The Ferroportin Metal Efflux Proteins Function in Iron and Cobalt Homeostasis in *Arabidopsis*
interfere with the assembly of iron-sulfur clusters (Ranquet et al., 2007) and heme (Watkins et al., 1980). As plants are sessile and possess a wide range of adaptations to soil stresses, it would seem likely that mechanisms exist to control cobalt levels and localization to minimize disruption of iron homeostasis.

To determine how the uptake and homeostasis of cobalt and other metals are controlled, the Arabidopsis Ionomics project uses a high-throughput approach combining inductively coupled plasma–mass spectrometry (ICP-MS) and bioinformatic analysis to identify lines that have altered elemental profiles (Baxter et al., 2007). Approximately 10,000 Arabidopsis mutant lines and natural accessions have been screened for variation in elemental composition (data can be accessed at www.ionomicshub.org). Here, we report on several accessions of Arabidopsis that accumulate cobalt and identify FPN2 as the gene mutated in these accessions. We also present data supporting a role for FPN1 in cobalt transport to the shoot as well as a role for both FPN1 and FPN2 in iron efflux.

RESULTS

Increased Shoot Cobalt Is Seen in fpn2-1 and Natural Accessions with Truncated FPN2

Shoot cobalt content was measured across 94 Arabidopsis accessions (Figure 1A). The Spanish accession Ts-1 was one of the top 10 accessions that showed elevated shoot cobalt relative to Columbia-0 (Col-0) (see Supplemental Table 1 online). Bulk segregant analysis mapped the high shoot cobalt locus in Ts-1 to the region of chromosome five containing the metal transporter gene FPN2 (Figure 1B). FPN2 was sequenced in Ts-1 and found to contain an adenine inserted after base pair

![Figure 1. Mapping the Shoot Cobalt Accumulation Locus to FPN2 in Ts-1.](image)

(A) Shoot cobalt content across 94 accessions. Histogram of shoot cobalt content in 94 Arabidopsis accessions (Nordborg et al., 2005). Black bars indicate lines having the insertion that produces a frameshift in FPN2. The black arrow denotes Col-0 cobalt content, and the gray arrow indicates Ts-1. Shoot cobalt concentrations are normalized so that the average of the Col-0, Fab-2, Ts-1, and Cvi-0 means included in each growth tray are equivalent across all trays. Plants were grown in soil for 5 weeks. Data represent median values (n = 6) for each accession.

(B) Bulk segregant analysis of the high shoot cobalt content in an F2 population from a Col-0 x Ts-1 cross. Data are presented as a scaled pool hybridization difference (SPHD), representing the difference between the hybridization of the two pools at the single feature polymorphisms (SFPs), scaled so that a pure Col-0 pool would be at 1 and a pure Ts-1 pool would be at −1. The pools were prepared from F2 plants with a low cobalt content (n = 30) and F2 plants with a high cobalt content (n = 28). SFPs were scored after hybridization of genomic DNA prepared from these pools to Affymetrix ATH1 DNA microarrays. Dotted lines denote the region where the signal is larger than the 95% confidence threshold for unlinked loci.

(C) Alignment of FPN2 showing adenine inserted after position 1228 of the Ts-1 genomic sequence.

(D) Cobalt content of Ws-2, fpn2-1, Ts-1, and F1 plants from Ws-2 x fpn2-1 and Ts-1 x fpn2-1. Data are shown as a five-number summary (the minimum, 1st quartile, median, 3rd quartile, and maximum) for each line with outliers indicated by small circles and is summarized from an average of 10 replicate plants for each line. Lowercase letters denote groups that are not significantly different from each other at P < 0.01. Plants were grown in soil for 5 weeks.
1228 of the genomic sequence (Figure 1C), upstream of five of the 10 predicted transmembrane domains (see Supplemental Figure 1C online; Aramemnon Plant Membrane Protein Database) and in the first third of the ferroportin domain shared with Hs FPN and At FPN1. This frame shift produces a stop codon 117 amino acids earlier than in Col-0 (see Supplemental Figure 1B online). Of the top seven cobalt accumulating accessions, five have the same adenine insertion after position 1228, resulting in truncation of FPN2 (see Supplemental Figure 1A online).

To confirm that this FPN2 truncation was the cause of the elevated shoot cobalt phenotype, Ts-1 was crossed to the 

Expression of FPN2 Is Iron Regulated, While FPN1 Expression Is Not

To understand how the loss of FPN2 could change cobalt homeostasis, we examined the tissue expression pattern of FPN2 and its paralog FPN1. Previous experiments have shown that FPN1 expression in the roots is low and not iron regulated (Colangelo and Guerinot, 2004; Dinneny et al., 2008); FPN2 transcript is present in iron-sufficient roots, and expression in roots is upregulated almost fourfold in response to iron deficiency (Colangelo and Guerinot, 2004; Schaaf et al., 2006; Dinneny et al., 2008). We fused the respective FPN promoters to the β-glucuronidase (GUS) reporter gene (Figure 2). The FPN1-GUS plants show staining in the vasculature of the root and shoot (Figures 2A to 2C). The FPN2-GUS plants show expression in the roots of iron-sufficient plants, with expression increasing under iron-deficient conditions (Figure 2D). Staining in the leaf veins also becomes evident in iron-deficient plants. When iron-deficient FPN2-GUS roots are stained with the fluorescent GUS substrate ImaGene Green, expression is limited to the outer two layers of the root (Figure 2E). Expression is strongest in the cortex but also present in the epidermis and root hairs and absent from

Figure 2. Localization of FPN1 and FPN2.

