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Figure 1

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Figure 2

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Figure 3

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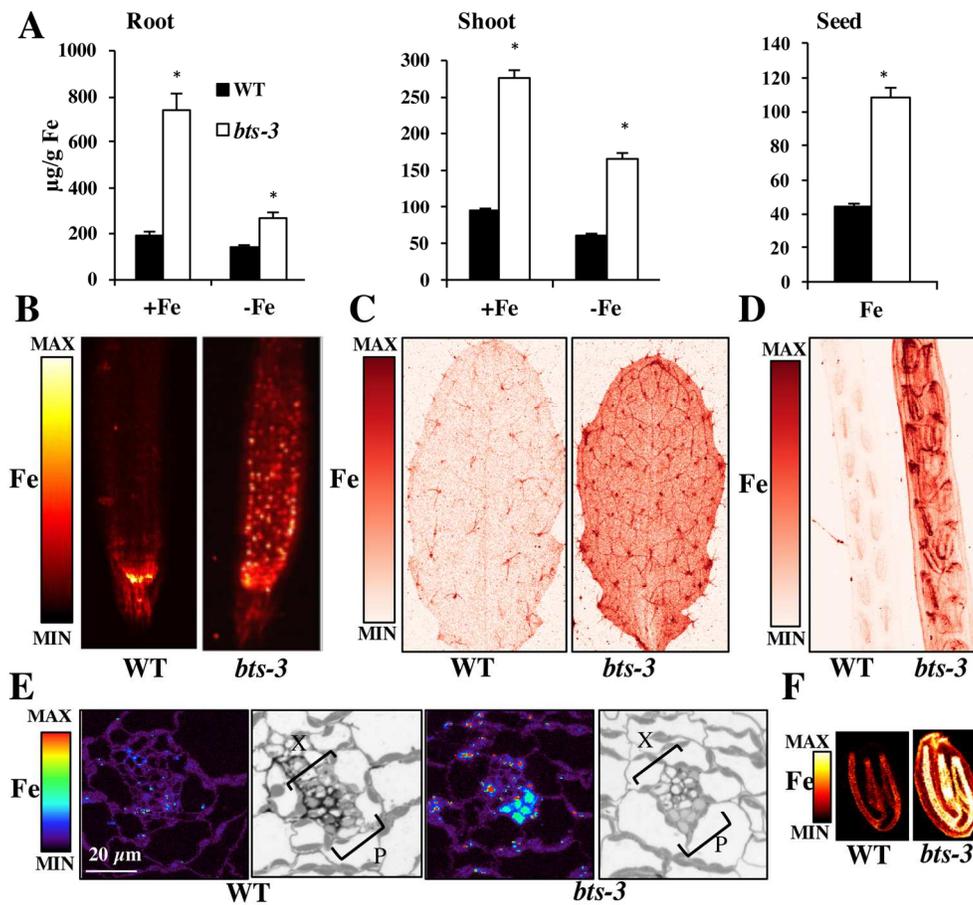
Figure 4

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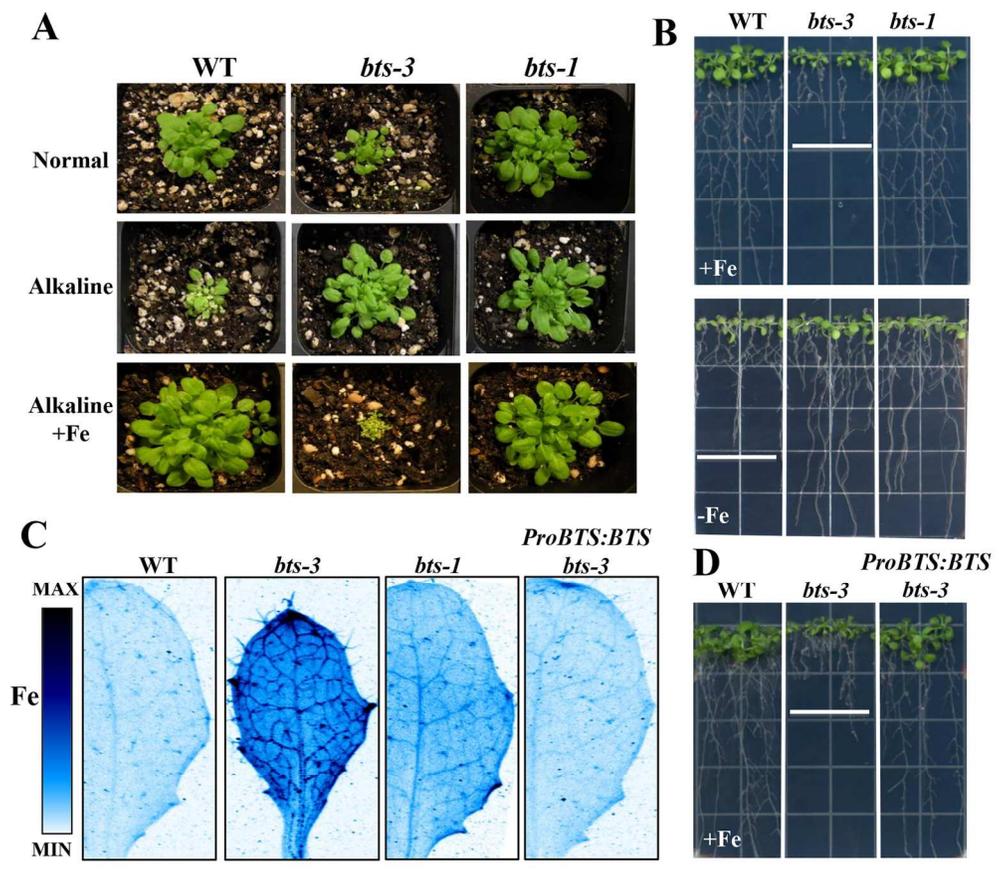
Figure 5

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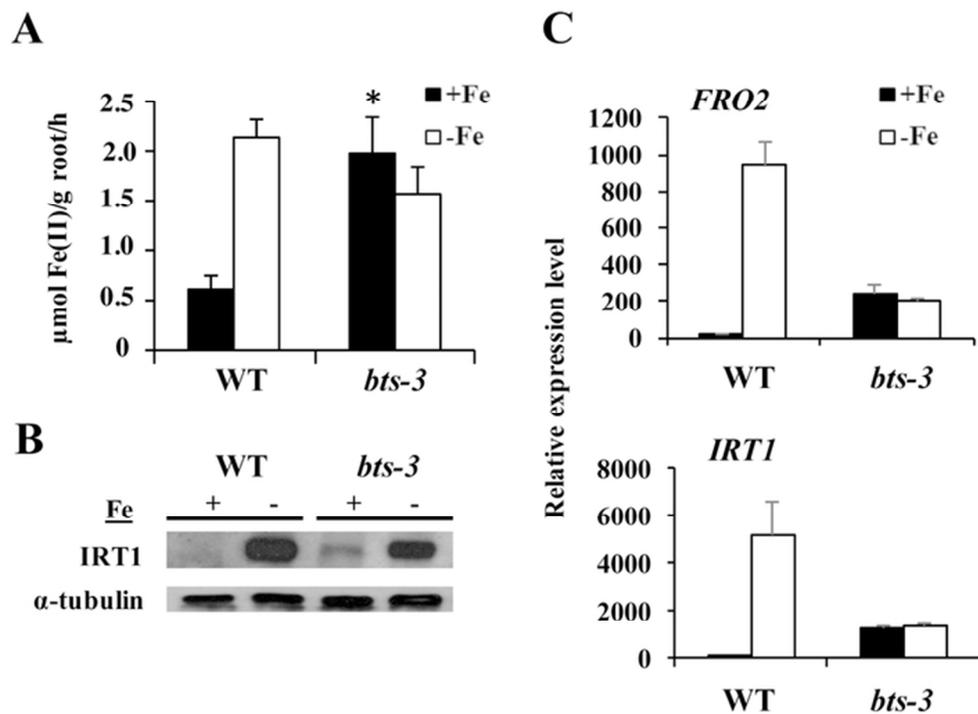
Figure 6

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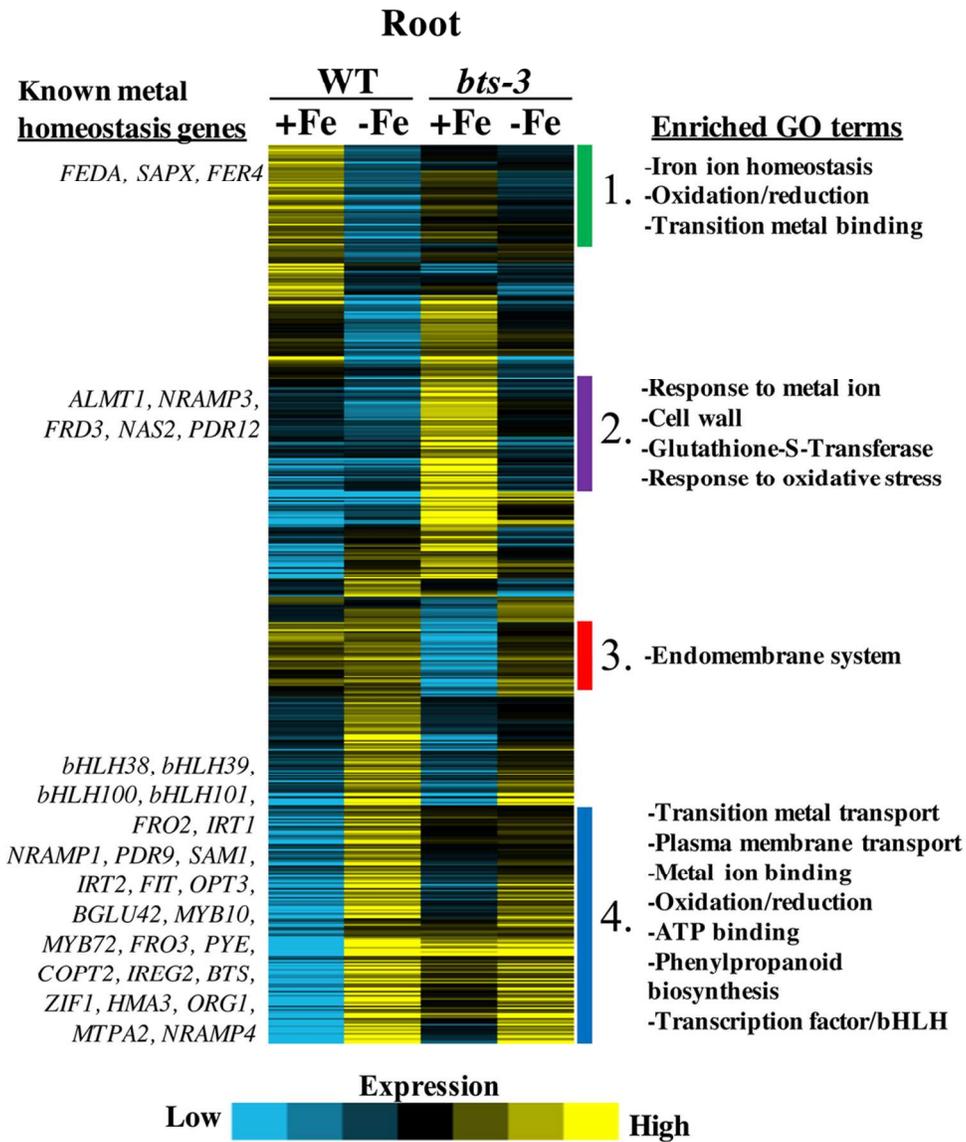
Figure 7

