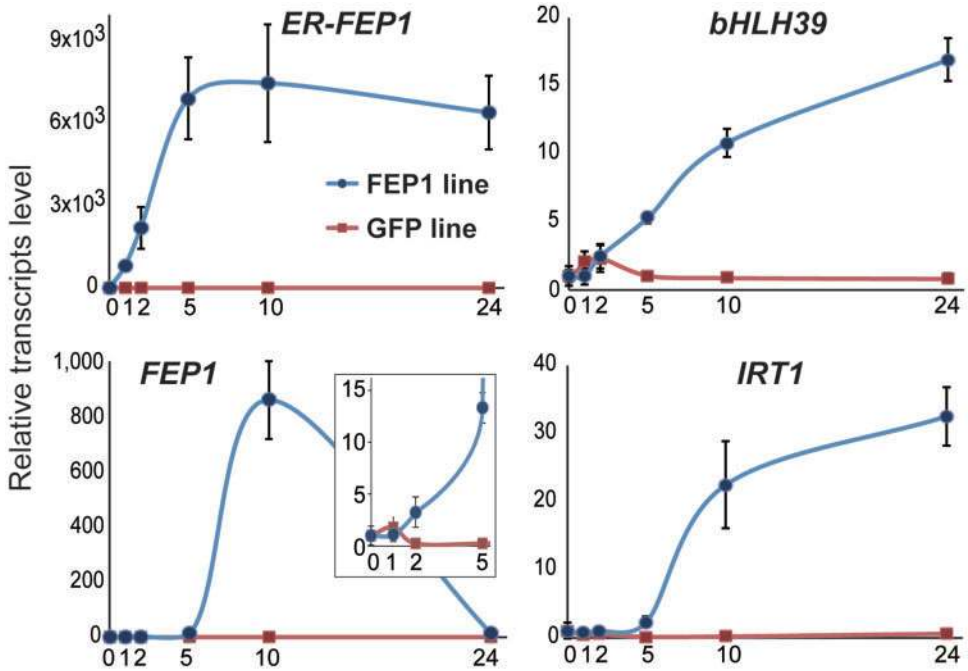

Fig. 7 Time-course analysis of gene expression upon *FEP1* induction. Two-week-old *ER-FEP1* (blue line) or *ER-GFP* (red line) transgenic plants were treated with 10 μM estradiol for the indicated period and total RNA was extracted from roots. qRT–PCR analysis was performed using RNA samples and gene-specific primers (Supplementary Table S2). The inset in the *FEP1* graph is the enlarged graph from 0 to 5 h. Error bars indicate the SD (n = 3).

inhibited the activation of *bHLH39* by FEP1 (**Fig. 8F**). Thus, we postulate that in the iron regulatory system, *FEP1* is positively regulated, presumably indirectly, by FEP1 itself and negatively regulated by its downstream factor bHLH39, as well as FIT, allowing a transient and strong amplification of the iron deficiency signal. This scenario is supported by the decreased expression of *FEP1* (and of *FEP2* and *FEP3*) in the *bHLH39*-overexpressing transgenic plants (Naranjo-Arcos et al. 2017). Activation of endogenous *FEP1* by the ER-inducible *FEP1* transgene was transient under iron-sufficient conditions (**Fig. 7**), while *FEP1* was activated during the iron deprivation treatments (**Fig. 2**). Therefore, the self-activation of *FEP1* might be dependent on the iron status of the cells.

Even under iron-sufficient conditions, the *fep1*-defective mutants had lower iron levels in shoots but the same iron levels in roots when compared with the wild type (**Fig. 4F**), suggesting that the translocation of iron from roots to shoots was reduced in the *fep1-1* mutant as in the *FRD3*-defective mutant (Green and Rogers 2004). However, in contrast to *frd3-7* wherein most of the iron deficiency response genes are up-regulated in both shoots and roots, *bHLH38*, *bHLH39* and *FEP2*, but not *FRO2* and *IRT1*, were up-regulated only in shoots of the *fep1* mutants. This difference suggests that *fep1* does not cause a simple defect in the iron translocation from roots to shoots. Regarding the normal iron levels and normal expression levels of iron deficiency response genes in roots, *fep1* might be defective in the regulation of iron re-distribution in roots or in the signaling of iron deficiency from shoots to roots. Ectopic or induced expression of *FEP1*, in contrast, activated the iron deficiency response genes including *FRO2* and *IRT1* in roots, but down-regulated them in shoots, as in *35S-FEP1* lines (**Table 1**; **Fig. 7**; Supplementary Fig. S7B); however, both roots and shoots had high iron contents in the ER-treated *ER-FEP1* lines (**Fig. 6**). The expression of known factors implicated in iron metabolism was not changed in *fep1*. Taking these data into consideration, it is likely that *FEP1* is involved in the regulation of iron distribution in Arabidopsis, although its molecular mechanism remains to be elucidated.