Eleven-day-old FPN1-GUS plants show staining in the stele ([A] and [B]), root-shoot junction, and veins of the cotyledons (C). Inset in (B) shows staining in stele of root cross section. Inset in (C) shows staining in the seedling root-shoot junction. When 2-week-old FPN2-GUS plants are transferred from B5 to -Fe minimal medium for 3 d, GUS staining is very dark in the root and is also present in the shoot (D). Staining with propidium iodide (red) and the fluorescent GUS substrate, ImaGene Green, shows FPN2-GUS expression primarily in the cortex but also in the epidermis and root hairs (E). When FPN1 and FPN2 are fused to GFP and transiently expressed in protoplasts, FPN1-GFP localizes to the plasma membrane (F), as does the plasma membrane marker AHA2-RFP (G); FPN2-GFP localizes to the vacuole (I), as does the vacuole marker dye FM4-64 (J). Overlays of GFP and the markers are shown in (H) and (K).
the inner layers. This agrees well with the elevated level of FPN2 expression detected via transcriptomics in the cortex during iron starvation (Dinneny et al., 2008).

**FPN1 and FPN2 Are Localized to Different Membranes**

FPN2 was previously found to localize to the vacuolar membrane (Schaaf et al., 2006). To determine the localization of FPN1, the gene was fused to green fluorescent protein (GFP) and transiently expressed in protoplasts. FPN1 localizes to the plasma membrane (Figures 2F to 2H), while FPN2 localizes to the vacuole as expected (Figures 2I to 2K). This suggests that while both ferroportins likely efflux metal from the cytoplasm, they play very different roles in metal homeostasis. FPN1 likely effluxes metals from the cytoplasm into the vasculature, allowing movement of metals from root to shoot; FPN2 effluxes into the vacuole, sequestering metals in the outer cell layers of the root, especially under iron deficiency.

**Cobalt Accumulation Changes in Ferroportin Mutants**

To confirm that plants carrying the fpn2-2 allele in the Col-0 background also accumulate cobalt, and to determine how the loss of FPN1, as well as the loss of both FPN1 and FPN2, would affect metal accumulation, ICP-MS was used to analyze the shoots of soil-grown plants (Figure 3A). The shoots of fpn2-1 and fpn2-2 have elevated cobalt, while fpn1-1, fpn1-2, and the fpn1 fpn2 double mutant have decreased shoot cobalt compared with the wild type. Despite the role FPN2 plays in nickel tolerance (Schaaf et al., 2006), no change in shoot nickel accumulation was observed in ferroportin mutants grown under our soil conditions (0.02 μmol Ni g⁻¹ dry weight soil). Because FPN2 localizes to the vacuoles in the root, it likely sequesters cobalt in the root; its loss allows cobalt to move to the shoot, as seen in fpn2-1, fpn2-2, Ts-1, and other Arabidopsis accessions that accumulate cobalt. FPN1 localizes to the plasma membrane of stele cells, suggesting that it loads cobalt into the vasculature; its loss reduces the amount of cobalt moving to the shoot. In the double mutant, cobalt is not sequestered in the vacuoles of the root via FPN2 but is unable to enter the vasculature via FPN1.

To determine how the changes in shoot cobalt related to metal levels in the roots, the mutant lines were grown hydroponically in medium supplemented with cobalt (to ensure a robust ICP-MS signal). The plants were harvested and the roots and shoots analyzed for cobalt accumulation and distribution using ICP-MS.
(Figure 3C). The loss of FPN2 produces a decrease in the concentration of cobalt in the root but an increase in the shoot. This supports the hypothesis that FPN2 sequesters cobalt in the root, and its loss allows cobalt to escape the root and accumulate in the shoot. The *fpn1* and *fpn1 fpn2* mutants have the reverse phenotype: both show increased cobalt concentrations in the root and decreased cobalt concentrations in the shoot. This further suggests that FPN1 loads cobalt into the root vasculature for transport to the shoot. We also measured the levels of Fe and Ni (see Supplemental Table 2 online). The only significant difference in Fe levels was for the *fpn2* mutant that accumulated more Fe than the wild type in its roots. There were no significant differences between the wild type and the *fpn* mutants for Ni in either roots or shoots.