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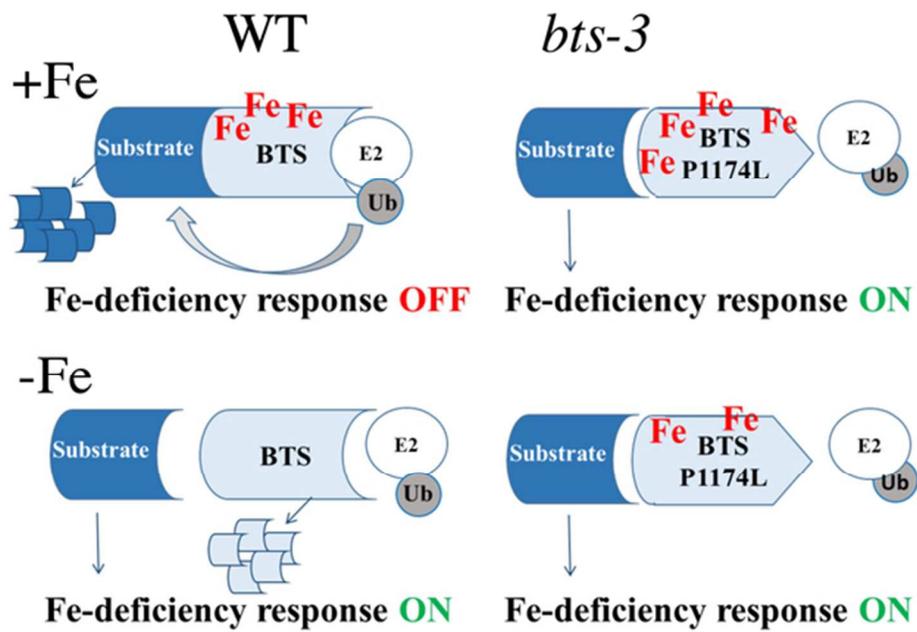
Figure 8

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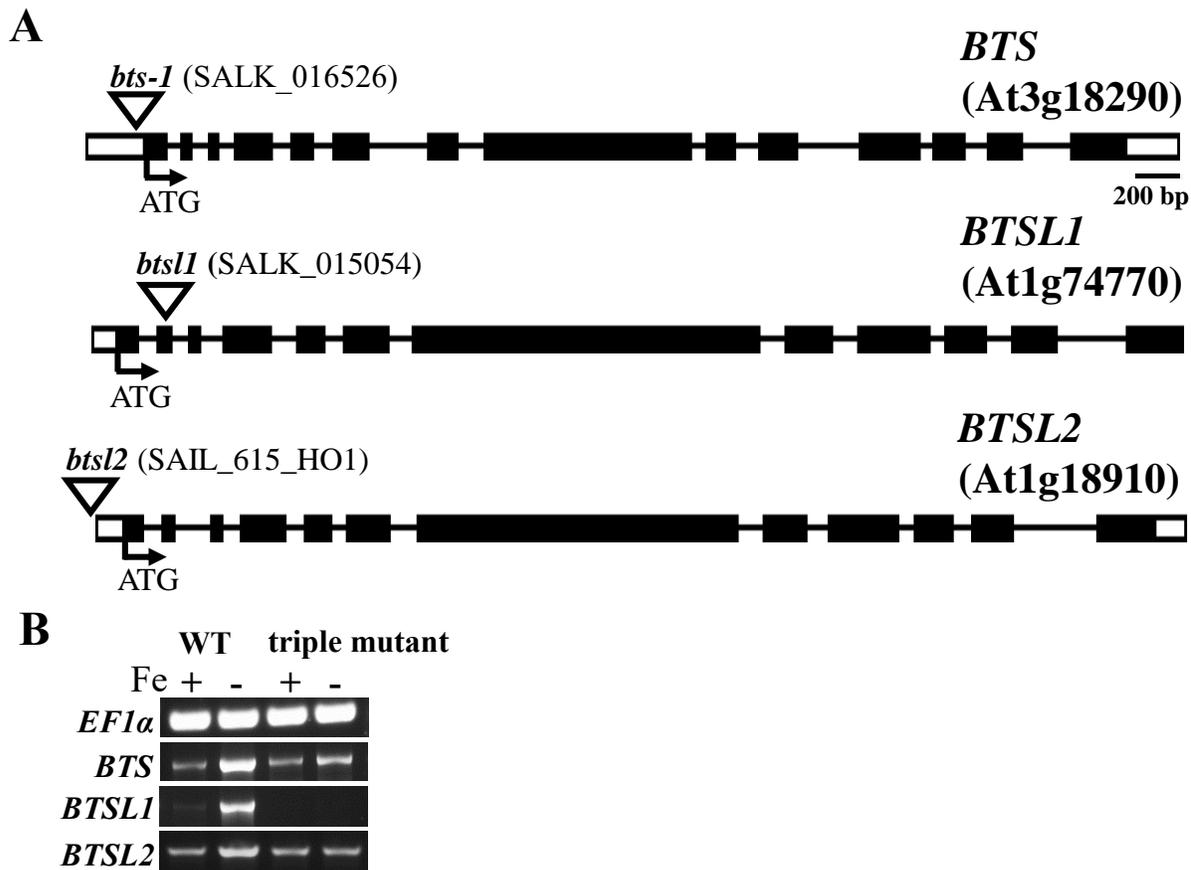
Supplemental Figure 1. Location of T-DNA insertions of *bts-1*, *btsl1* and *btsl2* mutants.
A. Arrows indicate position of T-DNA insertions within *BTS, BTSL1,* and *BTSL2* genes. Black boxes represent exons, lines indicate introns, and white boxes indicate UTRs. The *bts-1* insertion occurs in the 5'UTR, the insertion in *btsl1* occurs in the 2nd exon, and the insertion in *btsl2* occurs in the promoter just before the 5'UTR. **B.** Full length transcript amplified from cDNA of roots of plants grown for 2 weeks on B5 medium and then transferred to +Fe or –Fe medium for 3 days.
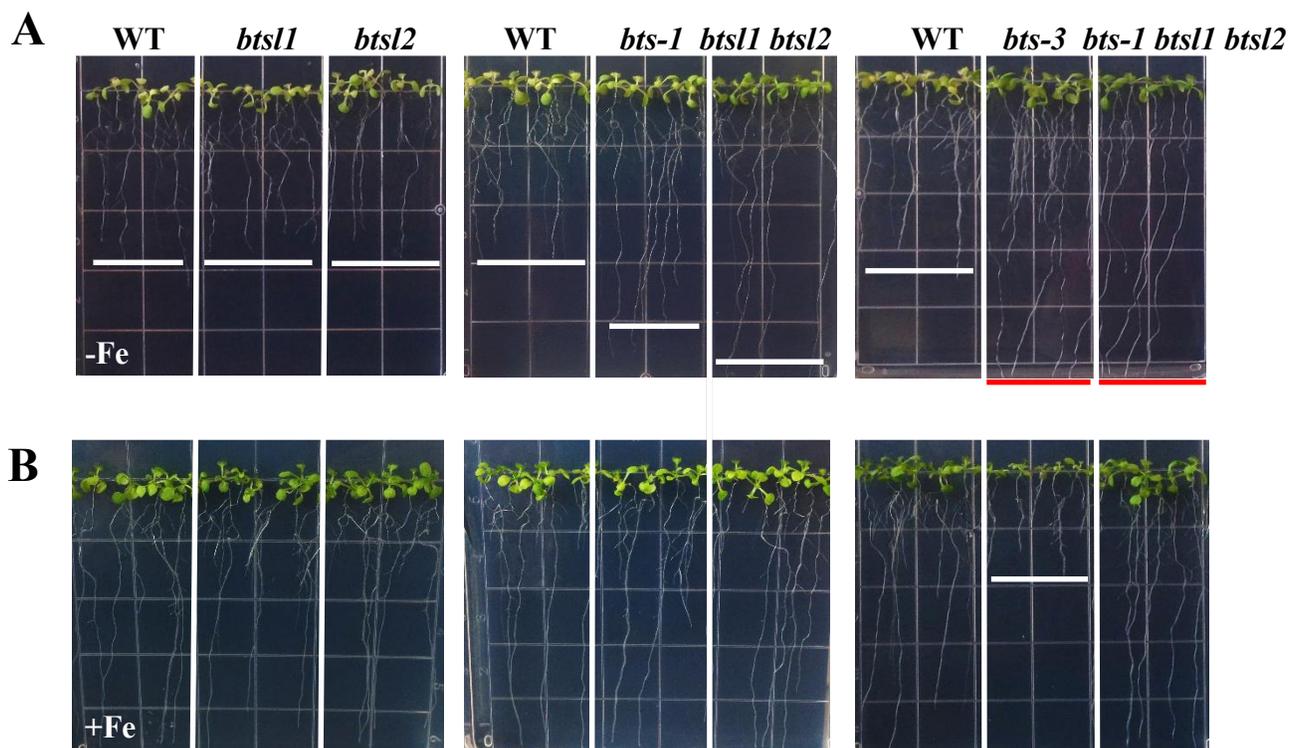
Supplemental Figure 2. *bts-1*, *btsl1 btsl2*, *bts-1 btsl1 btsl2*, and *bts-3* **mutants all exhibit tolerance to Fe-deficient growth conditions, but only** *bts-3* **is sensitive to Fe-sufficient conditions.**
Plants were grown vertically for 5 days on B5 medium and then transferred to +Fe (A) or –Fe medium (B) for 7 days.
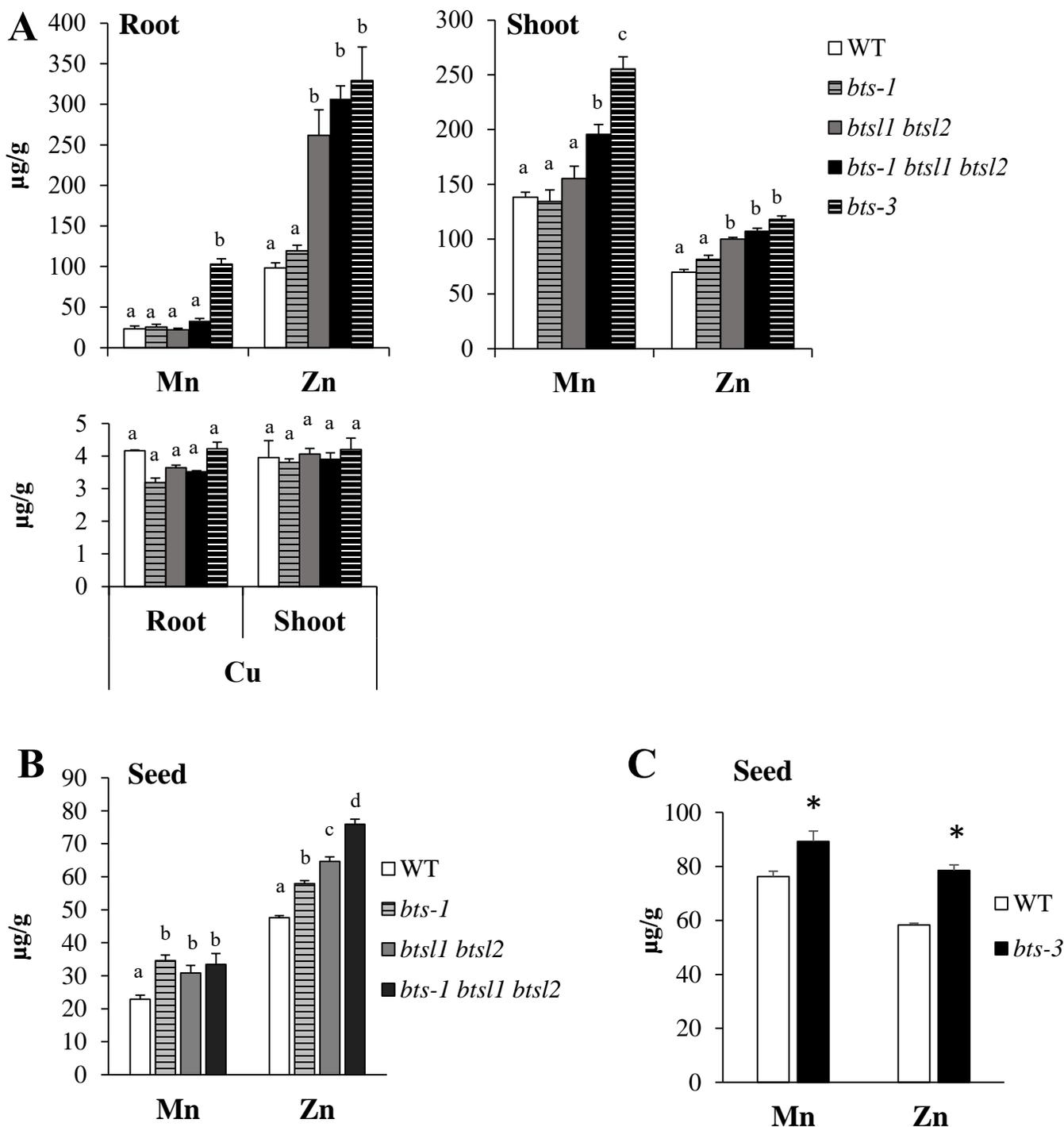
Supplemental Figure 3. BTS family E3 ligase mutants have higher Mn and Zn concentrations in roots, shoots and seeds compared to wild type.
A. Root and shoot ICP-MS measurements of plants grown for 2 weeks on B5 and then transferred to +Fe medium for 3 days. **B.** Seed ICP-MS measurements of harvest from soil grown plants (as in Figure 2). Lower case letters indicate significant differences at $p<0.05$ (ANOVA with Tukey's). **C.** Seed ICP-MS measurements of harvest from soil grown plants. * indicates $p<0.05$ compared to WT (Student's T-test).
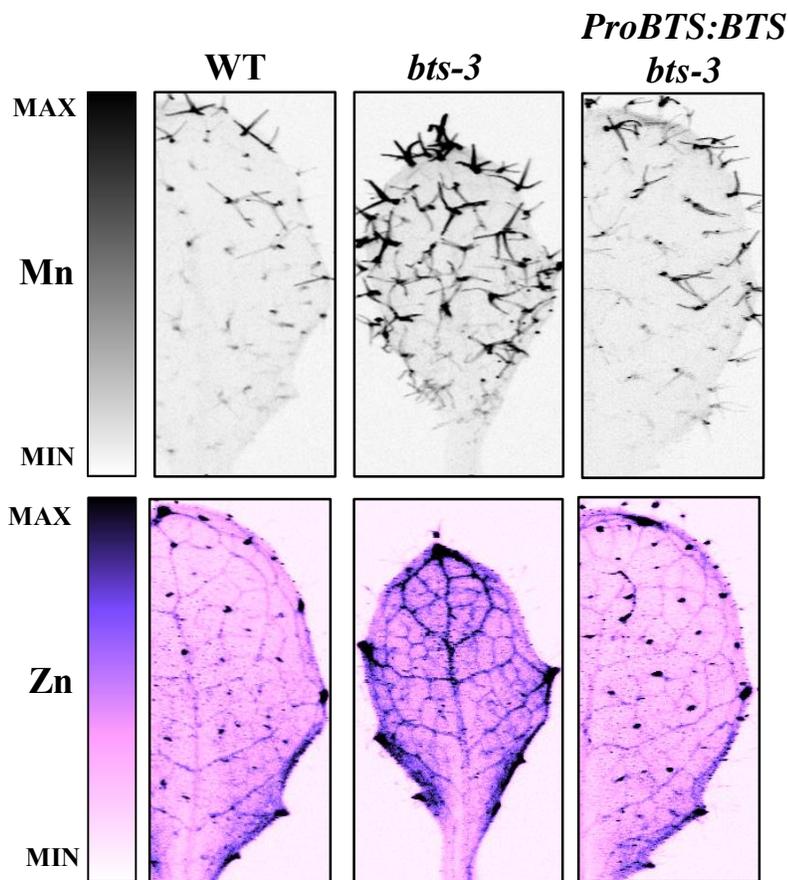
Supplemental Figure 4. Expression of *BTS* controlled by its endogenous promoter in *bts-3* returns Mn and Zn levels to wild type levels.
SXRF scans showing Fe localization in leaf #5 of plants grown for 2 weeks on B5 medium and then transferred to +Fe medium for 3 days. *ProBTS:BTS bts-3* is the complementation line where the *BTS* gene driven by its own promoter is expressed in the *bts-3* mutant.
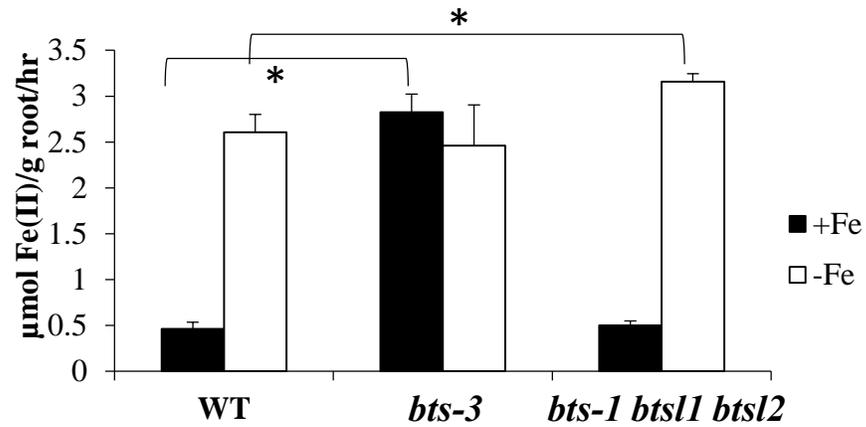
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Supplemental Figure 5. Unlike *bts-3*, *bts-1 btsl1 btsl2* mutants do not exhibit induction of ferric chelate reductase activity when grown under Fe-sufficient conditions.
Ferric chelate reductase activity of roots of plants grown for 2 weeks on B5 medium before transfer to +Fe or –Fe medium for 3 days. *p<0.05 (Student's t-test). Brackets indicate comparisons. Error bars indicate SE.

