BRUTUS-LIKE proteins moderate the transcriptional response to iron deficiency in roots

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Short title: BRUTUS-LIKE proteins target FIT for degradation

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ABSTRACT

Iron is an essential micronutrient but in excess is toxic inside cells. Under iron deficiency, the expression of iron uptake genes is increased, but it is not known how the transcriptional response is controlled to avoid uptake of too much iron. The hemerythrin E3 ligases BRUTUS (BTS) and BTS-LIKE (BTSL) have previously been identified as negative regulators of the iron deficiency response. Our phylogenetic analysis indicated that BTSL proteins are present in dicotyledonous plants only and form a separate clade from BTS homologs. *BTSL1* and *BTSL2* in *Arabidopsis thaliana* are in a network with nearly all iron uptake genes, whereas *BTS* is in a shoot-specific network. *BTSL1* and *BTSL2* are expressed predominantly in the root epidermis and cortex, separate from *BTS* in the root stele, shoot and embryos. Mutant analysis identified *BTSL2* as the dominant paralog of the otherwise redundant *BTSL* genes. The *btsl* double mutant had increased protein levels of FIT, the FER-like Iron deficiency-induced Transcription factor, and failed to switch off the transcriptional response upon iron resupply, leading to dramatic iron accumulation in roots and shoots. Protein interaction between the C-terminus of BTSL proteins and FIT indicate that FIT is a direct target for degradation. Taken together, our studies show that *BTSL1* and *BTSL2* control iron uptake in the epidermis and cortex, upstream of *BTS* in the vasculature and leaves.

INTRODUCTION

Iron (Fe) is the fourth most abundant element in the Earth's crust, but its bioavailability is greatly limited by the insolubility of Fe hydroxides. Plants have developed highly efficient Fe uptake systems, classically divided in Strategy I or reductive strategy, present in dicotyledonous plants, and Strategy II or chelating strategy in grasses (Römheld and Marschner, 1986; Tsai and Schmidt, 2017). The reductive strategy in plants such as *Arabidopsis* involves the activity of a Ferric Reductive Oxidase, *FRO2*, to reduce Fe^{3+} to Fe^{2+} which is then taken up by the Iron-Regulated Transporter *IRT1*. A key regulator of Fe uptake in Strategy I plants is the basic Helix-Loop-Helix (bHLH) transcription factor FIT (FER-like Iron deficiency-induced Transcription factor; Bauer et al., 2007). FIT forms heterodimers with one of four bHLH proteins from subgroup Ib, namely *bHLH38*, *bHLH39*, *bHLH100* and *bHLH101*. Mutant studies combined with transcriptomics have identified more than 400 genes that are controlled by FIT (Colangelo and Gueriot, 2004; Mai et al., 2016). These targets include *FRO2* and *IRT1* for which direct promoter binding by the FIT-bHLH Ib dimer has been shown (Yuan et al., 2008; Wang et al., 2013).

How the Fe status of the plant is communicated to the transcriptional regulators remains unknown. Recent studies have discovered a small family of hemerythrin E3 ligases in plants, including *BRUTUS* (*BTS*) in *Arabidopsis* (Long et al., 2010; Selote et al., 2015) and Hemerythrin motif-containing RING- and Zn-finger proteins (*HRZ1*, *HRZ2*) in rice (Kobayashi et al., 2013), that act as negative regulators of the Fe deficiency response. The expression of *HRZ1*, *HRZ2* and *BTS* is induced ~10-fold under Fe deficiency. Mutant lines of *BTS* or *HRZ* showed increased tolerance to Fe deficiency and accumulation of Fe in leaves and seeds. *BTS* / *HRZ* proteins contain three putative Fe-binding hemerythrin domains in the N-terminus, and a CHY/RING-type Zn finger domain with ubiquitin E3 ligase activity in the C-terminus (Kobayashi et al., 2013; Selote et al., 2015). This domain architecture bears similarity to mammalian *FBXL5* (F-box/LRR-repeat protein 5), which targets the Iron Regulatory Protein 2 (*IRP2*) for degradation (Vashisht et al., 2009; Salahudeen et al., 2009). The activity of *FBXL5* depends on Fe binding to its N-terminal hemerythrin domain and on oxygen levels (Salahudeen et al., 2009). Plants do not have a functional homolog of *IRP2*, however, *BTS* was shown to interact with, and affect the stability of *bHLH105* (*ILR3*) and *bHLH115*, two Fe-regulated transcription factors (Selote et al., 2015). A heterodimer of *ILR3* and *bHLH104* regulates the expression of *bHLH38* / *39* / *100* / *101*, encoding the main protein partners of FIT (Zhang et al., 2016).

BTS was first identified as *EMBRYO DEFECTIVE 2454* (*EMB2454*) in a screen for embryo lethal mutants (McElver et al., 2001; Tzafrir et al., 2004), and later re-named and described

as a negative regulator of Fe homeostasis (Long et al., 2010). Since *bts* loss-of-function mutants are lethal, studies have focused on viable alleles with T-DNA insertion in the promoter/UTRs or point mutations (Selote et al., 2015; Hindt et al., 2017). These *bts* mutant plants showed an enhanced Fe deficiency response, including increased root length, soil acidification and higher Fe reductase activities, and they are more tolerant to Fe limiting conditions. Promoter-GUS lines showed that *BTS* is expressed in leaves and embryos independent of the Fe status of the plant, and its expression is induced in the stele of the root under Fe deficiency (Selote et al., 2015). *BTS* is not the only hemerythrin-E3 ligase in Arabidopsis, as previously noticed by Kobayashi and colleagues (2013). Two homologous genes are found in the Arabidopsis genome, *BRUTUS-Like1* (*AT1G74770*) and *BRUTUS-Like2* (*AT1G18910*). Double *bts1 bts2* mutants have recently been reported to be more tolerant to Fe deficiency and to accumulate more Fe (Hindt et al., 2017). A triple mutant of *bts1*, *bts2* and a viable *bts* allele had an enhanced phenotype, and the authors concluded that the *BTSL* genes act as redundant negative regulators of Fe homeostasis together with *BTS* (Hindt et al., 2017).

Here we show that the function of the *BTSL* genes differs from *BTS*. *BTSL1* and *BTSL2* are expressed predominantly in the root epidermis and cortex and are part of a root-specific transcriptional network of genes involved in Fe uptake. In contrast, *BTS* is co-expressed with only a small number of shoot-specific Fe-regulated genes. We found that *BTSL1/2* function to control the levels of FIT protein and prevent excess Fe accumulation via the FRO2 / IRT1 uptake pathway.

RESULTS

***BTSL1* and *BTSL2* are co-expressed with Fe-responsive genes in the roots**

Co-expression analysis is a powerful tool for functional characterization because co-regulated genes are likely to function in the same biochemical pathway or biological process (Stuart et al., 2003). Previously we surveyed the transcriptional response to Fe deficiency in *Arabidopsis* and in *Medicago truncatula* (Rodríguez-Celma et al., 2013a). We considered the overlap between the two data sets working under the assumption that Fe homeostasis regulation should be conserved between plant species. We found the orthologous pair *AT1G18910-Medtr7g037040* up-regulated under Fe deficiency with the core genes of the Fe regulon. This gene has recently been annotated as *BTSL2* (Hindt et al., 2017). As an independent and unbiased approach, we used publicly available microarray data to build co-expression networks around *BTSL2* and the two homologous genes *BTSL1* and *BTS*. First, we found that *BTSL1* and *BTSL2* are co-regulated with each other but not with *BTS*. Second, we noticed that a large network could be built around *BTSL1* and *BTSL2* with root-specific data sets, but no co-regulated ‘root’ partners could be found for *BTS* (Supplementary Figure S1 and Supplementary Table S1). Correlations with *BTS* were only found when using shoot-specific data sets. Because *BTS*, *BTSL1* and *BTSL2* have been linked to the Fe deficiency response, the networks were trimmed to only those genes present in the so-called ferrome (Schmidt and Buckhout, 2011) (Figure 1A, B, Supplementary Table S2). We found that *BTSL1* and *BTSL2* are co-regulated with genes involved in Fe uptake, such as the Fe transporter *IRT1* and genes for the biosynthesis and export of coumarin-derived Fe chelators (*PDR9*, *4CL2* and *F6'H1*) (Figure 1A). Also co-expressed with *BTSL1* and *BTSL2* are the transcription factor *FIT* and its partner *bHLH39*, central regulators in the Fe-deficiency response (Figure 1B). Basically, the entire root Fe uptake regulon is found, except for *bHLH38* and *FRO2*, but these genes are not present on the micro-array chip (ATH1) that is most commonly used in the public database. The *BTS* network includes the transcription factor *POPEYE* (*PYE*), as previously documented (Long et al., 2010), and the ferric reductase *FRO3*. Also found is *AT5G05250*, a gene of unknown function which is highly induced during Fe deficiency (Rodríguez-Celma et al., 2013b).

Investigation of the expression of *BTS*, *BTSL1* and *BTSL2* in RNAseq data sets of the Fe deficiency response (Rodríguez-Celma et al., 2013b) confirmed that *BTSL1* and *BTSL2* were expressed mainly in the roots and strongly induced under Fe deficiency (Figure 1C). *BTS* is therefore the dominant family member expressed in the shoots, in accordance with the calculated networks.

Figure 1. *BTSL* and *BTS* are part of different gene expression networks.

(A, B) Co-expression analysis of Fe-responsive genes that are co-regulated with (A) *BTSL1* and *BTSL2* in roots, and (B) *BTS* in shoots. The edge thickness relate to the Pearson's correlation coefficient. See Supplemental Table S2 for descriptions of all genes and numerical values.

(C) Expression levels of *BTS*, *BTSL1* and *BTSL2* in roots and in shoots under Fe sufficient (+Fe) and Fe deficient conditions (-Fe). Data were obtained by RNAseq as described in Rodriguez-Celma et al., (2013). RPKM, Reads Per Kilobase Million. The bars represent the mean of $n = 3$ biological replicates $\times$ 10 seedlings $\pm$ SD (* $p < 0.05$ using a two-tailed *t*-test).

BTSL1 and BTSL2 proteins are specific to dicotyledonous plants

To study the evolutionary relationship between *BTS* and *BTSL*, we performed a phylogenetic analysis of homologous protein sequences across the tree of life. As noted previously (Kobayashi et al., 2013; Matthiadis & Long, 2016) homologs containing more than one hemerythrin (Hr) domain fused to a RING-domain E3 ligase are found only among plant species, including mosses. Interestingly, Arabidopsis *BTSL1* and *BTSL2* are in a separate clade unique to dicotyledonous species (Figure 2A).

BTSL1/2 proteins have two predicted Hr domains and a CHY/RING Zn-finger domain (Kobayashi et al., 2013). The two Hr domains of *BTSL1/2* correspond to the first and third Hr

domain in BTS. Further inspection of the amino acid sequence of BTSL1 and 2 revealed a third putative Hr domain (Figure 2B), not recognized by domain searching algorithms. Alignment of the individual Hr domains of the three proteins showed that in the gap between the two Hr domains of BTSL1 and 2 the amino acid sequence is highly homologous to the other Hr sequences, but lacks the four conserved histidine residues found in the canonical Hr domains. This domain is still predicted to form a four α -helical bundle typical of hemerythrins (Supplementary Figure S2) and additional histidines and acidic residues are present which may function as ligands for Fe. The C-terminal Zn finger domain is highly conserved, with 80% amino acid identity between BTSL1 and BTSL2, and 65% identity with BTS. This domain consists of a unique arrangement of Zn-fingers, a CHY/RING-type Zn finger domain found in the mammalian E3 ligase Pirh2 / RCHY1 (Leng et al., 2003) and in four other E3 ligases in Arabidopsis (Lee and Seo, 2016).

A

Figure 2. BTSL are uniquely found in dicotyledonous plants.

(A) Phylogenetic tree of BTS homologs from selected plant species. Sequences were found by BLASTing the amino acid sequences of Arabidopsis BTS, BTSL1, BTSL2 and rice HRZ1 in Ensembl Plants (<http://plants.ensembl.org>). Species used: Amborella, *Amborella trichocarpa*; At, *Arabidopsis thaliana*; Bo, *Brassica oleracea*; Hv, *Hordeum vulgare*; Mt, *Medicago truncatula*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Pt, *Populus trichocarpa*; Sl, *Solanum lycopersicum*; Ta, *Triticum aestivum*. Numbers next to branches indicate bootstrapping values for 100 replications. The scale bar indicates the rate of evolutionary change expressed as number of amino acid substitutions per site.

(B) Domain organization of BTSL, BTS and HRZ proteins.