In the *FEP1*-defective mutants, *bHLH38* and *bHLH39* were slightly up-regulated (~10× in shoots, ~5× in roots) under iron-sufficient conditions and further activated in iron-deficient conditions (**Fig. 5**; Supplementary Fig. S4). This result suggests that the iron deficiency response is not impaired completely in *fep1*, and that FEP1 is not the sole activator of these bHLH genes. Presumably, the effect of loss of function of FEP1 is partly compensated for by FEP2 and FEP3. Although our data showed that the functions of FEP1 and FEP2 are different (e.g. **Fig. 8A**), overexpression of *FEP2* or *FEP3* resulted in the activation of iron deficiency response genes (Supplementary Fig. S7B). On the other hand, *FRO2* and *IRT1* were not activated in *fep1* even in shoots (**Fig. 5**; Supplementary Fig. S4). The expression of these two genes has been shown to be activated by bHLH and FIT. Indeed, transgenic plants overexpressing *bHLH39* (~1,000×) have increased expression of *FRO2* and *IRT1* even under iron-sufficient conditions and accumulate more iron than wild-type plants (Naranjo-Arcos et al. 2017). In *frd3-7*, where *FRO2* and *IRT1* are constitutively activated, these bHLH genes were up-regulated 10 times more than *fep1*. Therefore, it is possible that the increased expression levels of bHLH genes in *fep1* mutants were not sufficient for activation of *FRO2* or *IRT1*. To clarify the discrepancy in the expression of bHLH genes and *IRT1* in *fep1*, further analyses on FEPs are required.