**Ferroportin Mutants Are More Sensitive to Cobalt and Nickel**

To determine if the observed changes in cobalt distribution alter metal tolerance, the mutant lines were grown on solid media supplemented with cobalt and nickel (Figure 4). Previously, *fpn2-2* was found to be more sensitive to nickel than wild-type Col-0 (Schaaf et al., 2006). On B5 medium alone, the mutant lines have no visible phenotype. However, when grown on B5 supplemented with 20 μM CoCl₂ or 50 μM NiCl₂, *fpn2-2* is more sensitive than the wild type. Similarly, *fpn2-1* is also more sensitive to cobalt and nickel than Ws (Figure 4B), as are Ts-1 and Se-0 (Figure 4A). The *fpn1-2* mutant is not visibly affected by cobalt or nickel, yet when crossed with *fpn2-2*, the double mutant is more sensitive than *fpn2-2* and dramatically more sensitive than the wild type. This demonstrates that FPN1 contributes to cobalt and nickel sensitivity, but this can only be seen in the absence of FPN2.

Combined with the shoot ICP-MS data, this indicates that without FPN2, cobalt is not sequestered in the root vacuoles, allowing its movement to the shoot, resulting in sensitivity to cobalt. In the double mutant, the loss of FPN1 prevents cobalt export to the shoot, causing its concentration to rise in the root. Because the double mutant also lacks a functioning FPN2, the increased root cobalt cannot be detoxified by compartmentalization into the vacuole. This results in the extreme sensitivity to cobalt observed in the double mutant *fpn1-2 fpn2-2*.

**FPN1 Is Required for Cobalt Movement from Root to Shoot**

Because the *fpn1* and *fpn1 fpn2* mutants have decreased cobalt accumulation in the shoot, we further investigated whether FPN1 loaded cobalt into the vasculature. To do this, we crossed *fpn1-2* with the *frd3-1* mutant. The *frd3* mutant expresses IRT1 constitutively and thus accumulates significantly more cobalt in the shoot (Lahner et al., 2003). If FPN1 is required for cobalt movement to the shoot, the loss of FPN1 in *frd3-1* would abolish the shoot cobalt accumulation phenotype. When shoots of soil-grown plants are analyzed with ICP-MS (Figure 5), *fpn1-2* shows significantly less shoot cobalt than the wild type, while *frd3-1* accumulates much more than the wild type. In the *fpn1 frd3* double mutant, shoot cobalt is much lower than the wild type, as is seen in *fpn1-2*. This confirms that FPN1 is essential for cobalt movement from root to shoot and indicates that FPN1 plays a significant role in cobalt loading into the vasculature.

**The Loss of FPN2 Leads to Chlorosis**

Because both ferroportins were found to affect cobalt movement from root to shoot, we examined whether the ferroportins also played a role in long-distance iron transport. To determine if iron was reaching the shoot efficiently, we assayed shoot chlorophyll concentrations; if a mutant had impaired iron movement, then it would likely become chlorotic and have lower chlorophyll levels. Plants were grown on B5 plates for 2 weeks and then transferred to minimal media that was either iron sufficient or deficient. Under both conditions, *fpn1* is more chlorotic than the wild type: chlorophyll and carotenoid levels were decreased by 13 to 18%
FPN1 is Required for Cobalt Movement to the Shoot

Shoots of soil-grown plants analyzed by ICP-MS. Mean ± se is shown. Significant differences from the wild type are indicated by * P < 0.0001. For Col-0, n = 12 plants; for fpn1-2, n = 36 plants; for frd3-1, n = 24 plants; and for fpn1 frd3, n = 36 plants.

Ferroportins Affect the Iron Deficiency Response

Because we found that FPN2 transports iron and that its expression is iron regulated, we examined how the loss of ferroportins affects the iron deficiency response. During iron deficiency in Arabidopsis, FRO2 activity is highly induced in the root epidermis to generate ferrous iron for uptake via IRT1 (Figure 9). The fpn1-2 mutant shows no change in ferric chelate reductase activity compared with the wild type (Figure 8A). The loss of FPN2, however, results in a significant decrease in ferric chelate reductase activity: fpn2-2 reduces about half as much ferric iron as Col (Figure 8A), while fpn2-1 reduces about half as much as Ws-2 (see Supplemental Figure 2 online). Two Spanish accessions with the frameshift mutation in FPN2, Ts-1 and Se-0, also show significantly less ferric chelate reductase activity than Col-0 and Ws-2 in response to iron deficiency. Overexpression of FPN2 using the 35S promoter produces the opposite phenotype: ferric chelate reductase activity under both iron-sufficient and iron-deficient conditions is >40% greater than in the wild type. This is similar to results seen with yeast where overexpression of
FPN2 results in cytoplasmic iron depletion as evidenced by induction of the iron-responsive FET3-lacZ reporter (see Supplemental Figure 3 online).