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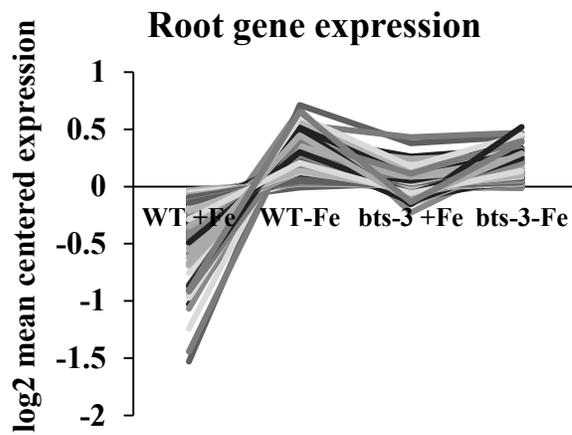
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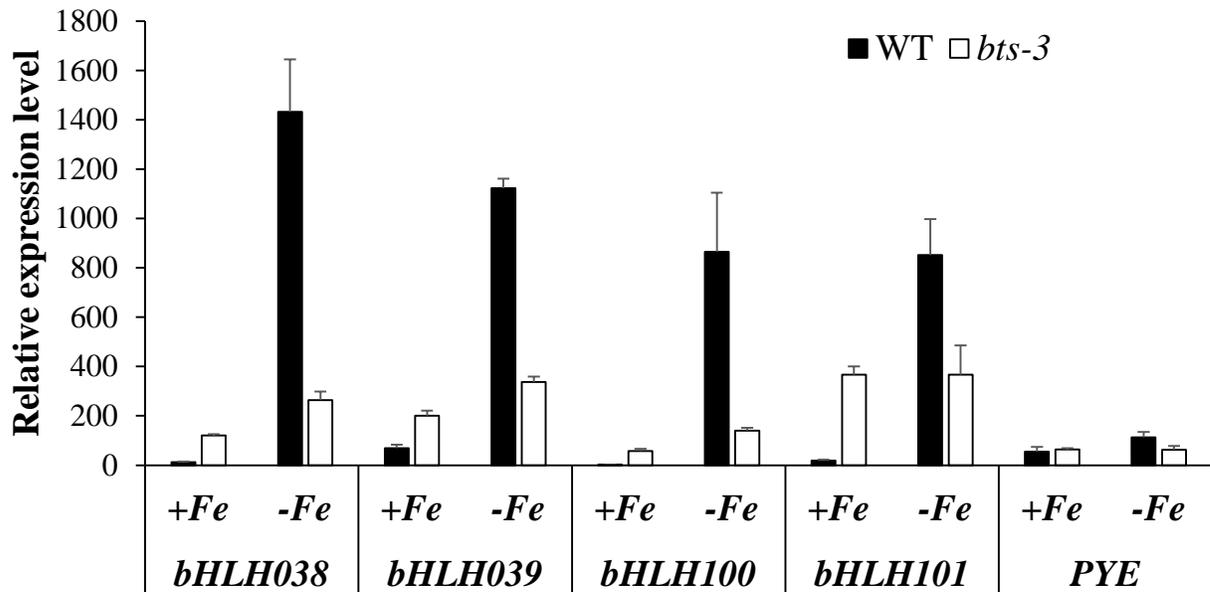
Supplemental Figure 6. *bts-3* roots exhibit constitutive expression of Fe deficiency genes.
A. Line graphs showing gene expression profiles of all genes from cluster 4 of root microarray analysis.
B. qPCR of bHLH transcription factor expression in roots of plants grown for 2 weeks on B5 medium and then transferred to +Fe or –Fe medium for 3 days. Expression is relative to EF1α . Error bars represent SE (n=3).

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Supplemental methods: SXRF analysis

Plants for NSLS leaf SXRF experiments were grown for 2 weeks on B5 and 3 days on +Fe or –Fe medium. Leaf #5 from plants of each genotype was detached and mounted on the sample stage on metal free Kapton$_{TM}$ tape just prior imaging. X26A and X27A use Kirkpatric-Baez (KB) mirror microprobes and a Ge detector. Incident energy for each leaf image was 11 keV, step size was 10 µm and dwell time was 100 milliseconds. Dry seeds from soil-grown plants imaged at NSLS were also mounted on Kapton$_{TM}$ tape and scanned with a 5 µm step size and 100 millisecond dwell time.

Roots, leaves, and siliques for SSRL experiments were detached and mounted on Kapton$_{TM}$ tape from plants just prior to analysis. Roots were from that were grown for 7 days on B5 medium and 3 days on –Fe medium. Leaf #8 for shoot images was from plants grown for 10 days on B5 medium. Green, developed siliques were from plants grown on soil. Beamline 2-3 also uses a KB mirror microprobe with a Ge detector. Root images had a step size of 1 µm and dwell time of 100 milliseconds. Leaves had a 7 µm step size and 30 millisecond dwell time. Siliques had a 7 µm step size and 50 millisecond dwell time.

Dry seeds imaged at XFM were from soil grown plants and mounted on metal free Ultralene thin window film®. A KB mirror microprobe was used with the Maia detector, a Si detector (Ryan et al., 2010). Incident energy was 11 keV, step size was 2 µm and dwell time was 2.6 milliseconds.

High resolution imaging of leaf sections were conducted at 2-ID-D of the Advanced Photon Source. Plants were grown on B5 medium for 10 days and leaf #1 was fixed for sectioning. Sample preparation was performed as described, using LR White embedding resin, 1µm thick sectioning, and mounting on silicon nitride windows (Punshon et al., 2012). The 2-ID-D beamline uses a zone plate insertion device to achieve a highly focused beam. Imaging was conducted using an incident energy of 10.1 keV, 0.15 µm step size, and 500 millisecond dwell time.

Data gathered at SSRL was analyzed with Sam's Microtool Anlaysis Kit (SMAK) (microtoolkit.sams-xrays.com), data from NSLS was processed with Xmap Plotter. (bnl.gov/x26a/comp_download.shtml) and data from the Australian Synchrotron was analyzed with Image J (http://imagej.nih.gov/ij/). Images are false colored to show fluorescence counts detected on a scale from minimum to maximum for specific elements. Images that are directly compared are scaled to the same minimum and maximum fluorescence counts.