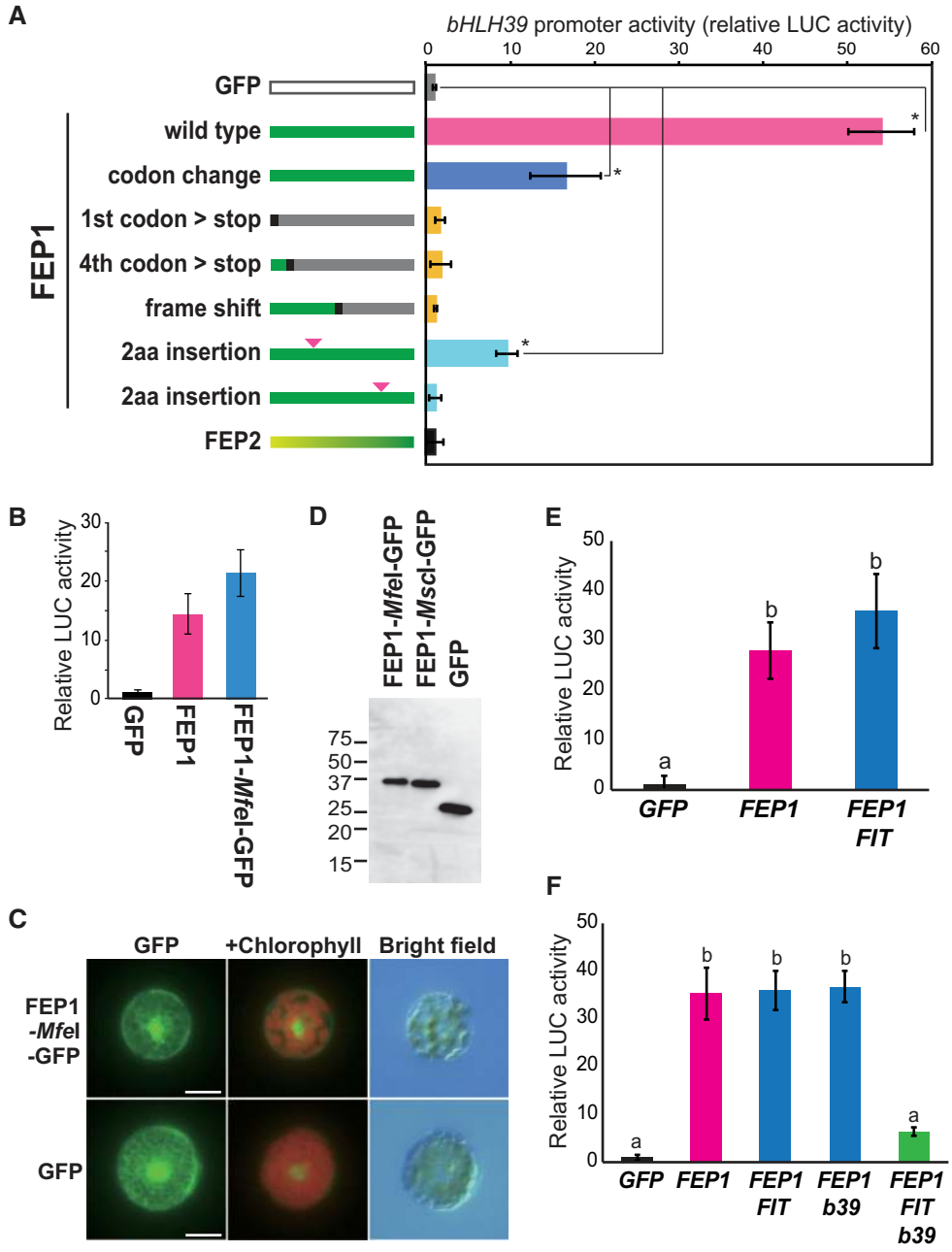

Fig. 8 FEP1 activates *bHLH39* in protoplasts. (A, B) Transfection experiment using protoplasts. The wild type or various *FEP1* recombinants (A) or FEP1–(*Mfe*I)–GFP (B) were co-transfected with the *probHLH39:LUC* reporter gene and the *pro35S:GUS* gene into Arabidopsis leaf protoplasts. LUC activity was normalized with GUS activity and shown as activity relative to that of the control (GFP). Error bars are the SD of three independent transfection reactions (*$P < 0.01$ by Student's *t*-test, $n = 3$). (C) Fluorescence of FEP1–(*Mfe*I)–GFP or GFP expressed in protoplasts. Scale bar = 20 μm. (D) Detection of FEP1–GFP fusion proteins. Total protein obtained from protoplasts expressing FEP1–(*Mfe*I)–GFP, FEP1–(*Msc*I)–GFP or GFP alone was immunoblotted. (E, F) Transfection experiments using *fit-2* mutant (E) or wild-type protoplasts (F). *pro35S:FEP1*, *pro35S:FIT* or *pro35S:bHLH39* was co-transfected with *probHLH39:LUC* and *pro35S:GUS* into the protoplasts. LUC activity was normalized with GUS activity and shown as activity relative to that of the control (GFP) (mean ± SD, $P < 0.05$ one-way ANOVA followed by Tukey's test, $n = 3$).

The emerging picture of the regulation of mineral metabolism suggests that peptides have pivotal functions in the regulation of mineral homeostasis. Small peptides are known to be involved in the systemic regulation of nitrogen acquisition in Arabidopsis (Tabata et al. 2014, Ohkubo et al. 2017). Our current results present a possibility that polypeptides of <50 amino acids are also involved in the systemic iron deficiency response. Most mobile signaling peptides are translated as precursor peptides and become mature, functional short peptides after going through several processing steps (Matsubayashi 2014). These mobile signaling peptides usually possess a signal sequence at the N-terminus for secretion. However, FEP1 and other FEPs do not have such a sequence. There are several small peptides without the signal sequence that function in extracellular signaling (Yamaguchi et al. 2006, Ohkubo et al. 2017). By analogy, it is possible that FEP1 is involved in long-distance signaling of iron

acquisition. The expression of *FEP1* was higher in leaf mesophyll cells, but its downstream gene, *bHLH39*, was not expressed in these cells in the wild type (Supplementary Fig. S1). This discrepancy in expression might be explained by the idea that FEP1 moves from mesophyll cells to the vasculature tissues of leaves and roots through cell to cell protein movement or via unconventional secretion pathways (Drakakaki and Dandekar 2013), which is consistent with the observation that the *fep1-1* mutant has a defect in iron distribution from roots to shoots. This idea is also supported by many studies showing that the iron deficiency signal is detected at the leaves and transduced to roots (García et al. 2013, Mendoza-Cózatl et al. 2014, Zhai et al. 2014, Gayomba et al. 2015, Kumar et al. 2017). Alternatively, FEP1 may function intracellularly, similarly to ENDO40, PORALIS, ROTUNDIFOLIA4 and DIP2 (Casson et al. 2002, Röhrig et al. 2002, Narita et al. 2004, Castelló et al. 2011). Immunostaining for GUS driven by the *FEP1* or *bHLH39* promoter indicated that *FEP1* and *bHLH39* are expressed in the same cells or tissues in the vasculature, supporting this alternative idea. Indeed, a peptide encoded by a gene that was previously annotated as a long non-coding RNA gene in insects and vertebrates has been found to regulate a calcium transporter through direct interaction (Magny et al. 2013). FEP1 might similarly regulate various kinds of factors including iron transporters. In any case, the molecular mechanism by which FEP1 regulates the expression of the genes is totally unknown. Further analysis of FEP1 is required to obtain more insights into iron homeostasis in plants.