To further characterize the role of ferroportins in the iron deficiency response, IRT1 protein levels were detected in the mutant backgrounds. IRT1 expression is induced by iron deficiency (Eide et al., 1996; Vert et al., 2002), and immunoblots using an IRT1-specific antibody show IRT1 protein accumulation in roots after 3 d on iron-deficient medium (Figure 8B). In fpn1-2, IRT1 levels are similar to the wild type. In fpn2-2, IRT1 levels are decreased, matching the decreased levels of ferric chelate reductase activity seen. The fpn1 fpn2 double mutant shows greatly increased levels of IRT1; combined with the ferric chelate reductase data, the double mutant clearly has an elevated iron deficiency response. The loss of both ferroportins causes a more extreme iron deficiency response, indicating that iron homeostasis is impaired. This likely exacerbates the increased cobalt and nickel sensitivity seen in the double mutant: increased IRT1 expression would bring more cobalt and nickel into the root, which lacks a means to sequester or efflux the metals. Thus, the changes in the iron deficiency response seen in the fpn2 and fpn1 fpn2 double mutants indicate that ferroportins are an integral part of iron homeostasis in Arabidopsis.

To better characterize how the loss of FPN2 alters the iron deficiency response, a time course comparing IRT1 induction in the wild type and the fpn2 mutant was performed (Figure 8C). Two-week-old plants were transplanted to iron-deficient medium, and roots were harvested every 24 h. IRT1 accumulates more slowly or to lower levels in fpn2-2. At 24 h, IRT1 protein is readily detected in wild-type roots, but much less apparent in

![Figure 7](image)

**Figure 7.** FPN2 Sequesters Iron and Cobalt in the Vacuole.

FPN2 expressed in yeast. FPN2-GFP localizes to vacuole membrane (A), and FPN2 expression rescues the iron-sensitive phenotype of the yeast Δccc1 mutant and the cobalt-sensitive phenotype of the Δcot1 mutant (B), presumably by loading iron and cobalt into the vacuole, respectively.

![Figure 8](image)

**Figure 8.** The Loss of Ferroportin Disrupts the Iron Deficiency Response.

(A) Plants were grown on B5 solid medium under constant light for 2 weeks at 21°C and then transferred to either +Fe (black bars) or –Fe (gray bars) minimal medium for 3 d. Ferric chelate reductase activity of a pool of six plants was measured, in triplicate, using the ferrozine assay. Data represent mean ± SE. Significant differences from the wild type are indicated by * P < 0.05.

(B) IRT1 protein levels in ferroportin mutants measured by immunoblot using IRT1 antibody. Plants were grown on B5 solid medium under constant light at 21°C for 2 weeks and then transferred to either +Fe or –Fe minimal medium for 3 d. Ten micrograms of total protein were loaded (bottom panel shows Coomassie blue staining).

(C) Plants were grown on B5 solid medium for 2 weeks under constant light at 21°C and then transplanted to –Fe minimal medium. Samples were taken every 24 h. After 72 h on –Fe minimal medium, the plants were transplanted to +Fe minimal medium and samples taken every 4 h. The presence of IRT1 protein was measured by immunoblots using IRT1 antibody.
Cobalt Accumulation in \( \text{fpn2} \) Shoots Is Iron Dependent

To examine the relationship between the iron deficiency response and the \( \text{fpn2} \) cobalt accumulation phenotype, we analyzed the shoot metal content of plants supplied with a range of iron supplementation levels (Figure 3B). Wild-type and \( \text{fpn2-1} \) plants watered with 30 \( \mu \text{M} \) Fe-HBED both accumulate very little cobalt. When plants are grown with decreasing levels of iron supplementation, a clear divergence in cobalt shoot accumulation is seen in the wild type. After 72 h, the iron-deprived plants were transferred to iron-sufficient medium and roots harvested every 4 h. After 4 h, IRT1 protein is still present in both wild-type and \( \text{fpn2} \) roots but after 8 h, IRT1 protein is no longer detected in \( \text{fpn2} \), while it is still present in the wild type. The loss of FPN2 likely mislocalizes iron that would otherwise be sequestered in the vacuoles of the outer root layers, altering either iron-sensing or the speed of iron remobilization during iron deficiency, delaying the induction or the strength of the response and ending it sooner.

**DISCUSSION**

**Ferroportins Are Required for Normal Cobalt Localization**

IRT1 is expressed in response to iron deficiency and transports iron into the root cytoplasm from the rhizosphere, along with zinc, manganese, cadmium, cobalt (Vert et al., 2002), and nickel (Schaaf et al., 2006). FPN2 is also expressed during iron deficiency, localizes to the vacuole in roots (Schaaf et al., 2006), and effluxes metals from the cytoplasm into the vacuole. Without FPN2, cobalt is not sequestered in the root vacuoles; instead, it is able to move to the shoot via FPN1, resulting in an increase in shoot cobalt and cobalt sensitivity (Figure 9). A similar increase in shoot zinc levels is seen in the \( \text{mtp3} \) loss-of-function mutant; like FPN2, it is expressed on the vacuolar membrane of iron-deficient roots, sequestering excess incoming zinc in the root (Arrivault et al., 2006). Schaaf et al. (2006) previously reported that FPN2 transported Ni into the vacuole and postulated that FPN2 expression was upregulated during iron deficiency to sequester the nickel that enters the root through IRT1. This explanation can now be extended to cobalt.