Supplemental References

Punshon, T., Hirschi, K., Yang, J., Lanzirotti, A., Lai, B., and Guerinot, M.L. (2012). The role of CAX1 and CAX3 in elemental distribution and abundance in Arabidopsis seed. Plant Physiol. **158,** 352-362.

Ryan, C.G., Kirkham, R., Hough, R.M., Moorhead, G., Siddons, D.P., de Jonge, M.D., Paterson, D.J., De Geronimo, G., Howard, D.L., and Cleverley, J.S. (2010). Elemental X-ray imaging using the Maia detector array: The benefits and challenges of large solid-angle. Nucl. Instrum. Meth. A. **619,** 37-43.
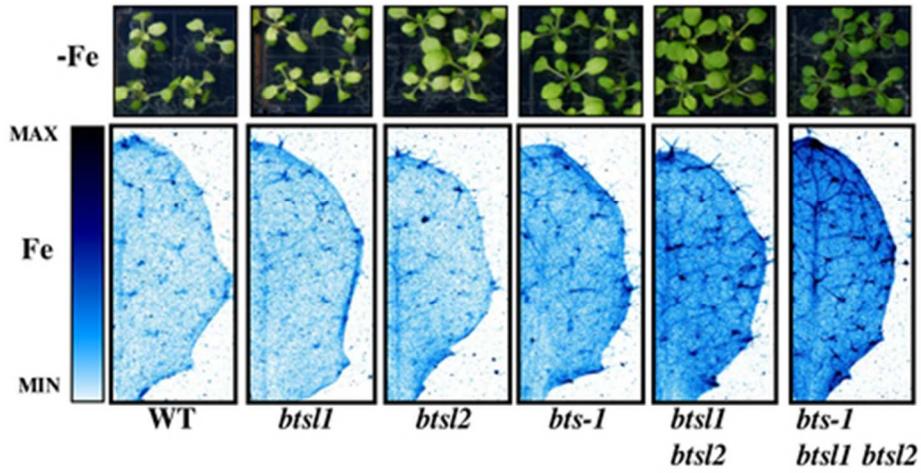
BTS family members negatively regulate the Fe deficiency response; mutants have increased Fe levels and tolerance to Fe deficiency.

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BRUTUS* and its paralogs, *BTS LIKE1* and *BTS LIKE2,* encode important negative regulators of the iron deficiency response in *Arabidopsis thaliana

Running title: E3 ligases mediate Arabidopsis Fe homeostasis

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Abstract

Iron (Fe) is required for plant health, but it can also be toxic when present in excess. Therefore, Fe levels must be tightly controlled. The *Arabidopsis thaliana* E3 ligase BRUTUS (BTS) is involved in the negative regulation of the Fe deficiency response and we show here that the two *A. thaliana BTS* paralogs, *BTS LIKE1* (*BTSL1*) and *BTS LIKE*2 (*BTSL2*) encode proteins that act redundantly as negative regulators of the Fe deficiency response. Loss of both of these E3 ligases enhances tolerance to Fe deficiency. We further generated a triple mutant with loss of both *BTS* paralogs and a partial loss of *BTS* expression that exhibits even greater tolerance to Fe-deficient conditions and increased Fe accumulation without any resulting Fe toxicity effects. Finally, we identified a mutant carrying a novel missense mutation of *BTS* that exhibits an Fe deficiency response in the root when grown under both Fe-deficient and Fe-sufficient conditions, leading to Fe toxicity when plants are grown under Fe-sufficient conditions.

Significance to *Metallomics*

Iron (Fe) deficiency commonly limits plant growth. If Fe homeostasis were understood, it might be feasible to engineer plants better able to grow in soils now considered marginal and to increase crop biomass on soils now in cultivation. Furthermore, as most people rely on plants as their dietary source of Fe, plants that serve as better sources of this essential nutrient would improve human health. In this study, we characterize the role of three closely related negative regulators of the Fe deficiency response. A triple mutant has increased tolerance to Fe deficient growth conditions and increased Fe accumulation without resulting toxicity.

Introduction

Iron (Fe) is essential for plant growth, crop yields and human health. Many people, particularly those in developing countries, rely on plants for dietary Fe. Unfortunately, Fe has a limited solubility in many neutral or basic soils and therefore Fe is not readily accessible in the rhizosphere [1]. This low solubility leads to a restricted Fe content in many plants and is a major factor contributing to the widespread prevalence of Fe deficiency anemia for people with plant-based diets. Thus, increasing plant Fe acquisition and storage may have profound impacts on plant and human nutrition. In order to manipulate plants to increase bioavailable Fe, it is first imperative we understand the genes and mechanisms governing Fe homeostasis in plants. When

faced with low Fe conditions, non-graminaceous plants such as *Arabidopsis thaliana* (Arabidopsis) employ a classic response to boost Fe mobilization and uptake from the soil. Root plasma membrane H^+-ATPases release protons to acidify and thus increase Fe solubility in the soil [2]. In addition to acidification, Fe^{3+} is reduced to Fe^{2+} by the membrane-bound ferric-chelate reductase enzyme FERRIC OXIDASE REDUCTASE 2 (FRO2) in Arabidopsis [3]. Fe deficiency also induces root secretion of phenolic compounds, particularly coumarins, which serve as direct Fe^{3+} reductants and also as Fe^{3+} and Fe^{2+} chelating compounds to improve Fe mobilization and reduction [4-7]. This series of mechanisms is known as the reduction strategy or alternatively Strategy I [1]. Reduced Fe is transported into the root by the plasma-membrane divalent cation transporter IRON-REGULATED TRANSPORTER 1 (IRT1) [8-11].

These activities to boost Fe mobilization under Fe-deficient conditions are controlled by several transcription factors in *Arabidopsis*. One major transcription factor identified in a well-characterized Fe deficiency network is a bHLH protein called FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT) [12-14]. Like *IRT1* and *FRO2, FIT* is induced in the root epidermis in Fe deficiency and expression of *IRT1* and *FRO2* requires FIT [12-14]. Other bHLH transcription factors which are induced in Fe-deficient conditions are those of the bHLH1b subfamily (bHLH38, bHLH39, bHLH100, bHLH101) and evidence suggests that FIT activity and activation of downstream Fe deficiency targets depends on heterodimerization of FIT with one of these bHLH transcription factors [14-17]. FIT also controls *MYB10* and *MYB72,* two other transcription factors essential for growth of plants on low Fe conditions [12, 18, 19].

Another set of transcriptional changes upon Fe deficiency occurs in the vasculature. A major player identified in this network is another bHLH transcription factor called POPEYE (PYE) [20]. *PYE* expression is highest in the root pericycle, but PYE protein is localized to the nuclei of all cells in Fe-deficient roots, suggesting that PYE may move throughout the root, linking it to the *FIT* network. PYE has been shown to negatively regulate the expression of known Fe deficiency targets *NICOTIANAMINE SYNTHASE 4* (*NAS4), FERRIC REDUCTASE OXIDASE 3* (*FRO3),* and *ZINC-INDUCED FACILITATOR* (*ZIF1*). Like FIT, PYE may require other bHLH proteins as binding partners to interact with downstream targets. Yeast two hybrid, bimolecular fluorescence complementation, and pulldown analyses have demonstrated that PYE can interact with bHLH104, bHLH115, and bHLH105 or IAA-LEUCINE RESISTANT 3 (ILR3), three bHLH transcription factors belonging to bHLH subclade IVc (of which only

bHLH115 has increased expression under Fe-deficiency) [20-24]. By coexpression analysis with

PYE, a gene encoding a RING E3 ligase with three hemerythrin/HHE Fe-binding domains called

BRUTUS (BTS) was also classified into this network. BTS is thought to act indirectly with PYE

because although it does not itself interact with PYE, it interacts with the same three IVc bHLH

transcription factors as PYE [20]. BTS was subsequently demonstrated to be a functional RING E3

ligase *in vitro*, and although it has not been confirmed *in planta*, the three IVc bHLH

transcription factors are proposed as targets for proteasomal degradation after ubiquitination by

BTS [23]. Two of these putative targets, bHLH104 and ILR3 (bHLH105), were recently identified

as positive regulators of the Fe deficiency response because loss-of-function mutants exhibit

decreased tolerance to Fe deficiency [22]. Further, bHLH104 and ILR3 regulate downstream

expression of *bHLH38/39/100/101* and *PYE*, genes from both the *FIT* and *PYE* networks [22]. *bts*

knockdown mutants, on the other hand, exhibit increased tolerance to Fe deficiency and thus

negatively regulate Fe uptake [20, 22, 23]. While a full length *BTS* construct can complement *bts-1*

mutant phenotypes under low Fe conditions, a deletion construct lacking the RING domain fails

to complement the mutant, suggesting the E3 ligase activity of BTS is critical for BTS'

repressive role in the Fe deficiency response [25]. Interestingly, complete loss-of-function *bts*

mutants exhibit embryo lethality [26].Taken together, current data suggests a model where BTS

acts as a negative regulator by targeting transcription factors important in positive regulation of

the Fe deficiency response for degradation.

Understanding the balance between positive and negative regulation of the Fe deficiency

response is essential for efforts to engineer plants with more Fe that do not experience Fe

toxicity. Although plants are often challenged with Fe deficiency, no environment remains

constant and Fe availability in the rhizosphere depends on many factors. When sufficient Fe is

available, it is crucial for plants to effectively suppress the Fe deficiency response to avoid

excessive uptake.

We found that in addition to BTS, two closely related RING E3 ligases that we named

BRUTUS LIKE1 and BRUTUS LIKE2 are important in negative regulation of the Fe deficiency

response. The double *btsl1 btsl2* mutant has increased Fe concentrations and tolerance to Fe

deficiency compared to wild type and the triple *bts-1 btsl1 btsl2* mutant has even higher

concentrations of Fe and tolerance to Fe deficiency, but without excessive Fe accumulation

leading to Fe toxicity. Additionally, we identified a novel allele of *BTS* in a mutagenesis screen

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for altered Fe accumulation. This mutant, *bts-3,* contains a point mutation in the RING E3 ligase domain of BTS, suggesting its E3 ligase activity may be impaired. We sought to explore the unique nature of this missense mutant compared to the other *bts* mutants, which are either embryo lethal or have a reduction in steady-state transcript levels and likely represent partial-loss-of BTS function. *bts-3* is more tolerant than wild type to Fe-deficient conditions. Further, *bts-3* is sensitive to Fe-sufficient conditions and accumulates excessive Fe. Using microarray analysis, we demonstrated that these *bts-3* phenotypes are associated with expression of the Fe deficiency response in the root under both Fe-sufficient and Fe-deficient growth conditions, despite accumulation of high concentrations of Fe in the root. Our characterization of *btsl1, btsl2,* and *bts* mutants increases our understanding of the role of BTS family members as negative regulators of the Arabidopsis Fe deficiency response.

Results

Identification of two *BTS* paralogs in Arabidopsis

To determine if there are any other proteins which may play a similar or overlapping role to BTS, we used the Ensembl Plants bioinformatics tool to identify genes encoding proteins in Arabidopsis containing both putative hemerythrin and RING domains [27]. This analysis identified two *BTS* paralogs, *At1g74770* (*BTSL1)* and *At1g18910* (*BTSL2*) (Figure 1A). Using an amino acid alignment and identity matrix calculated by ClustalW of the proteins encoded by *BTS*, *BTSL1* and *BTSL2*, we determined that BTSL1 and BTSL2 exhibit 72% amino acid identity and BTSL1 and BTSL2 each have 38% amino acid identity with BTS [28]. Analysis of domain architecture predicted by NCBI CD-Search revealed that while BTS contains three putative hemerythrin domains, BTSL1 and BTSL2 each contain two (Figure 1A) [29]. Each E3 ligase also contains a C-terminal Zf-CHY, RING, and Zinc ribbon domain. The RING domain of each protein has a conserved octet of Cys and His residues that form a canonical C3H2C3 RING structure for Zn^{2+} coordination and predicted RING E3 ligase enzymatic activity (Figure 1A). These proteins were also identified in a phylogenetic analysis of hemerythrin domain containing proteins in a study examining the role of the *BTS* orthologs in rice, *Oryza sativa* Haemerythrin motif-containing Really Interesting New Gene (RING)- and Zinc-finger proteins 1 and 2 (*OsHRZ1* and *OsHRZ2*) [30].