B

***BTSL1* and *BTSL2* promoters are activated by Fe deficiency in specific root tissues**

To investigate where in the roots and in which cell types *BTSL1* and *BTSL2* are expressed, the promoter regions of these genes were cloned into the vector pBGWFS7 upstream of an *eGFP-GUS* fusion reporter gene (Karimi et al., 2002). For the *BTSL1* promoter we used the intergenic region of 880 nt upstream of the ATG start codon. In the case of *BTSL2* the intergenic region was ~5 kb, and therefore we decided to use only 2097 nt upstream of the ATG start codon. Seeds were germinated on agar plates with minimum salts (see materials and methods section) supplemented either with 50 μ M FeEDTA (+Fe) or with 100 μ M ferrozine to effectively deplete Fe (-Fe). After 5 days of growth seedlings were analysed for GFP expression. Aside from some autofluorescence in the empty seed coat, no GFP fluorescence was observed in any part of the seedling under Fe sufficient conditions (+Fe). However, under Fe deficiency (-Fe), GFP was highly induced in the roots (Figure 3A), in agreement with RNAseq expression data (Figure 1C). At this early growth stage, GFP expression was similar in the *BTSL1* and *BTSL2* promoter constructs. In older seedlings that had developed lateral roots, the expression pattern of the *BTSL1* and *BTSL2* promoters became different: GUS staining showed that *BTSL1* is expressed in the upper half of the root under Fe deficiency, whereas *BTSL2* is expressed in the lower half of the root, predominantly in the differentiation zone (Figure 3B). *BTSL1* and *BTSL2* promoter-GUS activity was also found in the root cap (columella), but not in the root meristem or elongation zone. Cross-sections of the GUS-stained roots showed that both promoters are induced in the epidermis, including root hairs, and cortex cells. *BTSL2* was also expressed in the endodermis and stele in the differentiation zone (Figure 3B). The expression patterns of *BTSL1* and *BTSL2* contrast with the pattern observed for *BTS*. *BTS* promoter-GUS expression showed little promoter activity in the epidermis and cortex, but was strongly upregulated in the stele in response to Fe deficiency (Selote et al., 2015). Moreover, *BTS* is expressed in embryos which lack *BTSL1* and *BTSL2* promoter activity (Supplementary Figure S3).

In summary, the *BTSL1* and *BTSL2* promoters are activated by Fe deficiency in the upper and lower part of the root, respectively. Both genes are expressed in epidermis, cortex and columella cells.

***BTSL1* and *BTSL2* have partially redundant functions in maintaining Fe homeostasis**

To characterise the function of *BTSL* in Fe homeostasis, T-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Centre. Three independent mutant lines were selected for *BTSL1* (SALK_015054, SALK_032464 and SALK_117926; named *btsl1-1*, *btsl1-2* and *btsl1-3*, respectively), but only one line with a T-DNA insertion in the coding sequence was available for *BTSL2* (SALK_048470; *btsl2-2*) (Figure 4A). The *btsl1-1* was described as *btsl1* in Hindt et al., (2017) and the *btsl2* allele used by the same authors has an insertion in the promoter. Homozygous lines were selected by PCR, T-DNA insertion sites were confirmed by sequencing, and transcript levels were assessed by RT-qPCR. The expression of *BTSL1* in roots under standard and Fe deficient conditions was virtually abolished in the *btsl1-1* and *btsl1-3* alleles, but residual expression remained in *btsl1-2* (Figure 4B). Disruption of the *BTSL1* gene led to a 2-fold upregulation of *BTSL2* expression under Fe deficiency (Figure

4C). The *bts/2-2* mutant lacked detectable levels of *BTSL2* transcript (Figure 4C), while *BTSL1* expression was ~3-fold higher than wild type lacking Fe (Figure 4B). Double knock-out lines were produced by crossing *bts/1-1* and *bts/1-3* with the *bts/2-2* line. The *bts/1-1 bts/2-2* mutant was genetically complemented by either *BTSL1* or *BTSL2* (see below).

Mutant lines were tested for phenotypes on a range of Fe concentrations. Seedlings were germinated and grown on medium with 50 μ M FeEDTA for 10 days, followed by 4 days on plates with ferrozine (-Fe), or 4 days on Fe sufficient medium (50 μ M FeEDTA). The effect of toxic concentrations of Fe was tested by growing the seedlings on plates with 200 or 500 μ M FeEDTA for 14 days. Single insertion lines showed no obvious phenotype under any of the conditions tested. On the other hand, the *bts/1-1 bts/2-2* double mutant line showed subtle but noticeable phenotypes under conditions of both Fe deficiency and Fe excess (Figure 4D). After 4 days without Fe, the *bts/* double mutant appeared less chlorotic than wild type, which was confirmed by chlorophyll measurements (Figure 4E). The *bts/2-2* mutant allele also retained some chlorophyll, suggesting that *BTSL1* and *BTSL2* are not fully redundant. In the presence of excess Fe, growth of the *bts/* double mutant was significantly more impaired than wild type or the single mutants (Figure 4D; Supplementary Figure S4). Quantification of Fe levels in shoots showed a 2-fold accumulation of this metal in *bts/* plants grown on excess Fe (Figure 4F), indicating that this might be the cause of the observed growth impairment.

Figure 4. *BTSL1* and *BTSL2* have redundant functions in Fe homeostasis.

(A) Schematic representation of the *BTSL1* and *BTSL2* genes and mutant alleles. Exons are in black, 5' and 3'-UTRs in grey. T-DNA insertions are indicated by triangles: *btsl1-1*, SALK_015054; *btsl1-2*, SALK_032464; *btsl1-3*, SALK_117926; *btsl2-2*, SALK_048470. Arrows indicate the left border primer used for genotyping.

(B, C) Transcript levels of *BTSL1* (B) and *BTSL2* (C) in wild-type and mutant alleles under standard (+Fe, 50 μM FeEDTA) and Fe-deficient conditions (-Fe, 100 μM ferrozine). (* $p < 0.05$ using a two-tailed *t*-test).

(D) Growth of wild-type and *btsl* alleles on medium with varying Fe concentrations. Seeds were germinated on medium with 50 μM FeEDTA, and, after 10 days, transferred to medium depleted of Fe using 100 μM ferrozine (-Fe) or 50 μM FeEDTA; or grown in the presence of 200 or 500 μM FeEDTA for 14 days.

(E, F) Chlorophyll concentration (E) under -Fe and control conditions, and iron concentration in shoots (F) from wild type (WT) and *btsl* mutant alleles, grown under control and Fe toxicity conditions as in (D). The bars represent the mean of $n = 3$ biological replicates $\pm$ SD (* $p < 0.05$; ** $p < 0.01$ using a two-tailed *t*-test).

BTSL1 and BTSL2 limit Fe uptake after a period of Fe deficiency

Because *BTSL1* and *BTSL2* are only expressed in Fe-deficient roots, but phenotypes were seen under Fe excess, we tested whether the genes have a specific function in the transition from Fe deficiency to Fe sufficiency. Wild-type and *btsl* double mutant seedlings were grown up on medium with Fe (50 μ M FeEDTA) for ten days and then transferred to medium without Fe but with ferrozine for 3 days to induce the Fe deficiency response. After that, plants were resupplied with Fe by transferring them back to Fe sufficient conditions, and then sampled after 3 days. In the control treatment, seedlings were always grown on 50 μ M Fe for 10 + 3 + 3 days, with transfers to fresh medium. A diagram of the treatments and time points of sampling is presented in Figure 5A. For comparison, we also grew the *brutus-1* (*bts-1*) mutant line (Selote et al., 2015) under the same conditions. *bts-1* is a viable allele that lacks mRNA induction under Fe deficiency.

Figure 5. The *btsl* double mutant hyper-accumulates Fe after a period of deficiency.

(A) Diagram of the Fe treatments used in this study. Seedlings were germinated on agar plates with 50 μ M FeEDTA. On day 10, seedlings were transferred to a new plate (control treatment) or to medium depleted of Fe (100 μ M ferrozine). After 3 days, seedlings were transferred back to medium with 50 μ M FeEDTA. Samples were taken at the end of the treatment.

(B) Perls' Prussian Blue staining for Fe in roots in wild-type, *btsl1-1 btsl2-2* double mutant and *bts-1* seedlings following control (top) and Fe deficiency-resupply treatments (bottom) according to the diagram in (A). The images show a section of the differentiation zone. Scale bar is 50 μ m.

(C) Perls' Prussian Blue staining of leaves after the Fe deficiency-resupply treatment. The images are a close-up of the adaxial leaf surface with veins. Scale bar is 75 μ m.

(D) Immunoblot of ferritin (FER) protein levels in root samples of wild type, *btsl1-1 btsl2-2* double mutant and *bts-1* seedlings after control and resupply treatments.

(E) Quantification of Fe in the shoots of wild-type, *btsl1-1 btsl2-2* double mutant and *bts-1* seedlings after control and Fe deficiency-resupply treatments. Bars represent the mean of 3 biological replicates of 10 pooled plants each $\pm$ SD (* $p < 0.05$ using a two-tailed *t*-test).

None of the genotypes showed chlorosis or growth impairment after the Fe deficiency and resupply treatment. However, the *btsl* double mutant accumulated large amounts of Fe in the central core of the root and in leaf veins as shown by Perls' staining (Figure 5B, C). Iron did not accumulate when mutants were grown continuously on 50 μ M Fe. *bts-1* mutants behaved as wild-type plants and did not show increased Perls' staining in any part of the root or leaves after the Fe deficiency-resupply treatment.

To gain further insight into the management of the Fe influx, we analysed the levels of ferritin protein, which functions as a temporary Fe store in Arabidopsis roots and leaves (Ravet et al.,

2009). Ferritin protein levels followed the same trend as observed with Fe staining, with ferritin accumulating only in the *btsl* double mutant after the Fe deficiency-resupply treatment (Figure 5D).

When we measured the Fe content of the shoots, we found that wild-type plants, when subjected to deficiency and resupply, contained 3-fold more Fe compared to continuous growth on 50 μ M Fe (Figure 5E). It is likely that upregulation of the Fe uptake machinery during Fe deficiency leads to a sudden influx of Fe when this is resupplied. In wild-type seedlings, the increased Fe levels do not seem to be toxic as there is no growth impairment and Fe is evenly distributed (Figure 5B, C). In the *btsl* double mutant, the Fe content of shoots increased more than 5-fold after the deficiency-resupply treatment relative to the control conditions (Figure 5E), reaching similar levels to those observed in plants grown continuously with 500 μ M Fe (Figure 4F). In contrast to the *btsl* double mutant, *bts-1* seedlings had a similar Fe content to wild type after the Fe deficiency-resupply treatment (Figure 5E).

In summary, our data show that the *btsl* double mutant is unable to adapt to changes in Fe supply, accumulating toxic concentrations of Fe. Moreover, the lack of Fe accumulation in *bts-1* line under these conditions suggests that BTSLs and BTS have different functions or act in different tissues.

The *btsl1-1 btsl2-2* double mutant fails to switch off the Fe deficiency response

Next, we investigated the levels of FRO2 and IRT1, two key players in Fe uptake, during Fe deficiency and resupply in the wild-type and *btsl* double mutant. We followed the same treatment described above, and plants were sampled before the resupply from the control (+Fe) and Fe deficiency treatment (-Fe), and daily after Fe resupply (1, 2 and 3 d Fe resupply) (Figure 6A). Wild-type seedlings were able to regulate FRO2 enzyme activity as expected, showing a ~6-fold induction in response to Fe deficiency and returning down to basal levels one day after Fe resupply (Figure 6A). In *btsl* double mutant seedlings FRO2 enzyme activity remained high after Fe resupply: the activity was 4-fold induced after one day, 3-fold induced after two days and it was still nearly double the basal wild-type levels after three days (Figure 6A). The single insertion *btsl1-1* and *bts-1* mutant lines behaved like the wild type, but *btsl2-2* showed a slight delay in switching off FRO2 activity (Supplementary Figure S5A). The sustained induction of FRO2 activity in the *btsl* double mutant was restored by expressing either *35S:BTSL-YFP* or *35S:BTSL2-GFP* (Supplementary Figure S5B).

IRT1 protein levels in wild-type plants were strongly increased under Fe deficiency, and were nearly below detection after one day of Fe resupply. In the *btsl* double mutant, IRT1 was not detected under standard conditions (+Fe), but after three days of Fe deficiency was present

at much higher levels than in wild-type roots. Upon Fe resupply, the IRT1 protein levels fluctuated but remained high overall (Figure 6B). The sustained presence of FRO2 and IRT1 would explain the increased Fe accumulation in the *bts1* double mutant following the Fe-deficiency-resupply treatment.

Figure 6. The *bts1* double mutant maintains *FRO2* and *IRT1* upon Fe resupply.

(A) Ferric chelate reductase activity of wild-type and *bts1-1 bts2-2* mutants. Bars represent the mean of 3 biological replicates ($n=5$ seedlings/assay) $\pm$ SD (* $p < 0.05$ using a two-tailed *t*-test). (B) Immunoblot of IRT1 protein levels in root samples in the Fe resupply treatment. (C, D) Expression of *FRO2* (C) and *IRT1* (D) in roots from wild-type and *bts1-1 bts2-2* seedlings determined by quantitative RT-PCR. All values are relative to wild type +Fe. Values are the mean of 3 biological replicates $\pm$ SE (* $p < 0.05$ using a two-tailed *t*-test).