We found that *FEP1* was constitutively activated in the ABA-hypersensitive *ahg2-1* mutant that has compromised function of mitochondria. This mutant contained a lower level of heme under iron deficiency conditions. Thus, the *ahg2-1* mutation might disturb iron metabolism or iron homeostasis. It is likely that the level of heme or of other iron-co-ordinating molecules is one of the determinants for the induction of *FEP1*. Recently, a link between iron metabolism or heme and the ABA response was demonstrated in Arabidopsis and a red alga (Connolly et al. 2003, Schwarzländer and Finkemeier 2013, Lei et al. 2014, Kobayashi et al. 2016, Nishimura et al. 2018). In addition, another stress-related phytohormone, salicylic acid, that is also accumulated in *ahg2-1* is implicated in the iron deficiency response (Shen et al. 2016). Further analysis of the *ahg2-1* mutant will offer a better understanding of the physiological relevance of stress-related plant hormones in iron metabolism of higher plants.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia-0 (Col-0) was used in this study. Plants were grown on MS plates [1× Murashige and Skoog salt mix, 2% sucrose, 2.5 mM MES (pH 5.8) and 0.8% agar], on soil, or hydroponically with 1/10 Hoagland medium at 23°C under 16 h light/8 h dark cycles. The T-DNA *fit-2* mutant seeds (Salk_126020) and *frd3-7* mutant seeds (Salk_122235) were obtained from TAIR (Alonso et al. 2003).

RNA sequencing

Total RNA was prepared from 2-week-old plants of the wild type (Col-0) and *ahg2-1* mutants using the RNeasy kit according to the manufacturer's instructions (Qiagen). RNA quality (RIN >7) was confirmed with an Agilent Bioanalyzer (Agilent). rRNA was removed with the Ribo-Zero Kit (Illumina) and the remaining RNA sample was subjected to library construction with the TruSeq Stranded mRNA kit (Illumina). The sequence analysis was done with Illumina Hiseq, generating paired-end reads. Consequently, 60 million reads with Q30Bases >96.3% and MeanQS >37.3 were obtained. Raw data of RNA-seq analysis were mapped to the Arabidopsis genome sequence using the R package, QuasR (Gaidatzis et al. 2015). Annotation count, and count comparison among samples were performed with the R packages Rsamtools and edgeR, respectively (Robinson et al. 2010).

Heme measurement

About 20–50 mg (FW) of 12-day-old seedlings was frozen in liquid nitrogen and crushed to a fine powder by a mortar and pestle. The powder was then extracted in 1 ml of 80% neutral acetone (acetone:water = 80:20, v/v). After centrifugation at 10,000×*g* for 15 min at 4°C, the pellet was extracted with 0.75 ml of 80% acidic acetone (acetone:1.6 M HCl = 80:20, v/v). After centrifugation at 10,000×g for 15 min at 4°C, the supernatant (containing total non-covalently bound heme) was collected and used to determine heme content using the Hemin Colorimetric Assay Kit (BioVision #K672-100).

Producing *fep1* mutants with the CRISPR/Cas9 technique

The target sequences were selected using the FOCUS program (Osakabe et al. 2016). DNA segments corresponding to guide RNAs for selected target sites were synthesized by PCR and introduced into the *Bsa*I site of a vector, pEgP126-Paef1-2A-GFBSD2 (Osakabe et al. 2016). The obtained constructs were used for transformation of Arabidopsis with *Agrobacterium tumefaciens*. The *fep1* mutant lines were obtained only in the transgenic lines with the target1 guide RNA (Supplementary Fig. S2A). After confirmation by sequencing, the homozygous lines were established. The off-target site with the highest probability among predicted target sites was confirmed to be intact in the *fep1* mutants by sequence analysis of the PCR product around the predicted target site.