All three genes of the *BTS* family in Arabidopsis exhibit significantly increased expression in roots of plants exposed to Fe-deficient conditions compared to those grown under Fe-sufficient conditions (Figure 1B). *BTS* and *BTSL2* are expressed at similar levels in the roots and both exhibit approximately a 3-fold increase in expression in Fe-deficient conditions compared to Fe-sufficient conditions. *BTSL1*, on the other hand, exhibits an 8-fold increase in expression in Fe-deficient conditions. In the shoot, we found significant expression of *BTS*, which, like in roots, is increased in response to Fe-deficient growth conditions (approximately a 16-fold induction) (Figure 1B). *BTSL1* and *BTSL2* were not significantly expressed in shoots, regardless of Fe status.

Knockdown of *BTS* family E3 ligases increases tissue Fe concentrations and tolerance to Fe deficiency

A previously identified *bts-1* T-DNA insertion mutant has steady state mRNA levels 70% of those in wild type, and is more tolerant to Fe deficiency than wild type [20, 23]. Because *BTSL1* and *BTSL2* are also expressed under Fe-deficient conditions in the root, we wanted to determine if loss of *BTSL1* and *BTSL2* would affect tolerance to Fe deficiency. We obtained T-DNA insertion lines for each gene (Supplemental Figure 1A). The T-DNA insertion in exon two of *BTSL1* in SALK_015054 results in complete loss of full-length *BTSL1* transcript (Supplemental Figure 1B). In *BTSL2,* sequencing revealed that although the entire open reading frame is present, the T-DNA insertion in SAIL_615_H01 results in a chimeric transcript containing segments of T-DNA, promoter, and the 5'UTR fused upstream of the *BTSL2* open reading frame. This chimeric transcript contains several alternative start sites, making it unlikely that the native translation product is produced in this line (Supplemental Figure 1B).

Compared to wild type shoots which exhibit chlorosis when grown under Fe-deficient conditions, *bts-1* plants were greener and contained significantly more chlorophyll (a 44% increase compared to wild type) (Figure 2A), and as demonstrated previously, had longer roots than wild type when grown vertically under Fe-deficient growth conditions (Supplemental Figure 2A). By the same parameters, *btsl1* and *btsl2* single mutants were indistinguishable from wild type (Figure 2A, Supplemental Figure 2A). However, due to their high amino acid identity, we hypothesized that BTSL1 and BTSL2 function redundantly and thus we generated the double *btsl1 btsl2* mutant. Like *bts-1, btsl1 btsl2* had greener shoots, higher chlorophyll levels (a 92%

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increase compared to wild type), and increased root length when plants were grown under Fe-deficient conditions (Figure 2A, Supplemental Figure 2A) compared to wild type. Further, *bts-1* and the *btsl1 btsl2* double mutant had significantly higher Fe concentrations in roots (65% and 78% respectively) and shoots (33% and 25% respectively) of plants grown under Fe-sufficient conditions compared to wild type (Figure 2C). To assess whether both reduction of *BTS* and loss of *BTSL1* and *BTSL2* expression can further increase Fe content and tolerance to Fe deficiency, we created the triple *bts-1 btsl1 btsl2* mutant. Compared to the single *bts-1* mutant and the *btsl1 btsl2* double mutant alone, the triple *bts-1 btsl1 btsl2* mutant exhibited additional tolerance to Fe deficiency as seen by visibly greener shoots, significantly increased chlorophyll concentrations (236% increase compared to wild type), and longer roots when plants were grown on Fe-deficient conditions (Figure 2A, Supplemental Figure 2A). The triple mutant also had significantly higher Fe concentrations in roots and shoots of plants grown under both Fe regimes compared to wild type (Figure 2C). In addition, we found that triple mutant seeds have significantly higher concentrations of Fe than wild type (32% increase) (Figure 2D). Imaging Fe *in situ* in intact seeds revealed that although seed Fe concentrations are increased significantly in the triple mutant, Fe localization is not perturbed from wild type and is associated with the embryonic vasculature as previously described (Figure 2E) [31].

Increased Fe concentrations and storage in these mutants is not associated with Fe toxicity

Because increased Fe accumulation can be toxic to plants, we assessed *btsl1*, *btsl2*, *bts-1*, *btsl1 btsl2*, and *bts-1 btsl1 btsl2* for signs of Fe toxicity, such as stunted root growth or necrosis, when plants were grown under Fe-sufficient conditions. None of these mutants appeared different from wild type under these conditions (Supplemental Figure 2B). Thus, increased Fe concentrations in these mutants enhances tolerance to Fe-deficient growth conditions, but does not negatively impact growth under Fe-sufficient conditions. Because of this, we were interested in where these mutants store Fe to detoxify it. To address this question, we imaged Fe *in vivo* in intact leaves of our mutants compared to wild type plants grown under Fe-sufficient conditions. This *in vivo* elemental imaging also demonstrated increased Fe levels in mutants (Figure 2B), supporting data obtained by the bulk quantification of Fe concentrations in digested whole leaves (Figure 2B and Figure 2C). From a two-dimensional perspective, in wild type, *btsl1* and *btsl2* single mutants, Fe is distributed relatively evenly throughout the leaf surface with some

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enrichment in the main vein and in trichomes. In *bts-1* and *btsl1 btsl2*, Fe accumulation is increased throughout the leaf, the central vein, and trichomes compared to wild type. The triple mutant exhibits a further increase in Fe throughout the leaf compared to *bts-1* and *btsl1 btsl2* and increased enrichment of Fe in the central vein and trichomes and in minor veins (Figure 2B). These data suggest these regions are sites of Fe accumulation/storage in Arabidopsis leaves.

Elemental imaging of leaves from plants grown under Fe-deficient conditions demonstrates that in wild type, trichomes are depleted of Fe and no enrichment can be seen in the main vein as in plants grown under Fe-sufficient conditions (Figure 3 top panel). The double mutant has less Fe in the trichomes of plants exposed to Fe-deficient conditions compared to Fe-sufficient conditions, but still contains Fe in trichomes and the main vein, which is not seen in wild type (Figure 2B and Figure 3). In agreement with increased total leaf Fe concentrations (Figure 2C), the triple mutant has even more Fe in the trichomes, the main vein, and throughout the leaf tissue compared to the double mutant (Figure 3).

BTS family E3 ligase mutants also have increased concentrations of other metals

Because metals share and compete for uptake transporters, we wanted to determine if our mutants had higher concentrations of other essential micronutrient metals, particularly those transported by IRT1: Zn and Mn [8, 32]. We found that in roots, the double *btsl1 btsl2* and triple *bts-1 btsl1 btsl2* mutants had significantly higher Zn concentrations compared to wild type and *bts-1* (Supplemental Figure 3A). In shoots, the double and triple mutants had significantly higher concentrations of Zn compared to wild type and *bts-1* and the triple mutant had significantly higher concentrations of Mn compared to wild type, *bts-1*, and *btsl1 btsl2* (Supplemental Figure 3A). None of the mutants had elevated concentrations of Cu, a metal not transported by IRT1, in either roots or shoots. In seeds, we found significantly higher concentrations of Mn and Zn in *bts-1*, *btsl1 btsl2*, and *bts-1 btsl1 btsl2* compared to wild type (51%, 35%, and 46% increases of Mn compared to wild type, respectively and 22%, 36%, and 60% increases of Zn compared to wild type, respectively) (Supplemental Figure 3B).

We imaged Zn and Mn *in vivo* in intact leaves in shoots of the double and triple mutants compared to wild type (Figure 3 middle and bottom panels). We found that in wild type leaves, Zn enrichment is mainly found in hydathodes and veins. In *btsl1 btsl2* and *bts-1 btsl1 btsl2* mutants, Zn accumulates in the hydathodes, and more is seen in the veins and at the base of

trichomes. Mn is found mainly at the base of the trichomes in wild type and in the double and triple mutants, it can be seen in the stalk and branches of trichomes. These increases we see in Zn and Mn at specific sites in the leaf are in agreement with the increases in Zn and Mn concentrations we observe in digested whole leaf samples (Supplemental Figure 3A).

Identification of *bts-3*, an Fe overaccumulating *BTS* mutant

In an attempt to uncover additional regulators of the Fe deficiency response, we screened leaves from over 6,000 EMS mutagenized Arabidopsis plants following a high throughput elemental profiling approach we previously used [33] to successfully identify mutants with altered elemental profiles (*aka* ionome) [34-38]. In this screen, we identified a mutant that accumulated significantly more Fe than wild type. The mutant was backcrossed twice and the mutation determined to be recessive. Bulk segregant analysis with microarray-based detection of genetic markers on phenotyped pools of F2 plants from a *bts-3* x Ler outcross, following our previously developed approach [39-41], placed the causal mutation in a 1 Mb interval centered at 6 Mb on chromosome 3. Fine mapping of F2 recombinants using SNP-based markers was used to narrow down the mutation to a region of chromosome 3 between 6237000 and 6342000. Sequencing in this region revealed a C to T mutation at base pair 5753 of *BTS* genomic DNA. The single base pair change in this *bts-3* mutant confers a Pro to Leu change at amino acid 1174 in the RING domain of BTS (Figure 1A).

Elemental analysis revealed that *bts-3* contained significantly higher concentrations of Fe in roots and shoots when grown under Fe-sufficient and Fe-deficient conditions compared to wild type confirming the results obtained in the original screen (Figure 4A). *bts-3* seeds have Fe concentrations that are more than double than those of wild type, (a 244% increase) compared to the 32% increase in seed Fe of the *bts-1 bts1 btsl2* triple mutant compared to wild type (Figure 4A, Figure 2D). *bts-3* also had significantly higher concentrations of Fe in roots of plants grown under Fe-sufficient conditions and in shoots of plants grown under both Fe conditions compared to the triple mutant (Figure 2C and Figure 4A). We also performed *in vivo* elemental imaging on intact tissues and we found that increased Fe accumulation occurs in both roots and shoots in *bts-3* compared to wild type (Figure 4B-C), confirming our analysis of the total Fe concentration in whole roots and leaves (Figure 4A). In roots, where Fe uptake from the rhizosphere occurs, Fe in wild type was mainly localized to a distinct portion of the root meristem, perhaps the quiescent

center (Figure 4B). In *bts-3* roots, however, Fe was dispersed beyond this region in puncta, which may represent vacuolar Fe storage sites. In Fe-sufficient leaves, Fe was also greatly increased, particularly in the vasculature (including the central vein and minor veins) and trichomes (Figure 4C). We also examined *bts-3* alongside the previously imaged Fe-deficient leaves and found that *bts*-3 leaves have increased Fe storage in trichomes and throughout the leaf tissue compared to the double and triple mutants (Figure 3). Because our two-dimensional analysis revealed an increase in Fe associated with the vasculature in *bts-3*, we wanted to determine where Fe accumulates at the cellular level. Elemental imaging of manually cross-sectioned primary vasculature revealed that *bts-3* has higher levels of Fe in phloem compared to wild type (Figure 4E). Finally, *in vivo* elemental imaging of seeds within green siliques and of dry seed demonstrated that Fe is associated with the vasculature of the embryo at a much higher concentration in *bts-3* seed compared to wild type (Figure 4D, Figure 4F). Higher resolution elemental imaging reveals that Fe also accumulates in the seed coat of *bts-3* (Figure 4F).