To investigate which step of *FRO2* and *IRT1* expression is misregulated, we analysed transcript levels of *FRO2* and *IRT1* by RT-qPCR. Interestingly, under control conditions (+Fe), *FRO2* and *IRT1* transcripts levels were already increased 5 to 8-fold, respectively, in the *bts1* double mutant compared to wild type (Figure 6C, D), although no increase in enzymatic activity or protein levels was observed (Figure 6A, B). After three days of Fe deficiency, transcription of *FRO2* and *IRT1* was strongly upregulated by a similar magnitude in both mutant and in wild type. However, upon Fe resupply, *FRO2* and *IRT1* transcript levels remained high in the *bts1* double mutant, matching the sustained *FRO2* activity and IRT1 protein levels observed (Figure

6C). In contrast, in wild type, *FRO2* and *IRT1* transcripts were downregulated on day 2 and 3 after resupply compared to the initial levels (Figure 6C). Taken together, these data show that the transcriptional control of *FRO2* and *IRT1* is altered in the *bts1* double mutant and is disconnected from the Fe concentration in the medium.

The transcriptional regulator FIT-bHLH38/39 is mis-regulated in the *bts1* double mutant

The transcription of *FRO2* and *IRT1* is directly regulated by FIT together with one of four partially redundant bHLH transcription factors (bHLH38, bHLH39, bHLH100, bHLH101). To investigate if FIT is misregulated, its protein and transcript levels were followed in wild-type and mutant plants subjected to the Fe deficiency-resupply treatment. Under control conditions, FIT was not detected by protein blot analysis in either wild-type or *bts1-1 bts2-2*. FIT protein levels were strongly induced after three days of Fe deficiency in wild-type seedlings, and even more so in *bts1* double mutant seedlings (~3-fold more than wild type, Figure 7A). Upon Fe resupply, FIT protein levels fell below the limit of detection in wild type, but were partially sustained in *bts1* roots, which could explain the increase in *FRO2* and *IRT1* transcripts (Figure 6C). In contrast, the transcriptional regulation of *FIT* was similar in wild-type and *bts1* seedlings (Figure 7B). This suggests that the protein levels of FIT may be directly or indirectly controlled by BTSL1 and/or BTSL2 under Fe deficiency, as well as upon Fe resupply.

As already stated, FIT functions as a heterodimer, therefore we tested *bHLH38* and *bHLH39* transcript levels as a representative of all four redundant bHLH transcription factors from the subgroup Ib. Interestingly, under control conditions, *bHLH38/39* transcripts levels were increased 8-fold in the *bts1* double mutant compared to wild type (Figure 7B). After three days of Fe deficiency, the upregulation was similar in both genotypes, but upon Fe resupply, *bHLH38/39* transcript levels remained high in the *bts1* double mutant, matching the patterns observed for *FRO2* and *IRT1* transcripts (Figure 7B). In wild type plants, as soon as day 1 after resupply *bHLH38/39* transcripts were downregulated below initial levels (Figure 7C).

In summary, our data show that BTSL1 and 2 are required for FIT protein turnover, and for repressing transcription of *bHLH38/39*.

Figure 7. FIT protein and *bHLH38*, *bHLH39* transcript levels are misregulated in the *bts1* double mutant upon Fe resupply.

(A) Immunoblot of FIT protein levels in root samples of the Fe deficiency-resupply treatment.

(B) Expression of *FIT*, *bHLH38* and *bHLH39* in roots from wild-type and *bts1-1 bts2-2* seedlings determined by quantitative RT-PCR. All values are relative to wild type +Fe. Values are the mean of 3 biological replicates $\pm$ SE (* $p < 0.05$ using a two-tailed *t*-test).

BTSL1 and BTSL2 interact with FIT *in vitro*

Next, we tested if BTSL proteins can physically interact with FIT, a prerequisite for a direct target of ubiquitin-mediated protein degradation. A standard yeast 2-hybrid approach was not possible because of self-activation by the individual proteins. Therefore, we opted for an *in vitro* approach. The C-terminal CHY/RING Zn-finger domain of BTSL1 or BTSL2 was fused to MBP (maltose binding protein) for stabilization, and expressed in *E. coli*. FIT was also produced in *E. coli* with a Myc tag at the C-terminus. bHLH39:Myc and bHLH40:Myc were produced as controls. The protein interactions were studied by Far-Western blot analysis (Figure 8A) to overcome issues with the stability of the transcription factors. *E. coli* extracts with FIT, bHLH39 or bHLH40 were separated by SDS-PAGE and immobilized on nitrocellulose

membrane. Immuno-detection of the Myc tag confirmed the presence and protein levels of FIT, bHLH39 and bHLH40 (Figure 8B and Supplementary Figure S6, left panels). Purified MBP, MBP:FIT or MBP:BTSL proteins were incubated with the membrane, followed by antibody detection of the MBP tag. MBP:FIT bound strongly to bHLH39 (35 kDa band) and weakly to itself (55 kDa band), as previously reported (Yuan et al 2008). The immuno-signals were clearly separated from an aspecific band in the MBP control. MBP:BTSL1c and MBP:BTSL2c bound to FIT, but not to bHLH39. A separate set of experiments showed that the BTSL proteins also did not interact with bHLH40 (Supplementary Figure S6), better known as the transcription factor INDEHISCENT involved in abscission but unrelated to iron homeostasis (Liljegren et al., 2004). These data suggest that both BTSL1 and BTSL2 bind FIT to target it for degradation.

Figure 8. The CHY/RING Zn finger domain of BTSL proteins interact with FIT.

(A) Diagram of Far-Western blot analysis. Bait proteins with a Myc-tag were produced in *E. coli* and protein extracts separated by SDS-PAGE gel followed by transfer to nitrocellulose membrane. Blots were incubated with purified MBP-tagged proteins of interest, then labelled with anti-MBP antibodies.

(B) Far-Western blots analysis showing direct protein interactions between BTSL proteins and FIT. Bacterial protein extracts with FIT:Myc and bHLH39:Myc were immobilized on nitrocellulose, and the blots were incubated with recombinant MBP, MBP:FIT and the C-terminal domains of BTSL1 and BTSL2, fused to MBP (MBP:BTSL1c and MBP:BTSL2c). FIT is known to form a heterodimer with bHLH39. Ponceau staining shows equal protein loading.

DISCUSSION

In this study, we show that BTSL1 and BTSL2 have a clearly distinct function from BTS, and are not simply redundant paralogs. *BTSL* genes have evolved specifically in dicotyledonous plants, they are root specific and co-regulated with the Fe uptake machinery. We show that the partially redundant *BTSL* proteins negatively control Fe uptake in the plant, which is exacerbated under Fe deficiency-resupply conditions. Under these conditions, *BTS* does not play any role. A closer look at the expression patterns of the *BTSL1*, *BTSL2* and *BTS* genes suggest a demarcation by the Casparian strip, an effective barrier against nutrient overload of plants. The two *BTSL* genes are predominantly expressed in the root epidermis and cortex, whereas *BTS* is expressed inside the root stele and in the shoot. *BTSL2* is also expressed in the stele in the differentiation zone, but this is where the Casparian strip is not yet formed or still permeable (Nasser et al., 2012; Barberon et al., 2016). Moreover, the *BTSL2* promoter-GUS expression was analysed under Fe deficient conditions, when suberization of the Casparian strip is suppressed to maximize metal and nutrient uptake (Barberon et al., 2016). We propose a model where *BTSL* proteins act as a primary defense mechanism against excess Fe uptake in the root. A second defense barrier against Fe overload would be the *BTS* protein in the stele and leaf tissues, behind the Casparian strip (Figure 9).

Figure 9. Proposed mode of action of BTSL and BTS in different parts of the plant.

A model of how *BTSL* and *BTS* function to control Fe homeostasis. Fe levels are first sensed in the epidermal and cortex cells, where *BTSL* proteins respond by targeting FIT protein for degradation, thus controlling the uptake of Fe by FRO2 and IRT1 (and other proteins). A second checkpoint to protect plants from excess iron is present in the stele, stem and leaves, where *BTS* negatively controls ILR3 and bHLH104 protein levels (Selote et al., 2015). How iron affects the activity of *BTS/L* is not yet known (gray dashed arrows).

The different functions of *BTSL1/2* and *BTS* suggest that the E3 ligases target different proteins for degradation. Their C-termini are rather unique among E3 ligases, consisting of a combination of CHY/RING-type Zn finger domains, as shown in NMR structures of the

mammalian Pirh2/RCHY1 homolog (Sheng et al., 2008) which shares 45% amino acid identity with the C-termini of BTS/L. Pirh2/RCHY1 binds to the tetrameric form of the p53 transcription factor and targets it for degradation (Leng et al., 2003; Sheng et al., 2008). In Arabidopsis, 7 proteins share this conserved Pirh2-like domain: AT3G62970, AT5G18650, AT5G25560, AT5G22920; and BTSL1, BTSL2 and BTS. The best characterized is MYB30-INTERACTING E3 LIGASE 1 (MIEL1, AT5G18650), which targets the MYB30 transcription factor for degradation (Lee and Seo, 2016). Thus, it is likely that the unique CHY/RING-finger domain facilitates interaction with transcription factors for ubiquitination and degradation. BTS was found to interact and induce the degradation of the bHLH transcription factors ILR3 and bHLH115 (Selote et al., 2015), but direct ubiquitination of these two putative targets remains to be shown. It has recently been shown that HRZ1, the BTS homolog in rice, targets PRI1 (Positive Regulator of Iron homeostasis1) for degradation (Zhang et al., 2017). The bHLH transcription factor PRI1 is the homolog of Arabidopsis ILR3 and promotes the expression of *OsIRO2* and *OsIRO3*, which are orthologous to *bHLH38/39* and *PYE*, respectively (Kobayashi et al., 2013).

Our data suggest that the BTSL proteins target FIT for degradation. Firstly, the expression patterns of *FIT* and *BTSL* genes overlap in the epidermis and cortex (Figure 3; Colangelo and Gueriot, 2004; Jakoby et al., 2004). Secondly, FIT protein levels are increased in the *btsl* double mutant (Figure 7). Thirdly, the C-termini of BTSL1 and BTSL2 interact with FIT in vitro (Figure 8). It has previously been reported that FIT is regulated post-transcriptionally via proteasomal degradation (Sivitz et al., 2011), being turned over in response to ethylene (Lingam et al., 2011). Our data suggest that the BTSL proteins act as the E3 ligases responsible for ubiquitination of FIT which is then degraded by the 26S proteasome. At the moment, we cannot rule out that BTSL proteins may have other targets. The misregulation of *bHLH38* and *bHLH39* expression cannot be explained by lack of FIT protein degradation, because the transcriptional activators of bHLH38/39 are ILR3 and bHLH104 (Zhang et al., 2015). Either BTSL also target ILR3 and/or bHLH104 for degradation specifically in those root tissues where BTS is not expressed, or mutation of the *BTSL* genes has an indirect effect on the BTS regulon. A comprehensive study of the direct targets of BTSL and BTS proteins will clarify these questions, although the experimental approaches for determining E3 ligases targets are not straight forward (Iconomou and Saunders, 2016).

Where the Fe status is sensed is still under debate (Kobayashi and Nishizawa, 2014; Gayomba et al., 2015). The observation that *BTSL* and *BTS* are expressed in different tissues support the hypothesis that Fe is sensed independently in different parts of the plant. Our data suggest that BTSLs act as local Fe sensors controlling Fe uptake. FRO2 activity and transcript levels are misregulated in the *btsl* double mutant after Fe resupply. The same happens with

IRT1 protein and transcript levels, that remain high irrespective of the new Fe status. It is interesting to note the fluctuating levels of IRT1 protein after Fe resupply, which could be explained by its own internalization/degradation mechanism (Barberon et al., 2011; Shin et al., 2013). This protective mechanism seems to be disconnected from transcriptional regulation, with decreased levels of detectable IRT1 protein one day after resupply. However, the sustained transcript levels then seem to overrule the post-translational control mechanism, and two days later detectable protein levels are almost as high as under Fe deficiency. On the inside of the Casparian strip, BTS could sense the Fe status in the shoot and control systemic signals that regulate Fe homeostasis. Both local and systemic controls of Fe homeostasis have been reported (Grusak and Pezeshgi, 1996; Schikora and Schmidt, 2001; Vert et al., 2003).