Without FPN1, cobalt accumulates in the root and decreases in the shoot. When \( \text{fpn1-2} \) is crossed to the cobalt-accumulating \( \text{frd3-1} \) mutant, shoot cobalt accumulation is abolished. This confirms that FPN1, despite its low (but constant) expression level, is a primary route for cobalt movement from root to shoot. Cobalt sensitivity does not increase in \( \text{fpn1} \) because FPN2 is still able to sequester the increased root cobalt in the vacuole. Thus, in the \( \text{fpn1 fpn2} \) double mutant, cobalt is not dispersed into the shoot via FPN1 or sequestered in the root vacuole via FPN2. Instead, cobalt likely accumulates in the cytoplasm of root cells, resulting in a dramatic increase in cobalt sensitivity. Ultimately, it is unclear whether these ferroportin functions are cobalt-specific adaptations or whether they are a side effect of the broad substrate specificity of iron transporters.

**Ferroportins Are Required for Iron Homeostasis**

FPN2 is an iron effluxer, like its ortholog Hs FPN, but instead of localizing to the plasma membrane, FPN2 is on the vacuolar membrane, moving iron from the cytoplasm into the vacuole. This function is essential for normal iron homeostasis, as \( \text{fpn2} \) mutants have a diminished iron deficiency response. Although it seems counterintuitive to sequester iron in the vacuole during iron deficiency, FPN2 may buffer the influx of iron into the cytoplasm of the outer root layers. Expression of FPN2 could serve to sequester excess free iron that would otherwise not be chelated or transported out of the cell quickly enough. This buffering function would be similar to that of yeast zinc transporter ZRC1 that is expressed during zinc deficiency to sequester excess zinc in the vacuole, preventing zinc shock (MacDiarmid et al., 2003).

Iron homeostasis is disrupted in \( \text{fpn2} \) because iron that would otherwise be stored in the vacuole is now mislocalized. It seems likely that iron sensing occurs outside the vacuole; thus, it takes the \( \text{fpn2} \) mutant longer to perceive deficiency; when iron is restored, the cell is replenished more quickly because FPN2 is not moving iron into the vacuole or because the previously mislocalized iron is remobilized more quickly from nonvacuolar.
iron pools. The opposite phenotype is seen in the nramp3 loss-of-function mutant: when iron is not released from the vacuole by NRAMP3, the iron deficiency response is elevated (Thomine et al., 2003). While NRAMP3 is expressed in the vasculature, FPN2 is expressed in the root epidermis and cortex. Thus, during iron deficiency, FPN2 buffers the influx of iron into the cytoplasm in the outer tissue layers of the root, while NRAMP3 releases iron in the vascular tissue so that it can immediately move to the shoot.

The loss of FPN1 results in chlorosis, suggesting that FPN1 loads iron into the vasculature. Yet, fpn1 plants show no change in the iron deficiency response. The loss of both ferroportins, however, results in a greatly increased iron deficiency response, the opposite of the fpn2 phenotype. This could be caused by changes in metal concentration and localization within the root, at both the subcellular and root layer level, altering the kinetics of iron movement to the shoot. While the loss of FPN1 alone is not enough to alter iron sensing, the additional loss of the vacuolar iron buffering of FPN2 adds stress to an impaired system. With iron no longer sequestered in the vacuoles of the outer root layers, more iron likely moves toward the vasculature, which is now lacking FPN1. With increased cytosolic iron levels, competition for access to the remaining transporters and chelators would also increase, perhaps slowing iron movement to the vasculature. This could also be caused, or made worse, by cobalt and nickel accumulating in the root cytoplasm, especially when IRT1 is expressed. Cobalt and nickel are sterically similar to iron, and the presence of cobalt induces the iron deficiency response (see Supplemental Figure 4 online). Their mislocalization to the cytoplasm could competitively inhibit iron transport or bind up the chelators necessary for iron transport (e.g., citrate and NA). This would slow translocation of iron to the shoot, resulting in the more intense iron deficiency response as the shoot becomes iron starved. A similar phenotype was seen in mung bean seedlings treated with cobalt: iron uptake increased, but iron was unable to move from the root to the shoot (Liu et al., 2000).

To confirm these theories, we need to determine how iron and cobalt localization is changing in the ferroportin mutants at both the cellular and subcellular levels. Combining ICP-MS with the recently demonstrated technique of sorting cells by root layer markers (Dinneny et al., 2008) would allow us to determine if root cortex cells contained less iron and cobalt in fpn2 than the wild type. Additionally, improving microscope resolutions and metal binding dyes will eventually allow the real-time visualization of subcellular metal distribution. The ferroportin mutants would be ideal candidates for application of this technology, as we believe that iron and cobalt are mislocalized at the subcellular level.

**Cobalt Uptake Is Iron Regulated**

The amount of cobalt a plant takes up is dependent on its iron status: as it becomes more iron deficient, more IRT1 is expressed, through which more cobalt is transported. Expression of FPN2 is also iron regulated, increasing simultaneously with IRT1 levels, thus preventing increased cobalt accumulation in the shoot. Without FPN2, less cobalt is sequestered in the root, and each decrease in iron supplementation results in increases in shoot cobalt. Thus, cobalt uptake is iron regulated, while its localization between root and shoot is dependent on FPN2 expression.