bts-3 also accumulates high concentrations of other metals

We wanted to determine if *bts-3* had increased concentrations of other metals in addition to Fe. Bulk analysis of digested whole leaves revealed increases in Mn and Zn concentrations compared to wild type in both roots and shoots of *bts-3* plants (Supplemental Figure 3A). Compared to the triple mutant, *bts*-3 had similar concentrations of Zn in roots and shoots, but significantly higher concentrations of Mn in both tissues (Supplemental Figure 3A). In comparison with our *in vivo* imaging of Zn and Mn accumulation in shoots, we found that like the triple mutant, *bts-3* had Zn enrichment in the veins, hydathodes, and at trichome bases (Figure 3). *bts-3* trichomes had higher levels of Mn compared to trichomes of the triple mutant (Figure 3). In seeds, *bts-3* had significantly higher concentrations of Mn and Zn compared to wild type (Supplemental Figure 3C). We note that concentrations of Mn and Zn in wild type seeds were different for seeds shown in Supplemental Figure 3B compared to Supplemental Figure 3C. This is likely due to variation in metal content of the soil and/or water, as these experiments were performed independently.

bts-3 is sensitive to Fe supply, but thrives in Fe-deficient conditions

We next examined the effect of increased Fe accumulation in *bts-3* plants grown under Fe-deficient and Fe-sufficient conditions. When *bts-3* plants were grown on normal, Fe-

sufficient soil, they were smaller than wild type and *bts-1* plants (Figure 5A). However, when plants were grown on Fe-deficient alkaline soil, both *bts-1* and *bts-3* plants were larger and greener than wild type, which is small and chlorotic. The chlorosis that developed in wild type plants grown on alkaline soil could be rescued by watering plants with exogenous Fe. However, when *bts-1* and *bts-3* plants were watered with the same amount of Fe, *bts-3* plants did not grow beyond the seedling stage, while *bts-1* plants were visibly unaffected by Fe addition. In addition, we observed that *bts-3* had visibly shorter roots and small shoots compared to wild type plants grown on Fe-sufficient plates (Figure 5B). Although our bulk and imaging analyses of Fe revealed increased shoot Fe concentrations of *bts-1* plants grown under Fe-sufficient conditions (Fig 2C & Figure 5C), *bts-1* did not display the short root phenotype, highlighting the unique phenotype of *bts-3* (Figure 5B, See also Supplemental Figure 2B for comparison to previously discussed mutants of this study). On the other hand, in Fe-deficient conditions, *bts-3* grew longer roots than wild type (Figure 5A, Supplemental Figure 2A), similar to *bts-1* mutants.

We could restore to wild type levels *bts*-3 Fe sensitivity, leaf size, and Fe levels/localization when we transformed the mutant with a complementation construct containing the wild type Col-0 allele of *BTS* driven by its native promoter, which we named *ProBTS:BTS bts-3* (Figure 5C-D). This complementation line confirmed that the recessive mutation in *BTS* is responsible for the Fe-related phenotypes of *bts-3*. Mn and Zn were also restored to wild type levels and distribution patterns in complemented lines (Supplemental Figure 4).

bts-3 roots exhibit the Strategy I Fe deficiency response regardless of Fe growth conditions

Because *bts-3* accumulates more Fe than wild type, experiences Fe toxicity when grown under Fe-sufficient conditions, and thrives in Fe-deficient conditions, we hypothesized that *bts-3* has a constitutively active Fe deficiency response. At the transcriptional level, *bts*-3 exhibits increased expression of *FRO2* and *IRT1* in Fe-sufficient conditions compared to WT and decreased expression in Fe-deficient conditions compared to WT (Figure 6A). Assays of ferric-chelate reductase activity showed that unlike wild type, where significant Fe reduction occurred only in Fe-deficient conditions, *bts-3* reduced Fe^{3+} regardless of Fe status (Figure 6B). We also examined ferric chelate reductase activity in the *bts-1 btsl1 btsl2* Fe accumulating triple mutant; although we see an increase in ferric chelate reductase activity in Fe-deficient conditions compared to wild type, there is no increase in Fe reduction in the triple mutant in Fe-sufficient

conditions compared to wild type (Supplemental Figure 5). By western blotting, we showed that in wild type, IRT1 protein is only present in Fe-deficient conditions (Figure 6C). In *bts-3* however, IRT1 protein accumulation occurs in Fe-sufficient conditions as well as in Fe-deficient conditions. Although this IRT1 accumulation in *bts*-3 plants grown under Fe-sufficient conditions is aberrant compared to wild type, IRT1 protein levels still exhibit Fe regulation because concentrations in Fe-deficient conditions are higher than concentrations in Fe-sufficient conditions.

The *bts-3* transcriptome varies dramatically from wild type

Because we found the Fe uptake response to be active in *bts-3* roots regardless of Fe status, we wanted to understand at what level BTS might be acting, i.e. how upstream BTS acts and if *BTS* misregulation affects only a subset or multiple subsets of Fe-regulated genes. We were also interested in studying the transcriptome of the *bts-3* mutant to determine how high/toxic Fe concentrations influence gene expression. To address these questions, we performed a microarray analysis comparing roots of wild type to *bts-3* plants exposed to either Fe-sufficient or Fe-deficient growth conditions. We performed hierarchical clustering analysis on genes that were at least 1.5-fold upregulated or downregulated by Fe in either wild type or mutant. From top to bottom of heat map (Figures 7), four major defined subsets of genes were found in the cluster analyses: 1) genes which were more highly expressed in Fe-sufficient conditions compared to Fe-deficient conditions in wild type, 2) genes which were more highly expressed in *bts-3* in Fe-sufficient conditions compared to all other genotypes/treatments, 3) genes which were expressed at lower levels in *bts-3* Fe-sufficient conditions compared to other genotypes/treatments, and 4) genes which were more highly expressed in Fe-deficient conditions compared to Fe-sufficient conditions in wild type (Figure 7). We also performed Gene Ontology (GO) term enrichment analysis on each of these subsets to understand general features of *BTS* misregulation [42, 43]. We will briefly discuss major findings of each subset that are most relevant to our understanding of the role of BTS in Fe homeostasis.

Subset 1: Genes upregulated in Fe-sufficient wild type roots exhibited reduced expression in Fe-sufficient *bts-3* roots

The subset of genes that were more highly expressed in the roots in Fe-sufficient

conditions compared to Fe-deficient conditions in wild type were enriched in the following GO

terms: Fe ion homeostasis, oxidation/reduction, and transition metal binding, In general,

expression of these genes was not increased to reflect higher Fe levels in *bts-3* (Figure 7).

Included in this subset of genes were *FERREDOXIN* (*FEDA*)*, STROMAL ASCORBATE*

PEROXIDASE, (*SAPX*)*,* and *FERRITIN,* (*FER4*) (encoding the major Fe storage protein, FER).

Although *bts-3* roots contained significantly more Fe than wild type roots in Fe-sufficient

conditions, expression of many Fe sufficiency induced genes within this subset did not increase

accordingly and were actually reduced compared to wild type. When plants were grown on Fe-

deficient medium, expression of the genes within this subset was low in wild type roots, but

slightly increased in *bts-3* roots, which corresponded to the higher Fe concentration in *bts-3* roots

compared to wild type roots under Fe deficiency. However, *bts-3* roots grown under Fe

deficiency still contained more Fe than wild type roots under Fe sufficiency, and expression of

these genes did not reach wild type levels. As a whole, the *bts-3* root did not display an Fe-

sufficient gene expression profile that corresponded simply with its Fe status.

Subsets 2 and 3: High concentrations of Fe in *bts-3* roots corresponded with major transcriptional remodeling in Fe-sufficient conditions

There were a large number of genes that did not exhibit strong changes in expression

levels in response to Fe treatment in wild type and were expressed at similar levels to wild type

in *bts-3* grown under Fe-deficient conditions but had increased expression in *bts-3* grown under

Fe-sufficient conditions (Figure 7, Subset 2). Enriched GO terms included response to metal ion,

cell wall, glutathione-S-transferases, and response to oxidative stress. Included in this subset

were known metal homeostasis genes, including genes encoding Fe transporters called

NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 3 (*NRAMP3*) and *FERRIC*

REDUCTASE DEFECTIVE 3 (*FRD3*)*,* an Al transporter *ALUMINUM-ACTIVATED MALATE*

TRANSPORTER 1 (*ALMT1*)*,* a Pb transporter *PLEIOTROPIC DRUG RESISTANCE 12*

(*PRD12*)*,* and the gene encoding the enzyme for synthesis of the Fe chelator nicotianamine,

NICOTIANAMINE SYNTHASE 2 (*NAS2*)*.*

Subset 3 includes genes that were relatively unchanged by Fe status in wild type and

expressed at similar levels to wild type in *bts-3* Fe-deficient plants, but significantly

downregulated in *bts-3* Fe-sufficient roots (Figure 7). The significantly enriched GO term in this subset was endomembrane system.

Subset 4: *bts-3* roots expressed Fe deficiency responsive genes regardless of Fe status

　　　　In subset 4, many genes that were induced only in Fe-deficient conditions in wild type were expressed in *bts-3* roots grown under both Fe regimes (Figure 7, Supplemental Figure 6A). In most cases, expression of these genes did not reach the level of induction seen in wild type Fe-deficient roots, but they were still elevated compared to wild type Fe-sufficient roots. Further, many of the Fe deficiency responsive genes exhibited dampened expression in *bts-3* roots compared to wild type roots in plants grown under Fe-deficient conditions. In addition, *bts-3* Fe-deficient roots showed increased expression of these genes in Fe-deficient conditions compared to *bts-3* roots in Fe-sufficient conditions. Taken together, this indicates that *bts-3* roots did have some ability to regulate transcription in response to Fe status, but it was still greatly perturbed compared to wild type because roots were unable to completely repress gene expression in Fe-sufficient conditions as wild type roots did. For example, two known upstream Fe deficiency responsive genes, *FIT* and *PYE*, encoding bHLH transcription factors, exhibited increased expression in *bts-3* Fe-sufficient conditions compared to wild type. Similarly, other transcription factors involved in the Fe deficiency response that are normally induced only under Fe deficiency also showed expression in roots of *bts-3* plants grown under Fe-sufficient conditions. These were *bHLH38, bHLH39, bHLH100, bHLH101, MYB10,* and *MYB72. FIT* is required for activation of downstream targets *FRO2* and *IRT1,* which as previously mentioned are responsible for Fe reduction and uptake in the root. These genes were also induced in *bts-3* Fe-sufficient roots. *PYE* targets *FRO3* and *ZIF1* were also constitutively activated in *bts-3.* The downstream targets of MYB10 and MYB72, *NAS2* and *BETA GLUCOSIDASE 42* (*BGLU42*), were also induced regardless of Fe status in the *bts-3* mutant. Other Fe deficiency responsive genes that were activated in Fe-sufficient conditions in *bts-3* included *NRAMP1, NRAMP4, IRT2, ZIP8, COPPER TRANSPORTER 2* (*COPT2*)*, IRON REGULATED 2* (*IREG2*)*, OBP3-RESPONSIVE GENE 1* (*ORG1*)*,* and *HEAVY METAL ATPASE 3* (*HMA3*)*.* Finally, *BTS* itself and *BTSL1* and *BTSL2* were also expressed in *bts-3* roots regardless of Fe status. Only a small number of Fe deficiency responsive genes exhibited normal expression in *bts-3* roots, such as *At1g47400* and

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At3g56360, but the function of these genes is currently unknown. Select bHLH TFs were analyzed by qPCR to validate the results of the microarray (Supplemental Figure 6B).

Discussion

Characterization of *BTS* paralogs in the Fe deficiency response

We characterized the role of two new E3 ligases in the Fe-deficiency response, BTSL1 and BTSL2. We found that like *BTS*, *BTSL1* and *BTSL2* were expressed in roots of plants grown under Fe-deficient conditions. Single *btsl1* and *btsl2* mutants did not exhibit any Fe phenotypes compared to wild type, suggesting a redundant function for these two highly related E3 ligases. Because of this, we made a *btsl1 btsl2* double mutant and found increased Fe levels and increased tolerance to Fe deficiency compared to wild type. To assess whether reduction of *BTS* expression in the previously identified *bts-1* mutant could further increase Fe content and tolerance to Fe deficiency in *btsl1 btsl2* plants, we created the *bts-1 btsl1 btsl2* triple mutant. This triple mutant indeed exhibited a further increase in Fe levels and tolerance to Fe deficiency, without any resulting Fe toxicity. Overall, these results demonstrate the importance of these three E3 ligases in the regulation of the Fe-deficiency response. As noted, all three genes exhibited increased expression in Fe-deficient conditions compared to Fe-sufficient conditions in roots, while only *BTS* was significantly expressed in Fe-deficient conditions in the shoots. Future studies should further examine the unique role of BTS in shoots.