An open question still is of how the BTS/L proteins sense Fe and adjust their E3 ligase activity. The BTS/L proteins have conserved hemerythrin domains that are predicted to bind 2 Fe atoms per domain. Recombinantly expressed HRZ and BTS were found to bind 2 Fe and 1 - 5 Zn atoms (Kobayashi et al., 2013; Selote et al., 2015), which is far below the 6 Fe expected for 3 Hr domains and 9 Zn for the CHY/RING-type Zn finger domain. HRZ and BTS proteins seem to be rather unstable, and GFP fusions are not detected (Selote et al., 2015). Removal of the N-terminal domain with the three hemerythrin motifs led to a stable GFP fusion protein, suggesting that this domain regulates the stability of BTS. Based on *in vitro* data, Selote et al (2015) concluded that BTS protein is stabilized in the absence of Fe bound to the hemerythrin domains. However, this mechanism is the opposite of what is observed for FBXL5, where Fe binding to the Hr domain stabilized the protein (Salahudeen et al., 2009). The fact that the phenotypes in *bts/* mutants are particularly noticeable upon Fe resupply points towards a stabilization of BTSL protein by Fe, in a mechanism analogous to that of FBXL5. However, the changes in protein turnover in FIT and IRT1 under Fe deficiency suggest that BTSL proteins are also active under low Fe conditions. An in-depth biochemical characterization of the Hr domains is necessary to better understand the possible sensing mechanism of these proteins. Furthermore, the plant proteins have three hemerythrins as opposed to one in mammal FBXL5, suggesting cooperativity between the domains to fine tune the dynamic range of Fe binding affinities. BTSL and BTS may differ in this respect, given the different sequence of the second Hr domain (Supplementary Figure S2). Only when those questions are addressed will we be able to fully understand the Fe dependent regulation of the E3 ligase activity of BTS/BTSL/HRZ proteins.

METHODS

Co-expression analysis

The co-expression networks were produced using publicly available microarray data (NASCArrays; <http://affymetrix.arabidopsis.info/>) specific from roots and shoots using maccu software (<http://maccu.sourceforge.net/>). Networks were visualized using Cytoscape 3.0 (<http://www.cytoscape.org/>). BTSL1, BTSL2 and BTS were used as baits to retrieve co-expressed genes with a Pearson correlation coefficient >0.60 for BTSL1 and BTSL2, and >0.50 for BTS, and correlations between the retrieved genes were also calculated. Only those genes also present in the published ferrome (Schmidt and Buckhout, 2011) were retained.

Phylogenetic analysis

Homologous sequences were found by using BLAST, with Arabidopsis BTS, BTSL1, BTSL2 and rice HRZ1 as queries in the Ensembl Plants database (<http://plants.ensembl.org>). Amino acid alignments were performed using Clustal Omega. A phylogenetic tree was plotted with BioNJ with the Jones-Taylor-Thornton matrix and rendered using TreeDyn 198.3. Species used: *Amborella trichocarpa*, *Arabidopsis thaliana*, *Brassica oleracea*, *Hordeum vulgare*, *Medicago truncatula*, *Oryza sativa*, *Physcomitrella patens*, *Populus trichocarpa*, *Solanum lycopersicum* and *Triticum aestivum*.

Plant material

Arabidopsis thaliana ecotype Columbia (Col-0) plants were used as wild type. The T-DNA insertion lines were *bts1-1* (SALK_015054); *bts1-2* (SALK_032464); *bts1-3* (SALK_117926); *bts2-2* (SALK_048470); and *bts-1* (SALK_016526); from the Nottingham Arabidopsis Stock Centre. The T-DNA insertion sites were confirmed by sequencing. Double mutant lines were produced by crossing single insertion lines *bts1-1* and *bts1-3* with *bts2-2*. Promoter sequences of *BTSL1* (-880 nt) and *BTSL2* (-2097 nt) were isolated by PCR from genomic DNA using primers BTSL1prom_GWF and BTSL1prom_GWR (BTSL1), and BTSL2pro_GWR and BTSL2pro_GWF (BTSL2). The promoter fragments were cloned upstream of eGFP-GUS in pBGWFS7 (Karimi et al., 2002) and the constructs were stably expressed in wild-type Arabidopsis. pXB2FS7 containing the CaMV 35S promoter upstream of eGFP-GUS was used as a positive control. The *bts1-1 bts2-2* double mutant line was complemented with tagged versions of BTSL1 and BTSL2. Full coding sequences of *BTSL1* and *BTSL2* were cloned using the primer pairs BTSL1_B1-F and BTSL1_B1-R, and BTSL2_B1-F and BTSL2_B1-R, respectively. These sequences were cloned upstream eYFP and eGFP, respectively, in the

vectors pEarleyGate101 and pEarleyGate103 (Earley et al., 2006). All the sequences of the primers used are in Supplementary Table S3.

Plant growth conditions

For Fe treatments, plants were grown on half Hoagland solution solidified with 0.8% (w/v) agar. The medium was composed of (in mM): K (3.5), Mg (1), Ca (2.5), NO₃ (7.5), PO₄ (1), and SO₄ (1); and (in μM): Mn (9.14), B (46.3), Mo (0.12), Zn (2.4) and Cu (0.37). The pH was adjusted to 5.5 with 1mM 2-(N-morpholino)ethanesulfonic acid (MES). For Fe treatments Fe was added at the desired concentrations using FeEDTA. For Fe deficiency media, no Fe was added and medium was supplemented with 100 μM of 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate (ferrozine). For seed propagation, plants were grown on compost under long-day conditions, 16 h light at 22°C, 8 h dark at 20°C, with 80% humidity and a light intensity of 140 – 200 μmol m⁻² s⁻¹.

Promoter GUS studies

Glucuronidase activity was detected as described (Jefferson et al., 1987), keeping the reaction time constant for equivalent samples. Seedlings were cleared in 100% ethanol, or fixed with a solution of 3.7% (w/v) formaldehyde, 5% (v/v) acetic acid, 50% (v/v) ethanol, followed by embedding in TechnoVit (Heraeus Kulzer GmbH, Germany), per manufacturer instructions. Transverse root sections (10 μm) were imaged on a LEICA DM6000 light microscope. The objectives used were x20/0.7 air or x40/0.85 air and images were collected with a LEICA DFC420C colour camera and processed with LEICA LAS-AF software.

Plant phenotyping

Arabidopsis leaf pigments were extracted following a modified protocol from Abadía and Abadía (1993). Briefly, leaf pigments were extracted on ice with 80% acetone, and extracts were filtered and centrifuged to eliminate any particulate. Absorbance was measured at 470, 645, 662, and 750 nm wavelengths in a multiplate reader (ClarioStar; BMG Labtech, Germany). Mean values and standard deviations (SD) were obtained from three biological replicates.

Iron content in Arabidopsis leaves was determined by the Ferene method. Dried and powdered samples (4–8 mg) were mineralized at room temperature in 150 μL nitric acid (65%) for 2 days. One hundred and fifty microliters of H₂O₂ (30%) were added, and the solution was incubated 2 more days. The volume was adjusted to 500 μL with sterile water. A standard curve with Fe from 0 to 10 μg of Fe from ferric ammonium citrate was subjected to the same mineralization process. One hundred microliters of the mineralized samples were buffered

with 1 mL of 15% ammonium acetate and reduced with 100 μ L of 4% (w/v) ascorbic acid. Then, Fe(II) was chelated by adding 100 μ L of 1.5% w/v 3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazine (Ferene, SIGMA). Absorbance at 593 nm was measured in triplicates in a multi-plate reader (ClarioStar, BMG Labtech).

To localize Fe in tissues, plants always grown on Fe-sufficient media or following a resupply after Fe deficiency induction were incubated with Perls' stain solution (equal volumes of 2% [v/v] HCl and 2% [w/v] K-ferrocyanide) for 30 min. Seedlings were then washed three times with water, chlorophyll partially removed by incubation with 50% ethanol and then imaged with a Leica DM6000 microscope.

Fe chelate reductase activity was measured following Yi and Guerinot, (1996). Briefly, roots from 5 plants were blotted dried, weighed and incubated for 30 minutes in the dark with measuring solution containing 0.1 mM Fe(III)-EDTA and 0.3 mM ferrozine in distilled water. Fe²⁺ production was determined with a spectrophotometer measuring absorbance at 562 nm.

Quantitative reverse-transcription PCR

Total RNA was extracted using the Plant RNeasy kit (Qiagen), followed by DNase treatment (Turbo DNase kit, Agilent). The integrity of RNA in all samples was verified using agarose gels, and RNA sample purity was analysed by comparing 260/230 nm and 260/280 nm absorbance ratios (Nanodrop 2000, Thermo Fisher). Only samples which passed these quality control checks were used. RNA was quantified using a Qubit 2.0 fluorometer (Thermo Fisher). RNA (4 μ g) was reverse transcribed to cDNA using Superscript III (Thermo Fisher). RT-qPCR reactions were made using SensiFAST master-mix (Bioline), in 20 μ L volumes, each with 20 ng of cDNA. Reactions were measured in a Bio-Rad CFX-96 real-time PCR system and cycled as per the Bioline protocol. Data were analysed using the Bio-Rad CFX Manager 3.1 software, and were normalized using primer efficiency. All data points are from three or more independent biological replicates, measured in three technical replicates (n=9). The house-keeping genes SAND (*AT2G28390*) and TIP41-like (*AT4G34270*) were used as reference genes, as they are unaffected by Fe levels in Arabidopsis (Han et al., 2013).

Protein expression and immunoblot analysis

Roots from ten plants were ground in liquid nitrogen and suspended in acetone containing 10% (w/v) TCA, mixed thoroughly and precipitated for 2 hours at -20°C. Proteins were pelleted and washed three times with cold acetone and then dried prior solubilisation with a buffer containing 8 M urea, 0.5% SDS, 50 mM DTT and 1 mM PMSF. Proteins were separated on 12.5% SDS-PAGE gels, blotted into Nitrocellulose membranes and immunodetected using

anti-Ferritin, anti-FIT and anti-IRT1 (Fan et al., 2014) antibodies and ECL Plus chemiluminescence substrate. Anti-Ferritin antibody was produced using purified pea ferritin for rabbit immunization. For anti-FIT antibody production, the coding sequence of FIT was fused to MBP, purified from *E. coli* and used for rabbit immunization.

Recombinant protein production

For recombinant protein production, full coding sequences of *FIT* (AT2G28160), *bHLH39* (AT3G56980) and *bHLH40* (AT4G00120) were cloned from cDNA from Fe deficient Arabidopsis roots or genomic DNA, with a N-terminal His-tag and C-terminal Myc tag. Coding sequences were then inserted into the pET-15b vector using NdeI and BamHI restriction sites. The C-terminal coding regions of *BTSL1* (amino acids 933-1259) and *BTSL2* (amino acids 787-1254) were amplified from cDNA from Fe deficient Arabidopsis roots and cloned into the expression vector pMAL-c5X (NEB) using NotI-Sall and NcoI-Sall restriction sites, respectively. This generated an N-terminal fusion with maltose binding protein (MBP) to obtain MBP:BTSL1c and MBP:BTSL2c. A construct for MBP:FIT was produced in the same way. Plasmids were transformed into *Escherichia coli* Rosetta (NEB). Cells were grown in LB medium and protein expression induced with IPTG (Isopropyl β -D-1-thiogalactopyranoside) following manufacturer's instructions for 4 h at 37°C. The recombinant MBP fusion proteins were purified using amylose resin. All primers used are listed in Supplementary Table S3.

Far-western blot analysis

Soluble protein extracts of bacteria expressing FIT:Myc and bHLH40:Myc proteins were separated in a 10% SDS PAGE gel and blotted into a nitrocellulose membrane. Membranes were then blocked and incubated with purified MBP, MBP:FIT, MBP:BTSL1c or MBP:BTSL2c at 0.5 μ M in TBS-tween buffer with 5% dry milk as blocking agent. After washing, proteins bound to the membrane were detected with anti-Myc (ab18185, Abcam) and anti-MBP (ab23903, Abcam) antibodies, respectively.

Accession numbers

BTSL1 (AT1G74770); BTSL2 (AT1G18910); BTS (ATG3G18290);
 OsHRZ2 (LOC_Os05g47780); OsHRZ1 (OS01T0689451_02);
 TaHRZ1-A (TRIAE_CS42_3AL_TGACv1_195251_AA0646970);
 TaHRZ1-B (TRIAE_CS42_3B_TGACv1_220625_AA0712080);
 TaHRZ1-D (TRIAE_CS42_3DL_TGACv1_249432_AA0848520);
 TaHRZ2-A (TRIAE_CS42_1AL_TGACv1_000799_AA0019360);
 TaHRZ2-B (TRIAE_CS42_1BL_TGACv1_031945_AA0123190);
 TaHRZ2-D (TRIAE_CS42_1DL_TGACv1_061110_AA0185650).
 FIT / bHLH29 (AT2G28160); bHLH38 (AT3G56970); bHLH39 (AT3T56980); bHLH40
 (AT4G00120)

Supplementary data

Supplementary Table S1. ATG numbers, gene description and correlations between genes appearing the in the networks shown in Supplementary Figure S1.

Supplementary Table S2. ATG numbers, gene description and correlation between genes appearing in the networks shown in Figure 1.

Supplementary Table S3. List of primers.

Supplementary Figure S1. Full co-expression networks of BTSL1, BTSL2 and BTS.

Supplementary Figure S2. Alignment of hemerythrin domains and structure prediction for the second Hr domain of BTSL1.