**The Potential of Cobalt Interference with Iron Sensing**

An alternative interpretation of the mutant iron deficiency response phenotypes could center around cobalt localization rather than iron transport. Perhaps the *Arabidopsis* ferroportins primarily transport cobalt and only minor amounts of iron; the disruption of the iron deficiency response could be caused by changes in cobalt abundance and localization. The trigger of the iron deficiency response in plants is unknown, but if it involves iron binding, cobalt is a likely candidate for inappropriate binding and interference. Exposure to cobalt triggers the iron deficiency response in *Arabidopsis*, as FIT1 (the transcription factor that controls aspects of the iron deficiency response; Colangelo and Guerinot, 2004) expression increases (J. Morrissey and M.L. Guerinot, unpublished data), as does the amount of ferric iron reduced by the roots (see Supplemental Figure 4 online). This indicates that control of cobalt is essential to maintaining iron homeostasis. It is often speculated that the iron deficiency response has both root and shoot components, and it is possible that the higher shoot cobalt levels in fpn2 could suppress the iron deficiency response, while the concentration of mislocalized cobalt in the roots of fpn1 fpn2 could increase it.

**Differences and Similarities to Human Ferroportin**

*Arabidopsis* ferroportins transport cobalt in addition to iron. Our findings raise the obvious question whether the animal FPN proteins also transport cobalt. FPN would be a likely path for cobalt and nickel to move from the digestive tract into the bloodstream. Hopefully, the complete range of metals entering the bloodstream through FPN will be characterized in the future. It would also be interesting to investigate whether there are parallels in regulation of ferroportins in *Arabidopsis* and humans. Hemin is a small peptide that binds to FPN. It is required for normal function of Hs FPN, and the malfunction of either results in iron-related disease (Collins et al., 2008; Muckenthaler et al., 2008). A plant ortholog of hepcidin has not been found, but the number of small plant peptides is very likely underpredicted; in fact, a recent examination of antimicrobial small peptides in *Arabidopsis* found almost a quarter were not yet annotated (Silverstein et al., 2007). Hemin is a specific domain on an extracellular loop of FPN (De Domenico et al., 2008), leading to phosphorylation of two Tyr residues (De Domenico et al., 2007) and the internalization and degradation of FPN. Both FPN1 and FPN2 have a Tyr and Phe in place of the Cys residue essential for binding (which is located in the same position as the T5-1 frameshift mutation).

FPN1 and FPN2 likely efflux both iron and cobalt and are essential for cobalt tolerance and iron homeostasis. Cobalt uptake is controlled by iron status, while the tissue-specific accumulation of cobalt is dependent on FPN1 and FPN2. Ferroportin mutants show both increased cobalt sensitivity and
METHODS

Identification of Mutant Lines

The fnp1-1 and fnp-2 lines were acquired from the Arabidopsis Knockout Facility and screened using FPN1 (5’-GACCCACTCTAGAAAAAGT-3’) and FPN2-specific primers (5’-GAAAGAGGTATTTT-TTACAGTTGAACCA-3’), in combination with a T-DNA-specific primer (5’-CATTCTTATAGAAGGATCCTG-3’) (Krysan et al., 1999). The Col lines fnp-1 and fnp-2 (SALK_074442) were acquired from the Salk Collection via ABRC (Nonso et al., 2003). Homozygous insertion mutants were identified using PCR with primers flanking the insertion site for fnp1 (5’-TGAAAATATTTCTCATGCGC-3’) and 5’-TGAGGGTTGTTCATCAGGAAG-3’) and fnp2 (5’-GTTAAGCTGATCATCGTTGACG-3’) and 5’-GATTATGACATCGACCTTC-3’) (see Supplemental Figure 6 online).

Plant Materials and Growth Conditions

Seeds were surface sterilized and stratified for 2 d in the dark at 4°C. B5 medium was supplemented with 1 mM MES and 0.6% agar, pH 5.8. To test for metal sensitivity, B5 medium was amended with 20 μM CoCl2 or 50 μM NiCl2. Square, gridded Petri dishes were used; six seeds from each line were placed in the center of its own grid box. The plants were grown under a 16/8-h light-dark cycle at 21°C for 3 weeks.

Plants used for ICP-MS of roots and shoots were germinated on B5 and transferred after 2 weeks to quarter-strength Hoagland solution supplemented with 2.5 μM CoCl2. The plants were grown under a 16/8-h light-dark cycle at 21°C, and the hydroponic solution was changed weekly. After 3 weeks, the roots were washed three times in deionized water, and then the roots and shoots were harvested and dried for 48 h.

Ferric Chelate Reductase Assay

Plants were grown under constant light on B5 plates (as described above) for 2 weeks and then transferred to either +Fe or –Fe minimal media. The medium was made with macronutrients and micronutrients (Marschner et al., 1982), 0.6% agar, and 1 mM MES, pH 6.0, then supplemented with either 50 μM Fe(III)-EDTA (+Fe), or 300 μM ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate] (–Fe). After 3 d, pools of six plants were analyzed for ferric chelate reductase activity in triplicate, as previously described (Yi and Guerinot, 1996).