Identification of a novel *BTS* allele

In our screen for plants with altered Fe concentrations, we identified a *bts* mutant with a novel phenotype. Previous *bts* alleles *emb2454-1* and *emb2454-2* were identified in a screen for essential genes in Arabidopsis and exhibited embryo lethality and are thus putative complete loss-of-function alleles [26]. To date, only partial loss-of-function alleles have been used to examine the role of BTS in post-embryonic development. The *bts-1* line exhibits a steady state level of *BTS* mRNA approximately 70% that of wild type when grown under Fe-sufficient conditions. In *bts-1,* normal BTS protein is predicted, but at lower levels. *bts-3* has a proline to leucine mutation at amino acid 1174 in the BTS RING domain. Our data suggests this protein variant is perturbed in its function, resulting in a phenotype distinct from the reduction in

function of BTS found in the *bts-1* mutant. This unique mutant allowed us to further explore the role of BTS.

Possible models for BTS action in the root

The fact that *bts-3* performs better in Fe-deficient conditions compared to wild type supports the idea that BTS is a negative regulator of the Fe deficiency response. Given that BTS is an E3 ligase, it is likely that BTS acts in the ubiquitin-dependent degradation of positive regulator(s) of the Fe deficiency response, as has been suggested previously [22, 23, 25]. Because BTS is an E3 ligase containing Fe-binding hemerythrin domains, we were interested in whether it acts in a similar manner to the Fe sensor identified in mammals, FBXL5. *FBXL5* mRNA exhibits constitutive expression, but FBXL5 protein stability is controlled by Fe binding to the hemerythrin domains, where Fe binding stabilizes FBXL5 protein so that it can assemble with other subunits to form a complete SCF ubiquitin ligase and perform its function to ubiquitinate IRP2, leading to proteasomal degradation of IRP2 [44, 45]. In low Fe conditions, the hemerythrin domain is destabilized, resulting in FBXL5 degradation. If BTS acts in an analogous manner, Fe binding to hemerythrin domains would stabilize BTS protein, allowing for its activity in Fe-sufficient conditions (Figure 8). If target proteins are positive regulators of the Fe deficiency response, BTS could degrade these regulators when Fe becomes available after a period of Fe deficiency. The expression of *BTS* in Fe-deficient conditions would allow for a quick transition to Fe availability via Fe-mediated BTS protein stabilization. This could allow a rapid "switching off" of the Fe deficiency response when Fe uptake is no longer needed. In a scenario where BTS protein is mutated so that it is unstable and/or enzymatically inactive, as we believe to be the case in *bts-3*, a loss of ubiquitin ligase activity would result in lack of degradation of target proteins. Notably, there is strong support in the literature that the Pro to Leu change renders BTS P1174L incapable of interacting with its partner E2 enzyme because the conserved Pro has previously been identified as a residue important for this interaction in specific E2-E3 pairs [46-51]. This hypothesis is outlined in the model shown in Figure 8. Lack of degradation and resulting accumulation of positive regulators of the Fe deficiency response would result in a constitutive activation of the Fe deficiency response and excessive Fe uptake and resulting toxicity, as we see in *bts-3* (Figure 7). This can also explain why *bts-3* performed better than wild type in Fe-

deficient conditions. Because *bts-3* plants always take in Fe, even when there is sufficient Fe available for growth, they have stored Fe available when faced with low Fe conditions.

Although protein levels and ubiquitination status of putative BTS targets have not been studied in *bts* mutants *in planta,* protein interaction studies and *in vitro* cell-free degradation experiments suggest that BTS targets may include bLH104, bHLH115, and ILR3[20, 23]. bHLH104 and ILR3 have been shown to be positive regulators of the Fe deficiency response because loss-of-function mutants of these genes are sensitive to Fe-deficient conditions [22]. Further, these transcription factors have been shown to directly bind to promoters and influence expression of Fe deficiency responsive genes: *bHLH38, bHLH39, bHLH100, bHLH101,* and *PYE* [22]. We show that in *bts-3* roots, these genes and others are expressed regardless of Fe status, supporting the hypothesis that BTS acts upstream to control expression of these genes. As mentioned previously, bHLH1b subgroup transcription factors can form dimers with FIT. We demonstrated that downstream targets of FIT*, IRT1* and *FRO2,* are constitutively expressed and their protein products function to reduce and transport Fe into the root irrespective of Fe status of the plant (Figure 6, Figure 7).

Besides the possibility that BTS functions in a similar manner to FBXL5 with Fe binding to hemerythrin domains leading to protein stability (Figure 8), another study suggests the opposite is true. Protein stability assays using a wheat germ *in vitro* translation system suggest that BTS is less stable in the presence of Fe and thus the authors propose that BTS is active in Fe-deficient conditions, when *BTS* gene expression is induced [23]. This *in vitro* data further suggests that this Fe-dependent destabilization is dependent on specific Fe binding residues within the hemerythrin domains. However, MG132 proteasomal inhibitor sensitivity of this putative protein destabilization was not shown and thus it is difficult to conclude whether BTS degradation is occurring in response to Fe or whether Fe simply negatively regulates the translation of BTS protein in the wheat germ extract system. The authors of this study suggest that if BTS is more stable in Fe-deficient conditions, BTS acts under these conditions to "fine-tune" regulators of the Fe deficiency response [23]. A fine-tuning capability may serve to inhibit excessive Fe accumulation by preventing uncontrolled action of positive regulators of the Fe deficiency response. It is well known that Fe levels are tightly controlled because excess Fe can be very toxic to plants and therefore, this idea of fine-tuning is plausible. Alternatively, BTS may function in the constitutive turnover of positive regulators of the Fe deficiency response in order

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to keep them "fresh" so they can continually bind to target promoters to activate Fe deficiency responsive genes when processive transcriptional cycles are needed The requirement for "fresh" transcription factor production to replace "fatigued" transcription factors to promote continuous gene expression has been suggested for FIT protein regulation [52]. Alternatively, constant degradation of positive regulators by BTS may be necessary to ensure that once synthesized, positive regulators can be rapidly removed upon Fe resupply to prevent excess accumulation.

Unfortunately, we and others have been unable to detect full-length BTS protein in Fe-sufficient vs. Fe-deficient conditions *in planta*, making it difficult to conclude how BTS protein stability/activity is physiologically regulated by Fe availability [23].

Conclusions

Considering the models discussed, if a plant is reduced in its ability to fine-tune or appropriately regulate protein stability of positive regulators of the Fe deficiency response in Fe-deficient conditions, an increased activation of the Fe deficiency response and resulting increased tolerance to Fe-deficient conditions may occur. Our *bts-1*, *btsl1 btsl2,* and *bts-1 btsl1 btsl2* mutant phenotypes seem to fit this scenario. Because they probably do not completely lack their BTS E3 ligase activity due to the presence of some full-length *BTS* transcript in these mutants, we propose they retain some capacity to negatively regulate the Fe deficiency response. Proper balance of both positive and negative regulation of the Fe deficiency response is essential, especially from an applied perspective. The triple *bts-1 btsl1 btsl2* mutant we created represents what we consider an ideal Fe accumulator: it takes in more Fe than wild type to allow for enhanced growth and tolerance to Fe deficiency, but still retains the capacity to sufficiently repress Fe uptake in Fe-sufficient growth conditions to avoid toxicity.

BTS orthologs are found across many plant species including crop plants such as rice and soybean and thus represent a potential target for enhancing tolerance to Fe deficiency and increasing Fe bioavailability for human consumption. In rice, RNAi knockdown of *BTS* orthologs, the *HRZ*s, was demonstrated to increase tolerance to growth on Fe-deficient calcareous soils and increase Fe concentration in seeds without a yield penalty [30]. Besides genetic knockdowns and modifications, perhaps natural variants of these genes in crop species may be identified and used in traditional breeding efforts to generate cultivars with more Fe. We

hope that the work described here will lead to greater understanding of plant Fe homeostasis to inform efforts for improved crops.

Experimental Methods

Plant growth conditions

For plate grown plants, seeds were surface sterilized and stratified for three days at 4ºC in the dark. Gamborg's B5 medium (Sigma-Aldrich) was supplemented with 1mM MES, 0.5% sucrose, 0.6% type M agar, and adjusted to pH 5.8. +Fe and –Fe plates were made with macronutrients and micronutrients at 2 mM $Ca(NO_3)_2$, 0.75 mM K_2SO_4, 0.65 mM $MgSO_4$, 0.1 mM KH_2PO_4, 10 µM H_3BO_3, 0.1 µM $MnSO_4$, 0.05 µM $CuSO_4$, 0.05 µM $ZnSO_4$, 0.005 µM $(NH_3)6Mo_7O_{24}$, 1mM MES, 0.6% agar, adjusted to pH 6.0 and supplemented with either 50 µM Fe(III)-EDTA for +Fe plates or 300 µM ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate (HACH Chemical)] for –Fe plates as described [53, 54]. Alkaline soil was generated by adding approximately 7.8 gm CaO/kg soil to achieve a soil pH of 7.5-8. To supplement plants grown on alkaline soil, 500 µM FeEDDHA was added. All plants in this study were grown at 21ºC under a 16 hour light/8 hour dark cycle.

Mutant lines

BTS/At3g18290 (*bts-1*)*, BTSL1/At1g74770* (*btsl1*)*,* and *BTSL2/At1g18910* (*btsl2*) T-DNA insertion lines were obtained from ABRC (SALK_016526, SALK_015054, SAIL_615_HO1, respectively). Homozygous lines were identified using gene-specific primers and a T-DNA specific border primer. Location of T-DNA insertions are shown in Supplemental Figure 1. *bts-3* was identified in an EMS mutagenesis screen and backcrossed twice. *btsl1 btsl2* was generated by crossing *btsl* and *btsl2* and identifying a homozygous double mutant. *btsl1 btsl2* was crossed with *bts-1* to generate the *bts-1 btsl1 btsl2* mutant and a homozygous triple mutant was identified.

Array Mapping

The Fe over accumulating mutant was crossed to Ler and F2 plants were scored for sensitivity to Fe sufficient growth conditions. Two pools of plants were created, one with the Fe

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sensitivity mutant phenotype and one with the wild type phenotype. DNA extracted from the two

pools was hybridized to the AtSNPTile array and the mapping was performed as described in

Becker et al. [39]. The array files are available on GEO.

Plasmid construction and plant transformation

The endogenous promoter complementation line was generated by amplifying the region

including 1.9 kb upstream of the *BTS* ATG and the entire *BTS* gene and is named *ProBTS:BTS*

bts-3. This construct was subcloned into pCR8 (Invitrogen) and finally into pEARLEY GATE

301 using Gateway LR Clonase II (Invitrogen). *Agrobacterium tumefaciens* strain GV3101 was

transformed with the above construct and *bts-3* plants were transformed using the floral dip

method [55]. Transformants were isolated using BASTA selection at 25 mg/mL.

Chlorophyll assay

Plants were grown for 10 days on B5 medium and then transferred to –Fe medium for 7

days. The shoots were harvested and assayed for chlorophyll concentration by heating plants in

95% ethanol at 80ºC as previously described [56].