Supplementary Figure S3. GUS staining of embryos

Supplementary Fig S4. Fresh weight of WT and *btsl* mutant lines under Fe sufficiency and toxicity conditions.

Supplementary Figure S5. Fe reductase activity of *btsl1-1*, *btsl2-2*, *bts-1* and complementation lines

Supplementary Fig S6. The CHY/RING Zn finger domain of BTSL proteins do not interact with bHLH40.

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J.R.C. and J.B. conceived the project, performed experiments, analysed the data and wrote the paper. J.M.C., R.T.G. and I.K. performed experiments and analysed data. Y.C. and H.Q.L. contributed new analytical tools.

REFERENCES

- Abadía, J., and Abadía, A.** (1993). Iron and plant pigments. *In* LL Barton, BC Hemming, eds, Iron Chelation in Plants and Soil Microorganisms, Academic Press, San Diego, pp 327–343.
- Barberon, M., Vermeer, J.E.M., De Bellis, D., Wang, P., Naseer, S., Andersen, T.G., Humbel, B.M., Nawrath, C., Takano, J., Salt, D.E., and Geldner, N.** (2016). Adaptation of Root Function by Nutrient-Induced Plasticity of Endodermal Differentiation. *Cell* **164**: 447–459.
- Barberon, M., Zelazny, E., Robert, S., Conéjéro, G., and Curie, C.** (2011). Monoubiquitin-dependent endocytosis of the IRON-REGULATED TRANSPORTER 1 (IRT1) transporter controls iron uptake in plants. *Proc. Natl. Acad. Sci.* **108**: E450–E458.
- Bauer, P., Ling, H.Q., and Guerinot, M. Lou** (2007). FIT, the FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR in Arabidopsis. *Plant Physiol. Biochem.* **45**: 260–261.
- Bournier, M., Tissot, N., Mari, S., Boucherez, J., Lacombe, E., and Briat, J.** (2013). Arabidopsis Ferritin 1 (AtFer1) Gene Regulation by the Phosphate Starvation Response 1 (AtPHR1) Transcription Factor Reveals a Direct Molecular Link between Iron and. **288**: 22670–22680.
- Colangelo, E.P. and Guerinot, M. Lou** (2004). The Essential Basic Helix-Loop-Helix Protein FIT1 Is Required for the Iron Deficiency Response. *Plant Cell* **16**: 3400–3412.
- Earley, K.W., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K., and Pikaard, C.S.** (2006). Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* **45**: 616–629.
- Fan, H., Zhang, Z., Wang, N., Cui, Y., Sun, H., Liu, Y., Wu, H., Zheng, S., Bao, S., and Ling, H.Q.** (2014). SKB1/PRMT5-mediated histone H4R3 dimethylation of Ib subgroup bHLH genes negatively regulates iron homeostasis in *Arabidopsis thaliana*. *Plant J.* **77**: 209–221.
- Gayomba, S.R., Zhai, Z., Jung, H., and Vatamaniuk, O.K.** (2015). Local and systemic signaling of iron status and its interactions with homeostasis of other essential elements. *Front. Plant Sci.* **6**: 1–13.
- Grusak, M.A. and Pezeshgi, S.** (1996). Shoot-to-Root Signal Transmission Regulates Root

- Fe(III) Reductase Activity in the *dg1* Mutant of Pea. *Plant Physiol.* **110**: 329–334.
- Han, B., Yang, Z., Samma, M.K., Wang, R., and Shen, W.** (2013). Systematic validation of candidate reference genes for qRT-PCR normalization under iron deficiency in *Arabidopsis*. *BioMetals* **26**: 403–413.
- Hindt, M.N., Akmakjian, Garo Z. Pivarski, K.L., Punshon, T., Baxter, I., Salt, E., Gueriot, M. Lou, and Punshon, T.** (2017). BRUTUS and its paralogs, BTS LIKE1 and BTS LIKE2, encode important negative regulators of the iron deficiency response in *Arabidopsis thaliana*. *Metallomics* **2**.
- Iconomou, M. and Saunders, D.N.** (2016). Systematic approaches to identify E3 ligase substrates. *Biochem. J.* **473**: 4083–4101.
- Jakoby, M., Wang, H.Y., Reidt, W., Weisshaar, B., and Bauer, P.** (2004). FRU (BHLH029) is required for induction of iron mobilization genes in *Arabidopsis thaliana*. *FEBS Lett.* **577**: 528–534.
- Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W.** (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**: 3901–7.
- Karimi, M., Inze, D., and Depicker, A.** (2002). GATEWAY vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci.* **7**: 193–195.
- Kobayashi, T., Nagasaka, S., Senoura, T., Itai, R.N., Nakanishi, H., and Nishizawa, N.K.** (2013). Iron-binding haemerythrin RING ubiquitin ligases regulate plant iron responses and accumulation. *Nat. Commun.* **4**: 1–12.
- Kobayashi, T. and Nishizawa, N.K.** (2015). Intracellular iron sensing by the direct binding of iron to regulators. *Front. Plant Sci.* **6**: 1–4.
- Kobayashi, T. and Nishizawa, N.K.** (2012). Iron uptake, translocation, and regulation in higher plants. *Annual Rev. plant Biol.* **63**: 131–152.
- Lee, H.G. and Seo, P.J.** (2016). The *Arabidopsis* MIEL1 E3 ligase negatively regulates ABA signalling by promoting protein turnover of MYB96. *Nat. Commun.* **7**: 12525.
- Leng, R.P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J.M., Lozano, G., Hakem, R., and Benchimol, S.** (2003). Pirh2, a p53-Induced Ubiquitin-Protein Ligase, Promotes p53 Degradation. *Cell* **112**: 779–791.

- Liljegren, S.J., Roeder, A.H.K., Kempin, S.A., Gremski, K., Østergaard, L., Guimil, S., Reyes, D.K., and Yanofsky, M.F.** (2004). Control of fruit patterning in *Arabidopsis* by INDEHISCENT. *Cell* **116**: 843–853.
- Lingam, S., Mohrbacher, J., Brumbarova, T., Potuschak, T., Fink-Straube, C., Blondet, E., Genschik, P., and Bauer, P.** (2011). Interaction between the bHLH transcription factor FIT and ETHYLENE INSENSITIVE3/ETHYLENE INSENSITIVE3-LIKE1 reveals molecular linkage between the regulation of iron acquisition and ethylene signaling in *Arabidopsis*. *Plant Cell* **23**: 1815–1829.
- Long, T. a, Tsukagoshi, H., Busch, W., Lahner, B., Salt, D.E., and Benfey, P.N.** (2010). The bHLH transcription factor POPEYE regulates response to iron deficiency in *Arabidopsis* roots. *Plant Cell* **22**: 2219–36.
- Mai, H., Pateyron, S., and Bauer, P.** (2016). Iron homeostasis in *Arabidopsis thaliana* : transcriptomic analyses reveal novel FIT- regulated genes , iron deficiency marker genes and functional gene networks. *BMC Plant Biol.* **16**: 211–33.
- Matthiadis, A. and Long, T.A.** (2016). Further insight into BRUTUS domain composition and functionality. *Plant Signal. Behav.* **11**: e1204508.
- McElver, J. et al.** (2001). Insertional mutagenesis of genes required for seed development in *Arabidopsis thaliana*. *Genetics* **159**: 1751–1763.
- Naseer, S., Lee, Y., Lapierre, C., Franke, R., Nawrath, C., and Geldner, N.** (2012). Casparian strip diffusion barrier in *Arabidopsis* is made of a lignin polymer without suberin. *Proc. Natl. Acad. Sci.* **109**: 10101–10106.
- Ravet, K., Touraine, B., Boucherez, J., Briat, J.F., Gaymard, F., and Cellier, F.** (2009). Ferritins control interaction between iron homeostasis and oxidative stress in *Arabidopsis*. *Plant J.* **57**: 400–412.
- Rodríguez-Celma, J., Lin, W.-D., Fu, G.-M., Abadía, J., López-Millán, A.-F., and Schmidt, W.** (2013a). Mutually exclusive alterations in secondary metabolism are critical for the uptake of insoluble iron compounds by *Arabidopsis* and *Medicago truncatula*. *Plant Physiol.* **162**: 1473–85.
- Rodríguez-Celma, J., Pan, I.C., Li, W., Lan, P., Buckhout, T.J., and Schmidt, W.** (2013b). The transcriptional response of *Arabidopsis* leaves to Fe deficiency. *Front. Plant Sci.* **4**: 1–10.
- Römheld, V. and Marschner, H.** (1986). Evidence for a specific uptake system for iron

phytosiderophores in roots of grasses. *Plant Physiol.* **80**: 175–180.

Salahudeen, A.A., Thompson, J.W., Ruiz, J.C., Ma, H.-W., Kinch, L.N., Li, Q., Grishin, N. V., and Bruick, R.K. (2009). An E3 Ligase Possessing an Iron-Responsive Hemerythrin Domain Is a Regulator of Iron Homeostasis. *Science* (80-.). **326**: 722–726.

Schikora, A. and Schmidt, W. (2001). Iron Stress-Induced Changes in Root Epidermal Cell Fate Are Regulated Independently from Physiological Responses to Low Iron Availability. *Plant Physiol.* **125**: 1679–1687.

Schmidt, W. and Buckhout, T.J. (2011). A hitchhiker’s guide to the Arabidopsis ferrome. *Plant Physiol. Biochem.* **49**: 462–470.

Selote, D., Samira, R., Matthiadis, A., Gillikin, J.W., and Long, T.A. (2015). Iron-binding E3 ligase mediates iron response in plants by targeting basic Helix-Loop-Helix transcription factors. *Plant Physiol.* **167**: 273–286.

Sheng, Y., Laister, R.C., Lemak, A., Wu, B., Tai, E., Duan, S., Lukin, J., Sunnerhagen, M., Srisailam, S., Karra, M., Benchimol, S., and Arrowsmith, C.H. (2008). Molecular basis of Pirh2-mediated p53 ubiquitylation. *Nat. Struct. Mol. Biol.* **15**: 1334–1342.

Shin, L.-J., Lo, J.-C., Chen, G.-H., Callis, J., Fu, H., and Yeh, K.-C. (2013). IRT1 degradation factor1, a ring E3 ubiquitin ligase, regulates the degradation of iron-regulated transporter1 in Arabidopsis. *Plant Cell* **25**: 3039–3051.

Sivitz, A., Grinvalds, C., Barberon, M., Curie, C., and Vert, G. (2011). Proteasome-mediated turnover of the transcriptional activator FIT is required for plant iron-deficiency responses. *Plant J.* **66**: 1044–1052.

Stenkamp, R.E. (1994). Dioxygen and Hemerythrin. *Chem. Rev.* **94**: 715–726.

Stuart, J.M., Segal, E., Koller, D., and Kim, S.K. (2003). A Gene-Coexpression Network for Global Discovery of Conserved Genetic Modules. *Science* **302**: 249–255.

Tsai, H.-H. and Schmidt, W. (2017). Mobilization of Iron by Plant-Borne Coumarins. *Trends Plant Sci.* **22**: 538-48.

Tzafrir, I., Pena-muralla, R., Dickerman, A., Berg, M., Rogers, R., Hutchens, S., Sweeney, T.C., Mcelver, J., Aux, G., Patton, D., Meinke, D., and Oklahoma, I.T. (2004). Identification of Genes Required for Embryo Development in Arabidopsis. *Plant Physiol.* **135**: 1206–1220.

- Vashisht, A.A. et al.** (2009). Control of Iron Homeostasis by an Iron-Regulated Ubiquitin Ligase. *Science* (80-.). **326**: 718–721.
- Vert, G.A., Briat, J.-F., and Curie, C.** (2003). Dual Regulation of the Arabidopsis High-Affinity Root Iron Uptake System by Local and Long-Distance Signals. *Plant Physiol.* **132**: 796–804.
- Wang, N., Cui, Y., Liu, Y., Fan, H., Du, J., Huang, Z., Yuan, Y., Wu, H., and Ling, H.Q.** (2013). Requirement and functional redundancy of Ib subgroup bHLH proteins for iron deficiency responses and uptake in *Arabidopsis thaliana*. *Mol. Plant* **6**: 503–513.
- Yi, Y. and Guerinot, M. Lou** (1996). Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency. *Plant J.* **10**: 835–844.
- Yuan, Y., Wu, H., Wang, N., Li, J., Zhao, W., Du, J., Wang, D., and Ling, H.-Q.** (2008). FIT interacts with AtbHLH38 and AtbHLH39 in regulating iron uptake gene expression for iron homeostasis in Arabidopsis. *Cell Res.* **18**: 385–97.
- Zhang, H.M., Li, Y., Yao, X.N., Liang, G., and Yu, D.** (2017). POSITIVE REGULATOR OF IRON HOMEOSTASIS 1 (OsPRI1) positively regulates iron homeostasis in rice. *Plant Physiol.* **175**: 543-54.
- Zhang, J., Liu, B., Li, M., Feng, D., Jin, H., Wang, P., Liu, J., Xiong, F., Wang, J., and Wang, H.-B.** (2015). The bHLH transcription factor bHLH104 interacts with IAA-LEUCINE RESISTANT3 and modulates iron homeostasis in Arabidopsis. *Plant Cell* **27**: 787–805.