Immunodetection

Plants were grown under constant light on B5 plates for 2 weeks and then transferred to either +Fe or –Fe minimal media for 3 d. The roots were then harvested and prepped, and an immunoblot with IRT1 antibody was performed as previously described (Connolly et al., 2002).

Plasmid Construction and Plant Transformation

The FPN2 promoter was amplified from Col genomic DNA using the primers 5’-TCTGCAGAATATTGCTAGAAC-3’ and 5’-TTCTGTGAGCTAGACCTTC-3’. These primers added a PstI site at the 5’ end and a Ncol site at the 3’ end. The fragment was subcloned into the pGEM T-easy plasmid (Promega) and sequenced. The FPN1 promoter was then excised by Ncol and PstI digestion and subcloned into pCAMBIA 1302 (GFP) and 1304 (GFP/GUS) (CAMBIA). Agrobacterium tumefaciens strain GV3101 was transformed with these constructs and used to transform wild-type Col-0 using the floral dip method (Clough and Bent, 1998). Transformsants were isolated by plating the seeds on B5 with hygromycin (25 mg/mL).

The 3SS-FPN2 line was generated by cloning FPN2 from Col genomic DNA using the primers 5’-CATTCTGAGAAGACCGCGGGGACTTGA-CGGAGGAGGAAACGAAC-3’ and 5’-CTTCTCCTTTACTGCTA-GATCTACATCGATCAAGAAGAATGGTCTC-3’. This was cloned into the Ncol site of pCAMBIA 1302, downstream of the 3SS promoter, using the SLIC subcloning method (Li and Elleedge, 2007). Agrobacterium and Col-0 were transformed as described above.

The FPN1 promoter and the coding region for the first 52 amino acids of FPN1 was amplified from Col genomic DNA using primers 5’-CCCCGATCCCCATTCTCATCAGA-3’ and 5’-CCCAAGCTTGGCACTCGCATCGTACG-3’, which added BamHII and HindIII sites, respectively. The fragment was subcloned into pSK and sequenced. It was then excised by digestion with BamHII and HindIII and ligated into pCAMBIA1381xA (CAMBIA). The construct was then moved into Agrobacterium strain GV3101 and transformed into Col gl-1 plants using vacuum infiltration (Bent et al., 1994).

GUS Histochemical Staining

FPN2 promoter-GUS T1 lines were germinated on B5 with hygromycin (25 mg/mL). After 14 d, they were transferred to +Fe or –Fe minimal media for 3 d. The plants were incubated with the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as described (Jefferson et al., 1987). FPN1 promoter-GUS T2 lines were germinated on B5 amended with hygromycin (25 mg/mL) and stained after 11 d. To examine the FPN2 expression pattern in detail, roots expressing FPN2-GUS were incubated with a fluorescent GUS substrate, Imagen Green (Invitrogen), and the cell wall was stained with propidium iodide. The confocal image confirmed that FPN2-GUS expression was predominantly detected in the cortex and epidermis. All fluorescence images were taken with a Nikon Eclipse 80i microscope (Nikon USA) using a green HeNe laser line (543 nm) for the GFP and a red HeNe laser line (633 nm) for the red fluorescence from the propidium iodide staining.

GFP Localization

To check subcellular localization, FPN1-GFP and FPN2-GFP constructs were transformed into Arabidopsis thaliana protoplasts, and then a tonoplast marker dye, FM4-64, was used to stain the protoplasts after 18 h. AHA2-red fluorescent protein was used as a plasma membrane marker (Kim et al., 2001). All fluorescence images were taken with an epifluorescence microscope, Nikon Eclipse 80i (Nikon USA), using the filter sets 31001 (exciter, D480/20; dichroic, 505DCLP; emitter, D535/40) for GFP and 31003 (exciter, D546/10; dichroic, 560DCLP; emitter, D590/30) from Chroma Technology.

Chlorophyll Assay

Plants were grown on B5 medium for 2 weeks under constant light and then transferred to +Fe or –Fe minimal media for 3 d. The shoots were harvested and assayed for chlorophyll and carotenoid content as previously described (Lichtenthaler, 1987).

Real-Time Quantitative PCR

RNA was prepared from 3SS-FPN2 plants (germinated and grown on B5 for 2 weeks under constant light) using the RNAeasy kit and protocol (Qiagen) and cDNA synthesized using M-MLV reverse transcriptase and protocol (Invitrogen). Real-time quantitative PCR was performed on an ABI Model 7700 using SYBR Premix ExTaq (Perfect Real Time) reagents.
and protocol (Takara) and the primers 5′-TTTTGTTAGCGGCTGGGAC-3′ and 5′-TAGACCAACACTCGCGCTGATT-3′. Samples were run in quadruplicate, FPN2 levels were analyzed by EF1α (5′-CAGTCTATT-GATGGCCACAGAC-3′ and 5′-GTGGTGTCCTCCTCGAAATCCAGAG-3′), and arbitrary transcriptional units calculated.