Elemental concentration analysis

Root and shoot tissues were collected from plants and to remove trace surface metals,

tissues were incubated for 5 mins in 5 mM $CaSO_4$, 5 mins in 10 mM EDTA, and rinsed with

deionized water. Root and shoot tissues were dried before analysis by incubation in open

Eppendorf tubes at 70ºC for 24 hours. Seeds were harvested when fully dried. Dry tissues (shoot,

root and seed) were transferred into Pyrex test tubes (16 x 100 mm). After weighing the

appropriate number of samples (these masses were used to calculate the rest of the sample

masses after Danku et al. [57], trace metal grade nitric acid (J. T. Baker Instra-Analyzed; Avantor

Performance Materials; Scientific & Chemical Supplies Ltd, Aberdeen, UK) spiked with indium

internal standard was added to the tubes (1.00 mL). They were then digested in dry block heaters

(DigiPREP MS, SCP Science; QMX Laboratories, Essex, UK) at 115˚C for 4 hours. The

digested samples were diluted to 10.0 mL with 18.2 MΩcm Milli-Q Direct water (Merck

Millipore, Watford, UK) and aliquots transferred to 96-well deep well plates using adjustable

multichannel pipette (Rainin; Anachem Ltd, Luton, UK) for analysis. Elemental analysis was

performed with an inductively coupled plasma-mass spectrometry (ICP-MS) (PerkinElmer

NexION 300D equipped with Elemental Scientific Inc. autosampler and Apex HF sample

introduction system; PerkinElmer LAS Ltd, Seer Green, UK and Elemental Scientific Inc.,

Omaha, NE, USA, respectively) in the standard mode. Twenty elements (Li, B, Na, Mg, P, S, K,

Ca, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, and Cd) were monitored. Separate liquid

reference materials composed of pooled samples of the digested tissues (shoot, root and seed)

were prepared before the beginning of sample runs and were used throughout the samples runs.

They were run after every ninth sample in all ICP-MS sample sets, respectively, to correct for

variation between and within ICP-MS analysis runs [57]. Sample concentrations were calculated

using external calibration method within the instrument software. The calibration standards (with

indium internal standard and blanks) were prepared from single element standards (Inorganic

Ventures; Essex Scientific Laboratory Supplies Ltd, Essex, UK) solutions.

Synchrotron X-Ray Fluorescence

Two-dimensional SXRF analysis was performed at various X-ray microprobe beamlines:

X26A (leaves in Figure 2B, Figure 3, and Supplemental Figure 4) and X27A (dry seeds in Figure

2E) of National Synchrotron Light Source (NSLS), Beamline 2-3 of Stanford Synchrotron

Radiation Lightsource (SSRL) (roots, leaves, and siliques of Figure 3B-D), XFM of the

Australian Synchrotron, (dry seeds in Figure 4F), and beamline 2-ID-D of the Advanced Photon

Source (APS) (Leaf tissue sections of Figure 4E). More details and metadata are found in the

Supplemental Methods.

Real-time quantitative PCR

RNA was prepared from plants grown on B5 medium for 2 weeks before transfer to +Fe

or –Fe media for 3 days. RNA extraction was performed using TRIzol® (Life Technologies) and

the RNEasy mini kit and protocol (Qiagen). Quantitative real-time PCR was performed on Step

One Plus Real Time PCR System (Applied Biosystems Version 2.2.3) using SYBR Premix

ExTaq reagents and protocol (Takara). Each sample was run in triplicate. Relative transcript

levels were calculated by normalizing to *EF1a* housekeeping expression.

Microarray analysis

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RNA was extracted from three biological replicates as described above from root tissue from plants grown on B5 medium for two weeks before transfer to either +Fe or –Fe medium for 3 days. RNA was labeled and hybridized to the Arabidopsis Gene 1.0 ST Arrays (Affymetrix) by the Dartmouth Genomics and Microarray Laboratory. Raw data was RMA normalized using Affymetrix Expression Console software and downstream analyses were performed using BRB-Array Tools Version 4.2.1. BRB-ArrayTools is an integrated software package for the analysis of DNA microarray data which was developed by the Biometric Research Branch of the Division of Cancer Treatment & Diagnosis of the National Cancer Institute under the direction of Dr. Richard Simon. Differentially expressed genes were identified between two classes using a random-variance t-test with a p-value cutoff of 0.01. The random-variance t-test is an improvement over the standard separate t-test as it permits sharing information among genes about within-class variation without assuming that all genes have the same variance [58]. We also used a cutoff of 1.5 fold change up or down in response to Fe treatment for either wild type or *bts-3*. Biological replicates for root and shoot arrays were averaged for analysis of gene expression in each tissue. Hierarchical clustering was employed to generate heat maps for subsets of significant genes using the open source software Cluster/Treeview[59, 60]. GO term enrichment using the DAVID Go Ontology program Functional Annotation Clustering tool using an enrichment cutoff of >1.3 as previously described [42, 43]. Microarray data was made publically available in the GEO repository (http://www.ncbi.nlm.nih.gov/geo).

Ferric chelate reductase assay

Plants were grown for 2 weeks on B5 medium and then transferred to either +Fe or –Fe media for 3 days. 5 plants were pooled for root Fe(III)-chelate reductase activity measurements, as previously described [54].

Protein isolation and immunodetection

Wild type and *bts-3* plants were grown for 2 weeks on B5 medium and transferred to +Fe or –Fe media for 3 days. Total protein was extracted from roots using protein extraction buffer: 50 mM Tris, pH8, 5% glycerol, 4%SDS, 1%PVPP, 2 mM Pefabloc (Roche), 1X Protease

Inhibitor Cocktail (Roche). SDS-PAGE followed by western electroblotting was performed and blots were probed with an IRT1 antibody or α-tublulin antibody (AbCam).

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers At3g18290 (*BTS*), At1g74770 (*BTSL1*), and At1g18910 (*BTSL2*).

Acknowledgments

Figure legends

Figure 1. BTS, BTSL1, and BTSL2 are a family of closely-related RING E3 ligases regulated by Fe deficiency. A. BTS, BTSL1 and BTSL2 have very similar protein domain architectures including hemerythrin domains and a C-terminal RING E3 ligase domain. The RING domain is highly conserved among family members. Conserved Cys and His Zn^{2+}-binding residues for each C3H2C3 type RING are shown in red. The BTS Pro residue shown in blue is mutated to Leu in *bts-3* plants. Protein domain structure was predicted by NCBI's conserved domain database [29].

B. qPCR of *BTS, BTSL1* and *BTSL2* expression in roots and shoots of plants grown for 2 weeks on B5 medium and then transferred to +Fe or –Fe medium for 3 days. Expression is relative to EF1α. Error bars represent SE (n=3).

Figure 2. *bts* and *btsl1/2* mutants have increased Fe concentrations and tolerance to Fe deficiency.

A. Chlorophyll levels were measured on plants grown for 10 days on B5 and then transferred to –Fe medium for 7 days (n=3). Representative images of plants shown below. **B.** Representative

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SXRF scans showing Fe localization in leaf #5 of plants grown for 2 weeks on B5 and then transferred to +Fe medium for 3 days. **C.** Root and shoot ICP-MS measurements of plants grown for 2 weeks on B5 and then transferred to +Fe or –Fe medium for 3 days (n=3) **D.** Seed ICP-MS measurements from soil grown plants (n=5). E. Representative SXRF scan showing Fe localization in mature, dry seed. In panels A, C, and D, lower case letters indicate significant differences at p<0.05 (ANOVA with Tukey's). Error bars indicate SE.

Figure 3. *bts* and *btsl1/2* mutants store more Fe, Zn and Mn in veins, hydathodes and trichomes than wild type.

SXRF scans showing Fe, Zn, and Mn localization in leaf #5 of plants grown for 2 weeks on B5 medium and then transferred to –Fe medium for 3 days.

Figure 4. *bts-3* has high concentrations of Fe in roots, leaves, and seeds.

A. ICP-MS measurements of roots and shoots of plants grown for 2 weeks on B5 medium and then transferred to +Fe or –Fe medium for 3 days (n=3). ICP-MS measurements of seeds from soil-grown plants (n=5 plants). *p<0.05 between genotypes (student's t-test). Error bars indicate SE. **B.** Representative SXRF scans of root tips of plants grown for 7 days on B5 medium and then transferred to –Fe medium for 3 days. **C.** SXRF scan of leaf #8 showing Fe in red. **D**. SXRF scan showing Fe (red), Zn (green), Mn (blue) localization in developed, green siliques. **E.** SXRF scan showing Fe localization in resin-embedded shoot primary vein cross sections. Plants were grown on B5 medium for 2 weeks. Bright field light microscopy images of adjacent sections stained with toluidine blue are shown for reference. X indicates xylem and P indicates phloem **F**. SXRF images show Fe localization to the vasculature in individual seeds.

Figure 5. *bts-3* is tolerant to Fe-deficient growth conditions, but sensitive to Fe sufficient conditions and this phenotype can be complemented by *BTS* expression.

A. Plants were grown for 2 weeks on normal, alkaline, and alkaline +Fe soil. **B.** Plants were grown vertically for 5 days on B5 medium and then transferred to +Fe or –Fe medium for 7 days. **C.** SXRF scans showing Fe localization in leaf #5 of plants grown for 2 weeks on B5 medium and then transferred to +Fe medium for 3 days. *ProBTS:BTS bts-3* is the complementation line

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where the *BTS* gene driven by its own promoter is expressed in the *bts-3* mutant. **D.** Plants were grown vertically for 5 days on B5 medium and then transferred to +Fe or –Fe medium for 7 days.

Figure 6. The Strategy I Fe deficiency response is on under +Fe conditions in *bts-3*.

A. qPCR of FRO2 and IRT1 expression in roots of plants grown for 2 weeks on B5 medium and then transferred to +Fe or –Fe medium for 3 days. Expression is relative to EF1α. Error bars represent SE (n=3).

B. Root ferric chelate reductase assay of plants grown for 2 weeks on B5 medium before transfer to +Fe or –Fe medium for 3 days. *$p<0.05$ comparison between genotypes on +Fe conditions (Student's t-test). Error bars indicate SE (n=3).

C. Representative IRT1 western blot of roots of plants as grown in panel A. α-tubulin was used as a loading control.

Figure 7. Heat map of genes regulated by Fe in roots of wild type and *bts-3* plants.

Microarray analysis of plants grown for 2 weeks on B5 medium and then transferred to +Fe or –Fe medium for 3 days. Hierarchical clustering analysis was performed on genes which were 1.5 fold up or down regulated in response to Fe treatment in either genotype. Subsets 1-4 are numbered and enriched GO terms (right) and known metal homeostasis genes (left) are shown. The heat map was made with Java TreeView and GO terms predicted by DAVID 6.7 [42, 43, 60].

Figure 8. Model of BTS activity in roots of wild type vs. *bts-3*.

In wild type, Fe binding via HHE domains may stabilize BTS protein, allowing for assembly of E3 ligase machinery and subsequent target degradation. If the target is a positive regulator of the Fe deficiency response, the Fe deficiency response is turned off. In –Fe conditions, lack of Fe binding to HHE domains may lead to BTS protein degradation and accumulation and action of positive regulators of the Fe deficiency response. In *bts-3*, if the mutation prevents interaction with E2 enzymes, BTS protein cannot act as an E3 ligase, regardless of Fe status. In this case, its target would always be able to induce the Fe deficiency response.

Supplemental Data

Supplemental Figure 1. Location of T-DNA insertions of *bts-1*, *btsl1* and *btsl2* mutants.

Supplemental Figure 2. *bts-1, btsl1 btsl2, bts-1 btsl1 btsl2*, and *bts-3* mutants all exhibit tolerance to Fe-deficient growth conditions, but only bts-3 is sensitive to Fe-sufficient conditions.

Supplemental Figure 3. BTS family E3 ligase mutants have higher Mn and Zn concentrations in roots, shoots and seeds compared to wild type.

Supplemental Figure 4. Expression of *BTS* controlled by its endogenous promoter in *bts-3* returns Mn and Zn levels to wild type levels.

Supplemental Figure 5. Unlike *bts-3, bts-1 btsl1 btsl2* mutants do not exhibit induction of ferric chelate reductase activity when grown under Fe-sufficient conditions.

Supplemental Figure 6. *bts-3* roots exhibit constitutive expression of Fe deficiency genes.

Supplemental Methods. SXRF analysis.

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