FIGURE LEGENDS

Figure 1. *BTSL* and *BTS* are part of different gene expression networks.

(A, B) Co-expression analysis of Fe-responsive genes that are co-regulated with (A) *BTSL1* and *BTSL2* in roots, and (B) *BTS* in shoots. The edge thickness relate to the Pearson's correlation coefficient. See Supplemental Table S2 for descriptions of all genes and numerical values.

(C) Expression levels of *BTS*, *BTSL1* and *BTSL2* in roots and in shoots under Fe sufficient (+Fe) and Fe deficient conditions (-Fe). Data were obtained by RNAseq as described in Rodriguez-Celma et al., (2013). RPKM, Reads Per Kilobase Million. The bars represent the mean of n = 3 biological replicates x 10 seedlings $\pm$ SD (* p<0.05 using a two-tailed t-test).

Figure 2. *BTSL* are uniquely found in dicotyledonous plants.

(A) Phylogenetic tree of *BTS* homologs from selected plant species. Sequences were found by BLASTing the amino acid sequences of Arabidopsis *BTS*, *BTSL1*, *BTSL2* and rice *HRZ1* in Ensembl Plants (<http://plants.ensembl.org>). Species used: Amborella, *Amborella trichocarpa*; At, *Arabidopsis thaliana*; Bo, *Brassica oleracea*; Hv, *Hordeum vulgare*; Mt, *Medicago truncatula*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Pt, *Populus trichocarpa*; Sl, *Solanum lycopersicum*; Ta, *Triticum aestivum*. Numbers next to branches indicate bootstrapping values for 100 replications. The scale bar indicates the rate of evolutionary change expressed as number of amino acid substitutions per site.

(B) Domain organization of *BTSL*, *BTS* and *HRZ* proteins.

Figure 3. Promoter activity of *BTSL1* and *BTSL2* in Fe-deficient roots.

Promoter sequences of *BTSL1* (-880 nt) and *BTSL2* (-2097 nt) were inserted upstream of eGFP-GUS and the constructs were stably expressed in wild-type Arabidopsis.

(A) GFP fluorescence in seedlings grown on medium with 50 μ M FeEDTA (+Fe) or with 100 μ M ferrozine to deplete Fe (-Fe), as indicated, for 6 days. Scale bar is 1 mm.

(B) GUS activity staining in seedlings grown on medium depleted of Fe (100 μ M ferrozine) for 8 days. Scale bar is 0.5 mm for whole seedlings, 100 μ m for a close-up of the root tip, and 50 μ m for root cross sections.

Images are representative of 3 independent lines for each promoter construct.

Figure 4. *BTSL1* and *BTSL2* have redundant functions in Fe homeostasis.

(A) Schematic representation of the *BTSL1* and *BTSL2* genes and mutant alleles. Exons are in black, 5' and 3'-UTRs in grey. T-DNA insertions are indicated by triangles: *bts1/1-1*, SALK_015054; *bts1/1-2*, SALK_032464; *bts1/1-3*, SALK_117926; *bts2/2*, SALK_048470. Arrows indicate the left border primer used for genotyping.

(B, C) Transcript levels of *BTSL1* (B) and *BTSL2* (C) in wild-type and mutant alleles under standard (+Fe, 50 μ M FeEDTA) and Fe-deficient conditions (-Fe, 100 μ M ferrozine). (* $p < 0.05$ using a two-tailed t-test).

(D) Growth of wild-type and *btsl* alleles on medium with varying Fe concentrations. Seeds were germinated on medium with 50 μ M FeEDTA, and, after 10 days, transferred to medium depleted of Fe using 100 μ M ferrozine (-Fe) or 50 μ M FeEDTA; or grown in the presence of 200 or 500 μ M FeEDTA for 14 days.

(E, F) Chlorophyll concentration (E) under -Fe and control conditions, and iron concentration in shoots (F) from wild type (WT) and *btsl* mutant alleles, grown under control and Fe toxicity conditions as in (D). The bars represent the mean of $n = 3$ biological replicates $\times$ 10 seedlings $\pm$ SD (* $p < 0.05$; ** $p < 0.01$ using a two-tailed t-test).

Figure 5. The *btsl* double mutant hyper-accumulates Fe after a period of deficiency.

(A) Diagram of the Fe treatments used in this study. Seedlings were germinated on agar plates with 50 μ M FeEDTA. On day 10, seedlings were transferred to a new plate (control treatment) or to medium depleted of Fe (100 μ M ferrozine). After 3 days, seedlings were transferred back to medium with 50 μ M FeEDTA. Samples were taken at the end of the treatment.

(B) Perls' Prussian Blue staining for Fe in roots in wild-type, *btsl1-1 btsl2-2* double mutant and *btsl-1* seedlings following control (top) and Fe deficiency-resupply treatments (bottom) according to the diagram in (A). The images show a section of the differentiation zone. Scale bar is 50 μ m.

(C) Perls' Prussian Blue staining of leaves after the Fe deficiency-resupply treatment. The images are a close-up of the adaxial leaf surface with veins. Scale bar is 75 μ m.

(D) Immunoblot of ferritin (FER) protein levels in root samples of wild type, *btsl1-1 btsl2-2* double mutant and *btsl-1* seedlings after control and resupply treatments.

(E) Quantification of Fe in the shoots of wild-type, *btsl1-1 btsl2-2* double mutant and *btsl-1* seedlings after control and Fe deficiency-resupply treatments. Bars represent the mean of 3 biological replicates of 10 pooled plants each $\pm$ SD (* $p < 0.05$ using a two-tailed t-test).

Figure 6. The *btsl* double mutant maintains FRO2 and IRT1 upon Fe resupply.

(A) Ferric chelate reductase activity of wild-type and *btsl1-1 btsl2-2* mutants. Bars represent the mean of 3 biological replicates ($n=5$ seedlings/assay) $\pm$ SD (* $p < 0.05$ using a two-tailed t-test).

(B) Immunoblot of IRT1 protein levels in root samples in the Fe resupply treatment.

(C, D) Expression of *FRO2* (C) and *IRT1* (D) in roots from wild-type and *btsl1-1 btsl2-2* seedlings determined by quantitative RT-PCR. All values are relative to wild type +Fe. Values are the mean of 3 biological replicates $\pm$ SE (* $p < 0.05$ using a two-tailed t-test).

Figure 7. FIT protein and *bHLH38*, *bHLH39* transcript levels are misregulated in the *btsl* double mutant upon Fe resupply.

(A) Immunoblot of FIT protein levels in root samples of the Fe deficiency-resupply treatment.
 (B) Expression of *FIT*, *bHLH38* and *bHLH39* in roots from wild-type and *btsl1-1 btsl2-2* seedlings determined by quantitative RT-PCR. All values are relative to wild type +Fe. Values are the mean of 3 biological replicates $\pm$ SE (* $p < 0.05$ using a two-tailed t-test).

Figure 8. The CHY/RING Zn finger domain of BTSL proteins interact with FIT.

(A) Diagram of Far-Western blot analysis. Bait proteins with a Myc-tag were produced in *E. coli* and protein extracts separated by SDS-PAGE gel followed by transfer to nitrocellulose membrane. Blots were incubated with purified MBP-tagged proteins of interest, then labelled with anti-MBP antibodies.

(B) Far-Western blots analysis showing direct protein interactions between BTSL proteins and FIT. Bacterial protein extracts with FIT:Myc and bHLH39:Myc were immobilized on nitrocellulose, and the blots were incubated with recombinant MBP, MBP:FIT and the C-terminal domains of BTSL1 and BTSL2, fused to MBP (MBP:BTSL1c and MBP:BTSL2c). FIT is known to form a heterodimer with bHLH39. Ponceau staining shows equal protein loading.

Figure 9. Proposed mode of action of BTSL and BTS in different parts of the plant.

A model of how BTSL and BTS function to control Fe homeostasis. Fe levels are first sensed in the epidermal and cortex cells, where BTSL proteins respond by targeting FIT protein for degradation, thus controlling the uptake of Fe by FRO2 and IRT1 (and other proteins). A second checkpoint to protect plants from excess iron is present in the stele, stem and leaves, where BTS negatively controls ILR3 and bHLH104 protein levels (Selote et al., 2015). How iron affects the activity of BTS/L is not yet known (gray dashed arrows).

Supplementary data

A

B

Supplementary Figure S1. Full co-expression networks of *BTSL1*, *BTSL2* and *BTS*.

(A, B) Co-expression analysis of genes that are co-regulated with (A) *BTSL1* and *BTSL2* in roots, and (B) *BTS* in shoots. The edge thickness relate to the Pearson's correlation coefficient. See Supplemental Table S1 for descriptions of all genes and numerical values.

Supplementary Figure S2. Alignment of hemerythrin domains and structure prediction for the second Hr domain of BTSL1.

(A) Alignment of hemerythrin domains of FBXL5, BTS, BTSL1 and BTSL2.

(B) Predicted structure for the second Hr domain of BTSL1 using Phyre2 server.

Supplementary Figure S3. GUS staining of embryos

GUS activity staining of embryos from constitutive CaMV35S:GUS, promBTSL1:GUS and promBTSL2:GUS. Data for promBTS:GUS for comparison from Selote et al., 2015. Scale bar 200 μ m.

Supplementary Fig S4. Fresh weight of WT and *bts/* mutant lines under Fe sufficiency and toxicity conditions

Shoot fresh weight of shoots from wild type (WT) and *bts/* mutant alleles, grown under control and Fe toxicity conditions as in Fig 4D. The bars represent the mean of n = 3 biol. reps x 10 seedlings $\pm$ SD. (* p < 0.05 using a two-tailed *t*-test).

Supplementary Fig S5. Fe reductase activity of *bts1-1*, *bts2-2*, *bts-1* and complementation lines

(A) Ferric chelate reductase activity of *bts1-1*, *bts2-2* and *bts-1* single insertion mutants under the Fe-deficiency and resupply treatment.

(B) Complementation of mutant alleles based on ferric chelate reductase activity. Wild type, *bts1-1 bts2-2* double mutant and *bts1-1 bts2-2* lines expressing 35S:BTSL1-YFP or 35S:BTSL2-GFP were grown under Fe sufficiency, Fe deficiency and resupplied with Fe for 1 day. The results are the mean value of 3 biological replicates $\pm$ SE (*, $p < 0.05$ using a two-tailed Student *t*-test).

Supplementary Fig S6. The CHY/RING Zn finger domain of BTSL proteins do not interact with bHLH40.

Far-Western blots analysis showing no protein interactions between BTSL proteins and bHLH40. Bacterial protein extracts with FIT:Myc and bHLH40:Myc were immobilized on nitrocellulose, and the blots were incubated with recombinant MBP, MBP:FIT and the C-terminal domains of BTSL1 and BTSL2, fused to MBP (MBP:BTSL1c and MBP:BTSL2c). FIT is known to not form a heterodimer with bHLH40.

Supplementary Table S1. ATG numbers and gene description of genes appearing in the correlation networks shown in Supplemental Figure S1.