**Plant Growth Conditions of Soil Grown Plants Analyzed by ICP-MS**

Plants used for elemental profiling by ICP-MS analysis were grown in a controlled environment, 8 h light/16 h dark (90 μmol m−2 s−1 light intensity) and 19 to 22°C (Lahner et al., 2003). Briefly, seeds were sown onto moist soil (Sunshine Mix LB2; Carl Brebob and Son) with various elements added at subtoxic concentrations (As, Cd, Co, Li, Ni, Pb, and Se; Lahner et al., 2003) and stratified at 4°C for 3 d. Plants were bottom-watered twice per week with 0.25× Hoagland solution in which iron was replaced with 10 μM Fe-HBED [N,N-di(2-hydroxybenzyl)ethylenediamine-N,N′-diacetic acid monohydrochloride hydrate; Strem Chemicals]. For elemental analysis after 5 weeks, plants were nondestructively sampled by removing one or two leaves. The plant material was rinsed with 18 MΩ water and placed into Pyrex digestion tubes.

**Tissue Elemental Analysis**

Tissue samples were dried at 92°C for 20 h in Pyrex tubes (16 × 100 mm) to yield ~2 to 4 mg of tissue for elemental analysis. After cooling, seven of ~100 samples from each sample set were weighed. All samples were digested with 0.7 mL of concentrated nitric acid (OmniTrace; VWR Scientific Products) and diluted to 6.0 mL with 18 MΩ water. Elemental analysis was performed with an ICP-MS (Elan DRCe; Perkin-Elmer) for Li, B, Na, Mg, P, S, K, Ca, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, and Cd. All samples were normalized to calculated weights, as determined with an iterative algorithm using the best-measured elements, the weights of the seven weighed samples, and the solution concentrations, implemented in the PIIMS database (Baxter et al., 2007).

**DNA Microarray-Based Bulk Segregant Analysis**

DNA microarray-based bulk segregant analysis was performed as previously described (Borevitz et al., 2003; Hazen et al., 2005). Briefly, SFPs were identified between Col-0 and Ts-1 by hybridizing labeled genomic DNA from each one of the accessions to Affymetrix ATH1 microarrays and comparing them to Col-0 hybridizations downloaded from http://www.naturalvariation.org/xam. Two genomic DNA pools from an F2 population of a cross between Ts-1 and Col-0 were created and hybridized to separate DNA microarrays. Each one of the pools contained plants with either shoot Co contents similar to Col-0 (control pool) or high shoot Co contents similar to Ts-1 (High Co pool). At loci unlinked to the high Co phenotype, the pools should have equivalent amounts of each genotype, and the hybridization signal at each SFP should be intermediate between the two parent accessions, for an average difference between the two DNA microarrays of zero. At linked loci, the difference between the two DNA pools should be approximately two-thirds the difference between the parent accessions. By smoothing the signal across multiple SFPs, noise is reduced and the peak of the differences in hybridization signal will correspond to the chromosomal region of the loci controlling the high Co trait. Raw hybridization data (.CEL files) for each probe on the ATH1 DNA microarrays used in these experiments have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) for public distribution (Barrett et al., 2007).

**Yeast Strains, Plasmids, and Growth Conditions**

Δccc1::HIS3, Δtrc1::HIS3, and their wild-type strains were in the W303 background (Li et al., 2001). △cot2::KanMX and its wild-type strain were in the BY4743 background (Conklin et al., 1992). Yeast strains were grown in CM medium (yeast nitrogen base, amino acids, and dextrose). For assay of metal toxicity, different concentrations of metals were added to the medium and serial dilutions of cells were spotted. Plant FPN1 or FPN2 genes were cloned into the yeast expression vector M3385 reference with a MET25 promoter and a C-terminal GFP epitope tag. To induce expression, cells were grown in medium lacking Met.

**GFP Protein Localization in Yeast**

Cells with plant FPN1/2-GFP expression plasmids were grown overnight in medium lacking Met. Cells were plated on 1 mg/mL Concanavalin A-coated glass slides. GFP images were captured by an Olympus BX501 epifluorescence microscope using a ×100 1.4 aperture.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or the EMBL/GenBank databases under accession numbers At2g38460 (FPN1) and At5g03570 (FPN2).

**Supplemental Data**

The following materials are available in the online version of this article.

- **Supplemental Figure 1.** Some Arabidopsis Accessions Contain a Frameshift Mutation in FPN2.
- **Supplemental Figure 2.** fpn2-1 Roots Have Less Ferric Chelate Reductase Activity Than Wild-Type Ws.
- **Supplemental Figure 3.** Overexpression of FPN2 Induces Iron Deficiency.
- **Supplemental Figure 4.** Cobalt Triggers the Iron Deficiency Response.
- **Supplemental Figure 5.** Alignment of FPN Domains from Humans and Arabidopsis.
- **Supplemental Figure 6.** Ferroportin Alleles.
- **Supplemental Table 1.** Fe, Co, and Ni Levels in the 10 Accessions with the Highest Levels of Co.
- **Supplemental Table 2.** ICP-MS Data from Hydroponically Grown Plants.

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