Gene expression patterns in response to metals deficiency

Plants were grown in a 1/10 Hoagland solution (pH 5.8) in a room with a 14 h light/10 h dark cycle at 23°C. The nutrient solution was changed once every 2 d. The nutrient solution contained the macronutrients (mM) KNO_3 (0.5), $Ca(NO_3)_2$ (0.5), $MgSO_4$ (0.2) and $(NH_4)H_2PO_4$ (0.1), and the micronutrients (μM) NaFeEDTA (20.0), H_3BO_3 (3.0), $MnCl_2$ (0.5), $CuSO_4$ (0.2), $ZnSO_4$ (0.4) and $(NH_4)_6Mo_7O_{24}$ (1.0). The nutrient solution was changed once every 2 d. After about 5 weeks of culture, the seedlings were exposed to a 1/10 Hoagland solution (pH 5.8) without Fe for 1, 3 or 5 d, or without Mn, Cu or Zn for 5 d. Whole roots and shoots were sampled and frozen immediately for extracting RNA for gene expression.

qRT–PCR

Total RNA was isolated from plant materials using Sepasol reagent (Nacalai Tesque) and used for cDNA synthesis. cDNA was synthesized using the PrimeScripts cDNA synthesis kit (TAKARA BIO INC.) with random primers. qRT–PCR was performed on a LightCycler (Roche Diagnostics) in a total volume of 20 μl containing 10 μl of TB Green Primer Ex Taq II (TAKARA BIO INC.), 8 pmol of each primer and a cDNA mixture. The amplification program consisted of 40 or 50 cycles of 95°C for 10 s and 60°C for 1 min. The relative transcript level was deduced with the comparative Ct method using the expression of endogenous *ACT2* as a control. Primers used for qPCR or qRT–PCR experiments are listed in Supplementary Table S2.

Immunostaining

After being grown in MS plates for 2 weeks, the seedlings possessing *proFEP1:GUS* or *probHLH39:GUS* 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) with or without Fe for 5 d. Different tissues were harvested

for immunostaining using an anti-GUS antibody as described previously (Yamaji and Ma 2007).

Protoplast experiments

Arabidopsis leaf mesophyll protoplasts were obtained according to the methods described previously by Yoo et al. (2007) or Wu et al. (2009). Nearly 10^4 protoplast cells were incubated with approximately 10 μg of each plasmid DNA. A DNA segment for the promoter region of *bHLH39* was amplified by PCR with specific primers (Supplementary Table S2) using genomic DNA of Arabidopsis Col-0 as a template, and placed upstream of the LUC gene. Transfected protoplasts were incubated at 23°C for 20 h in the dark and subjected to experiments. For LUC and GUS assays, protoplasts were collected by centrifugation at 200×*g* for 2 min and resolved in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl and 5 mM EDTA, and the supernatant was used for these assays. For construction of the FEP1–GFP gene, the *GFP* gene was inserted in-frame into the *Mfe*I site [FEP1–(*Mfe*I)–GFP] or the second *Msc*I site [FEP1–(*Msc*I)–GFP] (Supplementary Fig. S6A).

Fe accumulation in transgenic Arabidopsis lines

Plants were grown in a 1/10 Hoagland solution (pH 5.8) in a room with a 14 h light/10 h dark cycle at 23°C. The nutrient solution was changed once every 2 d for about 6 weeks. The plants were exposed to a 1/10 Hoagland solution (pH 5.8) with or without Fe and 0 or 1 μM ER for 7 d. Whole leaves were then harvested directly. Some root tips were sampled and frozen immediately for extracting RNA for gene expression. The remainder of the roots were harvested and washed with cold 1 mM $CaCl_2$ solution four times. Dried samples were digested with concentrated HNO_3 (60%) at 135°C. The concentration of the elements in the digest solution was determined by ICP-MS.

Constructing transgenic plants

The cDNA clone for *FEP1*, *FEP2* or *FEP3* (from the start codon to the stop codon) was fused to the *35S* promoter of a binary vector or the estrogen-inducible promoter of pER8 (Zuo et al. 2000). The DNA segment for the promoter region of *bHLH39* or *FEP1* was amplified by PCR with specific primers (Supplementary Table S2) using the genomic DNA of Arabidopsis Col-0 as a template and fused to the *GUS* coding gene of a binary vector. The obtained plasmid constructs were used for generating transgenic Arabidopsis via *Agrobacterium*-mediated transformation (Clough and Bent 1998).

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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