Root co-expression network			Correlation to	
ATG	Symbol	Description	BTSL1	BTSL2
Genes co-expressed to BTSL1 and BTSL2				
AT3G24290		ammonium transporter 1;5	0.780	0.731
AT1G07560		Leucine-rich repeat protein kinase family protein	0.771	0.711
AT3G01190	IREG2	Peroxidase superfamily protein	0.766	0.784
AT5G03570		iron regulated 2	0.765	0.711
AT1G47480		alpha/beta-Hydrolases superfamily protein	0.746	0.760
AT4G26010		Peroxidase superfamily protein	0.745	0.690
AT4G20460		NAD(P)-binding Rossmann-fold superfamily protein	0.745	0.802
AT5G55170		small ubiquitin-like modifier 3	0.739	0.719
AT2G45430		AT-hook motif nuclear-localized protein 22	0.736	0.826
AT3G62040		Haloacid dehalogenase-like hydrolase (HAD) superfamily protein	0.734	0.675
AT1G51790		Leucine-rich repeat protein kinase family protein	0.733	0.754
AT2G39530		Uncharacterised protein family (UPF0497)	0.730	0.749
AT3G55150		exocyst subunit exo70 family protein H1	0.728	0.752
AT2G21020		Unknown	0.727	0.722
AT4G21600		endonuclease 5	0.727	0.828
AT1G05250		Peroxidase superfamily protein	0.726	0.691
AT5G48290		Heavy metal transport/detoxification superfamily protein	0.721	0.661
AT4G35030		Protein kinase superfamily protein	0.718	0.646
AT3G23190		HR-like lesion-inducing protein-related	0.718	0.771
AT1G05260		Peroxidase superfamily protein	0.718	0.721
AT1G08325		Unknown	0.714	0.694
AT2G35270		Predicted AT-hook DNA-binding family protein	0.712	0.794
AT3G13760		Cysteine/Histidine-rich C1 domain family protein	0.711	0.676
AT5G61250	GUS1	glucuronidase 1	0.711	0.688
AT1G30900		VACUOLAR SORTING RECEPTOR 6	0.711	0.842
AT2G21045		Rhodanese/Cell cycle control phosphatase superfamily protein	0.711	0.699
AT5G43180		Protein of unknown function, DUF599	0.707	0.657
AT3G25930		Adenine nucleotide alpha hydrolases-like superfamily protein	0.706	0.670
AT5G40510		Sucrase/ferredoxin-like family protein	0.703	0.764
AT2G28250		Protein kinase superfamily protein	0.703	0.665
AT1G49310		Unknown	0.702	0.676
AT3G13610	F6H1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.702	0.793

AT2G16750		Protein kinase protein with adenine nucleotide alpha hydrolases-like domain	0.701	0.640
AT5G17820		Peroxidase superfamily protein	0.699	0.650
AT5G10580		Protein of unknown function, DUF599	0.699	0.663
AT3G54040		PAR1 protein	0.698	0.733
AT4G15500		UDP-Glycosyltransferase superfamily protein	0.697	0.680
AT5G10280		myb domain protein 92	0.697	0.662
AT1G73270		serine carboxypeptidase-like 6	0.697	0.747
AT3G58710		WRKY DNA-binding protein 69	0.695	0.796
AT1G51800		Leucine-rich repeat protein kinase family protein	0.694	0.671
AT5G51060		NADPH/respiratory burst oxidase protein D	0.693	0.821
AT4G20110	VSR7	VACUOLAR SORTING RECEPTOR 7	0.690	0.731
AT5G57740		XB3 ortholog 2 in Arabidopsis thaliana	0.689	0.650
AT5G67400		root hair specific 19	0.688	0.659
AT1G13300		myb-like transcription factor family protein	0.688	0.726
AT4G25090		Riboflavin synthase-like superfamily protein	0.686	0.680
AT3G06300		P4H isoform 2	0.686	0.805
AT4G15093		catalytic LigB subunit of aromatic ring-opening dioxygenase family	0.684	0.738
AT1G03210		Phenazine biosynthesis PhzC/PhzF protein	0.683	0.765
AT5G52830		WRKY DNA-binding protein 27	0.683	0.737
AT3G13710		prenylated RAB acceptor 1.F4	0.683	0.630
AT1G15210		pleiotropic drug resistance 7	0.680	0.823
AT2G28970		Leucine-rich repeat protein kinase family protein	0.678	0.663
AT1G61590		Protein kinase superfamily protein	0.678	0.612
AT5G44480		NAD(P)-binding Rossmann-fold superfamily protein	0.678	0.734
AT4G02270		root hair specific 13	0.676	0.628
AT4G16350		calcineurin B-like protein 6	0.676	0.673
AT1G30110		nudix hydrolase homolog 25	0.675	0.700
AT1G29280	WRKY65	WRKY DNA-binding protein 65	0.675	0.785
AT3G60330	HA7	H(+)-ATPase 7	0.675	0.719
AT5G50560		Protein of unknown function (DUF 3339)	0.675	0.619
AT5G01610		Protein of unknown function, DUF538	0.674	0.632
AT5G25160		zinc finger protein 3	0.674	0.673
AT4G21230		cysteine-rich RLK (RECEPTOR-like protein kinase) 27	0.673	0.696
AT5G63600	FLS5	flavonol synthase 5	0.672	0.672
AT5G40980		Protein of unknown function (DUF 3339)	0.671	0.659
AT5G53250		arabinogalactan protein 22	0.671	0.688
AT1G49390		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.671	0.694
AT2G42350		RING/U-box superfamily protein	0.669	0.680
AT4G23690		Disease resistance-responsive (dirigent-like protein) family protein	0.669	0.688
AT1G78090		trehalose-6-phosphate phosphatase	0.669	0.618
AT5G26655		Unknown	0.668	0.633
AT1G26250		Proline-rich extensin-like family protein	0.668	0.695

AT4G11290		Peroxidase superfamily protein	0.667	0.695
AT5G06800		myb-like HTH transcriptional regulator family protein	0.667	0.645
AT3G27190		uridine kinase-like 2	0.667	0.799
AT4G35380		SEC7-like guanine nucleotide exchange family protein	0.667	0.850
AT1G77330		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.666	0.767
AT2G34940		VACUOLAR SORTING RECEPTOR 5	0.665	0.670
AT3G58810	MTPA2	metal tolerance protein A2	0.664	0.643
AT1G08670		ENTH/VHS family protein	0.664	0.624
AT5G06840		Unknown	0.663	0.717
AT5G24230		Lipase class 3-related protein	0.663	0.691
AT1G30870		Peroxidase superfamily protein	0.663	0.623
AT1G74660		mini zinc finger 1	0.663	0.726
AT3G22570		Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	0.662	0.620
AT1G33800		Protein of unknown function (DUF579)	0.662	0.761
AT1G67330		Protein of unknown function (DUF579)	0.662	0.654
AT4G32650		potassium channel in Arabidopsis thaliana 3	0.661	0.766
AT4G30670		Putative membrane lipoprotein	0.660	0.665
AT3G23430		phosphate 1	0.658	0.613
AT4G00700		C2 calcium/lipid-binding plant phosphoribosyltransferase family protein	0.658	0.631
AT1G72300		Leucine-rich receptor-like protein kinase family protein	0.657	0.639
AT3G15300		VQ motif-containing protein	0.656	0.795
AT1G11540		Sulfite exporter TauE/SafE family protein	0.656	0.689
AT3G47040		Glycosyl hydrolase family protein	0.656	0.704
AT3G05920		Heavy metal transport/detoxification superfamily protein	0.656	0.674
AT5G05500		Pollen Ole e 1 allergen and extensin family protein	0.655	0.628
AT5G05790		Duplicated homeodomain-like superfamily protein	0.654	0.657
AT2G29750		UDP-glucosyl transferase 71C1	0.654	0.684
AT1G68150		WRKY DNA-binding protein 9	0.652	0.815
AT5G61340		Unknown	0.652	0.633
AT1G76760		thioredoxin Y1	0.651	0.770
AT4G18430		RAB GTPase homolog A1E	0.651	0.755
AT5G26310		UDP-Glycosyltransferase superfamily protein	0.650	0.642
AT1G02900		rapid alkalinization factor 1	0.650	0.650
AT5G42860		Unknown	0.649	0.683
AT5G15180		Peroxidase superfamily protein	0.649	0.626
AT3G61410		Unknown	0.649	0.632
AT2G28160	FIT	FER-like regulator of iron uptake	0.649	0.748
AT4G11530		cysteine-rich RLK (RECEPTOR-like protein kinase) 34	0.647	0.690
AT3G14280		Unknown	0.647	0.652
AT1G72120		Major facilitator superfamily protein	0.647	0.741
AT4G18610		Protein of unknown function (DUF640)	0.646	0.623

AT1G09090	respiratory burst oxidase homolog B	0.646	0.622
AT3G01720	Unknown	0.645	0.732
AT4G13235	embryo sac development arrest 21	0.644	0.646
AT5G67450	zinc-finger protein 1	0.643	0.694
AT2G03450	purple acid phosphatase 9	0.643	0.692
AT5G27920	F-box family protein	0.643	0.733
AT2G32270	ZIP3 zinc transporter 3 precursor	0.643	0.665
AT4G08690	Sec14p-like phosphatidylinositol transfer family protein	0.642	0.710
AT1G68940	Armadillo/beta-catenin-like repeat family protein	0.641	0.697
AT4G17800	Predicted AT-hook DNA-binding family protein	0.641	0.745
AT5G64100	Peroxidase superfamily protein	0.641	0.665
AT3G21710	Unknown	0.640	0.711
AT1G01750	actin depolymerizing factor 11	0.640	0.691
AT3G27150	Galactose oxidase/kelch repeat superfamily protein	0.639	0.758
AT3G23200	Uncharacterised protein family (UPF0497)	0.639	0.709
AT5G25890	indole-3-acetic acid inducible 28	0.638	0.685
AT5G48560	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	0.638	0.801
AT1G65610	Six-hairpin glycosidases superfamily protein	0.638	0.727
AT4G22212	Arabidopsis defensin-like protein	0.637	0.616
AT5G09690	magnesium transporter 7	0.636	0.761
AT1G49860	glutathione S-transferase (class phi) 14	0.636	0.640
AT1G51850	Leucine-rich repeat protein kinase family protein	0.635	0.662
AT3G05890	Low temperature and salt responsive protein family	0.634	0.720
AT3G22850	Aluminium induced protein with YGL and LRDR motifs	0.634	0.791
AT1G12010	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.633	0.695
AT5G59090	subtilase 4.12	0.633	0.610
AT4G30170	Peroxidase family protein	0.633	0.663
AT1G47840	hexokinase 3	0.631	0.756
AT3G09490	Tetratricopeptide repeat (TPR)-like superfamily protein	0.631	0.711
AT1G08990	plant glycogenin-like starch initiation protein 5	0.631	0.621
AT4G10380	NOD26-like intrinsic protein 5;1	0.630	0.721
AT2G37440	DNAse I-like superfamily protein	0.629	0.667
AT5G40730	arabinogalactan protein 24	0.629	0.605
AT4G20140	Leucine-rich repeat transmembrane protein kinase	0.629	0.693
AT2G30210	laccase 3	0.629	0.612
AT2G02960	RING/FYVE/PHD zinc finger superfamily protein	0.628	0.694
AT1G14080	fucosyltransferase 6	0.627	0.769
AT1G09740	Adenine nucleotide alpha hydrolases-like superfamily protein	0.627	0.701
AT5G65210	bZIP transcription factor family protein	0.627	0.695

AT4G26060		Ribosomal protein L18ae family	0.627	0.721
AT5G54140		IAA-leucine-resistant (ILR1)-like 3	0.626	0.740
AT1G70690		Receptor-like protein kinase-related family protein	0.626	0.640
AT1G51860		Leucine-rich repeat protein kinase family protein	0.626	0.684
AT3G18560		Unknown	0.626	0.659
AT2G39420		alpha/beta-Hydrolases superfamily protein	0.625	0.775
AT3G22600		Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	0.625	0.666
AT3G60070		Major facilitator superfamily protein	0.625	0.656
AT1G70990		proline-rich family protein	0.625	0.613
AT5G53110		RING/U-box superfamily protein	0.624	0.640
AT3G11550		Uncharacterised protein family (UPF0497)	0.623	0.607
AT5G20550		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.622	0.714
AT1G13970		Protein of unknown function (DUF1336)	0.622	0.746
AT3G62780		Calcium-dependent lipid-binding (CaLB domain) family protein	0.622	0.631
AT2G34830		WRKY DNA-binding protein 35	0.621	0.731
AT3G07570		Cytochrome b561/ferric reductase transmembrane with DOMON related domain	0.621	0.648
AT2G38390		Peroxidase superfamily protein	0.621	0.603
AT2G21550		Bifunctional dihydrofolate reductase/thymidylate synthase	0.620	0.760
AT3G04570		AT-hook motif nuclear-localized protein 19	0.620	0.746
AT1G12160		Flavin-binding monooxygenase family protein	0.620	0.755
AT5G25640		Rhomboid-related intramembrane serine protease family protein	0.620	0.738
AT1G66280		Glycosyl hydrolase superfamily protein	0.620	0.748
AT5G63990		Inositol monophosphatase family protein	0.620	0.809
AT1G30750		Unknown	0.619	0.613
AT1G69450		Early-responsive to dehydration stress protein (ERD4)	0.619	0.716
AT1G18970	GLP9	germin-like protein 4	0.619	0.610
AT4G14630		germin-like protein 9	0.618	0.722
AT3G49840		Uncharacterized conserved protein (DUF2215)	0.618	0.686
AT2G04430		nudix hydrolase homolog 5	0.616	0.657
AT3G19390		Granulin repeat cysteine protease family protein	0.616	0.843
AT3G21770		Peroxidase superfamily protein	0.614	0.631
AT3G47210		Plant protein of unknown function (DUF247)	0.613	0.706
AT1G66440		Cysteine/Histidine-rich C1 domain family protein	0.613	0.632
AT5G50200		nitrate transmembrane transporters	0.613	0.677
AT3G62280		GDSL-like Lipase/Acylhydrolase superfamily protein	0.612	0.719

AT3G20960	cytochrome P450, family 705, subfamily A, polypeptide 33	0.612	0.691
AT1G47290	3beta-hydroxysteroid-dehydrogenase/decarboxylase isoform 1	0.611	0.666
AT2G36890	Duplicated homeodomain-like superfamily protein	0.610	0.623
AT4G15070	Cysteine/Histidine-rich C1 domain family protein	0.610	0.673
AT4G13580	Disease resistance-responsive (dirigent-like protein) family protein	0.609	0.602
AT2G28670	Disease resistance-responsive (dirigent-like protein) family protein	0.609	0.603
AT2G27660	Cysteine/Histidine-rich C1 domain family protein	0.609	0.712
AT3G56950	small and basic intrinsic protein 2;1	0.608	0.683
AT2G30840	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.607	0.636
AT1G63220	Calcium-dependent lipid-binding (CaLB domain) family protein	0.607	0.685
AT3G26770	NAD(P)-binding Rossmann-fold superfamily protein	0.606	0.722
AT1G79530	glyceraldehyde-3-phosphate dehydrogenase of plastid 1	0.606	0.775
AT5G45480	Protein of unknown function (DUF594)	0.606	0.658
AT4G13660	pinorensin reductase 2	0.606	0.642
AT1G28680	HXXXD-type acyl-transferase family protein	0.605	0.672
AT2G41480	Peroxidase superfamily protein	0.604	0.636
AT2G16970	Major facilitator superfamily protein	0.604	0.607
AT5G12880	proline-rich family protein	0.603	0.762
AT4G15380	cytochrome P450, family 705, subfamily A, polypeptide 4	0.602	0.615
AT5G60950	COBRA-like protein 5 precursor	0.602	0.772
AT4G27270	Quinone reductase family protein	0.601	0.606
AT4G13130	Cysteine/Histidine-rich C1 domain family protein	0.601	0.719
AT2G18980	Peroxidase superfamily protein	0.601	0.605

Genes co-expressed only with BTSL1

AT5G19970	Unknown	0.705
AT1G72200	RING/U-box superfamily protein	0.695
AT4G19690	IRT1 iron-regulated transporter 1	0.687
AT3G01260	Galactose mutarotase-like superfamily protein	0.667
AT4G15390	HXXXD-type acyl-transferase family protein	0.661
AT1G62280	SLAC1 homologue 1	0.656
AT2G48130	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	0.653
AT4G30450	glycine-rich protein	0.647
AT4G00680	actin depolymerizing factor 8	0.642
AT3G09290	telomerase activator1	0.641

AT3G61380		Phosphatidylinositol N-acetylglucosaminyltransferase subunit P-related	0.639
AT4G31910		HXXXD-type acyl-transferase family protein	0.635
AT1G13830		Carbohydrate-binding X8 domain superfamily protein	0.634
AT1G48690		Auxin-responsive GH3 family protein	0.633
AT1G08590		Leucine-rich receptor-like protein kinase family protein	0.631
AT2G36100		Uncharacterised protein family (UPF0497)	0.631
AT2G27370		Uncharacterised protein family (UPF0497)	0.629
AT1G31050		basic helix-loop-helix (bHLH) DNA-binding superfamily protein	0.628
AT5G56540		arabinogalactan protein 14	0.627
AT5G24410		6-phosphogluconolactonase 4	0.626
AT1G34040		Pyridoxal phosphate (PLP)-dependent transferases superfamily protein	0.624
AT4G16400		Unknown	0.622
AT5G27100		glutamate receptor 2.1	0.621
AT4G12550		Auxin-Induced in Root cultures 1	0.619
AT4G26320		arabinogalactan protein 13	0.618
AT4G29800		PATATIN-like protein 8	0.618
AT5G07080		HXXXD-type acyl-transferase family protein	0.616
AT3G26610		Pectin lyase-like superfamily protein	0.615
AT4G22120		ERD (early-responsive to dehydration stress) family protein	0.614
AT4G17280		Auxin-responsive family protein	0.612
AT5G65970		Seven transmembrane MLO family protein	0.611
AT1G12080		Vacuolar calcium-binding protein-related	0.610
AT4G07820		CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein	0.608
AT1G68710		ATPase E1-E2 type family protein / haloacid dehalogenase-like hydrolase family protein	0.608
AT3G45920		Protein kinase superfamily protein	0.608
AT3G56980	bHLH39	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	0.607
AT1G77690		like AUX1 3	0.607
AT5G25810		Integrase-type DNA-binding superfamily protein	0.605
AT1G32450	NRT1.5	nitrate transporter 1.5	0.604
AT2G37180		Aquaporin-like superfamily protein	0.603
AT2G29660		zinc finger (C2H2 type) family protein	0.603
AT3G49750		receptor like protein 44	0.603
AT5G42500		Disease resistance-responsive (dirigent-like protein) family protein	0.602
AT5G56080	NAS2	nicotianamine synthase 2	0.600

Genes co-expressed only with BTSL2

AT5G05460		Glycosyl hydrolase family 85	0.817
AT5G43060		Granulin repeat cysteine protease family protein	0.790
AT4G39270		Leucine-rich repeat protein kinase family protein	0.789
AT4G26470		Calcium-binding EF-hand family protein	0.786
AT2G43610		Chitinase family protein	0.782
AT2G17720		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.780
AT5G15130		WRKY DNA-binding protein 72	0.779
AT5G17330		glutamate decarboxylase	0.778
AT1G29330		ER lumen protein retaining receptor family protein	0.775
AT3G51330		Eukaryotic aspartyl protease family protein	0.769
AT2G35000		RING/U-box superfamily protein	0.767
AT2G39200		Seven transmembrane MLO family protein	0.767
AT5G58730		pfkB-like carbohydrate kinase family protein	0.766
AT5G62260		AT hook motif DNA-binding family protein	0.765
AT4G12080		AT-hook motif nuclear-localized protein 1	0.764
AT2G44380		Cysteine/Histidine-rich C1 domain family protein	0.762
AT2G24570		WRKY DNA-binding protein 17	0.762
AT5G06750		Protein phosphatase 2C family protein	0.762
AT2G41220		glutamate synthase 2	0.761
AT5G63930		Leucine-rich repeat protein kinase family protein	0.755
AT2G46140		Late embryogenesis abundant protein	0.753
AT1G74030		enolase 1	0.753
AT5G10820		Major facilitator superfamily protein	0.751
AT3G09940		monodehydroascorbate reductase	0.746
AT2G31390		pfkB-like carbohydrate kinase family protein	0.745
AT4G38830	CRK26	cysteine-rich RLK (RECEPTOR-like protein kinase) 26	0.744
AT2G24610		cyclic nucleotide-gated channel 14	0.743
AT3G60450		Phosphoglycerate mutase family protein	0.742
AT3G20510		Transmembrane proteins 14C	0.737
AT2G16595		Translocon-associated protein (TRAP), alpha subunit	0.735
AT1G50560		cytochrome P450, family 705, subfamily A, polypeptide 25	0.735
AT4G35500		Protein kinase superfamily protein	0.734
AT2G17290		Calcium-dependent protein kinase family protein	0.734
AT4G21850		methionine sulfoxide reductase B9	0.732
AT5G44610		microtubule-associated protein 18	0.731
AT1G55730		cation exchanger 5	0.731
AT5G04860		Unknown	0.731
AT3G57480		zinc finger (C2H2 type, AN1-like) family protein	0.731
AT2G28890		poltergeist like 4	0.730
AT4G28890		RING/U-box superfamily protein	0.727

AT1G02530	P-glycoprotein 12	0.725
AT3G24240	Leucine-rich repeat receptor-like protein kinase family protein	0.724
AT3G03610	ELMO/CED-12 family protein	0.724
AT2G20780	Major facilitator superfamily protein	0.723
AT1G71400	receptor like protein 12	0.723
AT5G54780	Ypt/Rab-GAP domain of gyp1p superfamily protein	0.721
AT5G43370	phosphate transporter 2	0.719
AT4G39390	nucleotide sugar transporter-KT 1	0.719
AT3G11340	UDP-Glycosyltransferase superfamily protein	0.718
AT1G69810	WRKY DNA-binding protein 36	0.718
AT5G58940	calmodulin-binding receptor-like cytoplasmic kinase 1	0.717
AT1G07630	pol-like 5	0.717
AT3G51440	Calcium-dependent phosphotriesterase superfamily protein	0.717
AT3G01220	homeobox protein 20	0.716
AT5G02100	Oxysterol-binding family protein	0.715
AT4G15400	HXXXD-type acyl-transferase family protein	0.715
AT3G54960	PDI-like 1-3	0.714
AT2G27000	cytochrome P450, family 705, subfamily A, polypeptide 8	0.713
AT3G05580	Calcineurin-like metallo-phosphoesterase superfamily protein	0.713
AT5G10720	histidine kinase 5	0.712
AT5G41990	with no lysine (K) kinase 8	0.710
AT2G28960	Leucine-rich repeat protein kinase family protein	0.710
AT5G39970	catalytics	0.708
AT5G58700	phosphatidylinositol-speciwc phospholipase C4	0.707
AT5G43520	Cysteine/Histidine-rich C1 domain family protein	0.707
AT5G14150	Protein of unknown function, DUF642	0.705
AT4G30850	heptahelical transmembrane protein2	0.704
AT2G47140	NAD(P)-binding Rossmann-fold superfamily protein	0.704
AT5G18780	F-box/RNI-like superfamily protein	0.703
AT1G49050	Eukaryotic aspartyl protease family protein	0.703
AT2G42060	Cysteine/Histidine-rich C1 domain family protein	0.702
AT3G53780	RHOMBOLD-like protein 4	0.701
AT3G13320	cation exchanger 2	0.701
AT2G35120	Single hybrid motif superfamily protein	0.701
AT2G46620	P-loop containing nucleoside triphosphate hydrolases superfamily protein	0.700
AT5G24270	Calcium-binding EF-hand family protein	0.700
AT3G49860	ADP-ribosylation factor-like A1B	0.700
AT2G44450	beta glucosidase 15	0.700
AT3G11320	Nucleotide-sugar transporter family protein	0.700

AT1G02360		Chitinase family protein	0.700
AT4G08770		Peroxidase superfamily protein	0.698
AT2G39410		alpha/beta-Hydrolases superfamily protein	0.698
AT2G20010		Protein of unknown function (DUF810)	0.698
AT4G12010		Disease resistance protein (TIR-NBS-LRR class) family	0.697
AT1G21370		Unknown	0.697
AT5G59680		Leucine-rich repeat protein kinase family protein	0.697
AT1G29180		Cysteine/Histidine-rich C1 domain family protein	0.696
AT5G19520		mechanosensitive channel of small conductance-like 9	0.696
AT4G32870		Polyketide cyclase/dehydrase and lipid transport superfamily protein	0.696
AT5G41180		leucine-rich repeat transmembrane protein kinase family protein	0.696
AT5G01040		laccase 8	0.695
AT5G12970		Calcium-dependent lipid-binding (CaLB domain) plant phosphoribosyltransferase family protein	0.695
AT5G59930		Cysteine/Histidine-rich C1 domain family protein	0.694
AT3G21510		histidine-containing phosphotransmitter 1	0.694
AT3G53480	PDR9	pleiotropic drug resistance 9	0.693
AT1G23850		Unknown	0.693
AT1G19360		Nucleotide-diphospho-sugar transferase family protein	0.692
AT5G24290		Vacuolar iron transporter (VIT) family protein	0.690
AT1G50580		UDP-Glycosyltransferase superfamily protein	0.689
AT5G03630		Pyridine nucleotide-disulphide oxidoreductase family protein	0.689
AT2G14100		cytochrome P450, family 705, subfamily A, polypeptide 13	0.688
AT1G63460		glutathione peroxidase 8	0.688
AT1G17500		ATPase E1-E2 type family protein / haloacid dehalogenase-like hydrolase family protein	0.688
AT2G44370		Cysteine/Histidine-rich C1 domain family protein	0.687
AT5G18860		inosine-uridine preferring nucleoside hydrolase family protein	0.686
AT5G60280		Concanavalin A-like lectin protein kinase family protein	0.684
AT4G01750		rhamnogalacturonan xylosyltransferase 2	0.683
AT1G32120		Unknown	0.683
AT2G30340		LOB domain-containing protein 13	0.683
AT4G18340		Glycosyl hydrolase superfamily protein	0.683
AT2G21850		Cysteine/Histidine-rich C1 domain family protein	0.681
AT3G02880		Leucine-rich repeat protein kinase family protein	0.681
AT3G52200		Dihydrolipoamide acetyltransferase, long form protein	0.681

AT1G75390	basic leucine-zipper 44	0.680
AT3G48410	alpha/beta-Hydrolases superfamily protein	0.680
AT2G16570	GLN phosphoribosyl pyrophosphate amidotransferase 1	0.680
AT5G25770	alpha/beta-Hydrolases superfamily protein	0.680
AT5G14000	NAC domain containing protein 84	0.680
AT5G38630	cytochrome B561-1	0.679
AT1G07520	GRAS family transcription factor	0.679
AT1G08350	Endomembrane protein 70 protein family	0.678
AT1G71010	FORMS APLOID AND BINUCLEATE CELLS 1C	0.678
AT2G37980	O-fucosyltransferase family protein	0.678
AT1G56145	Leucine-rich repeat transmembrane protein kinase	0.678
AT2G02680	Cysteine/Histidine-rich C1 domain family protein	0.677
AT3G53670	Unknown	0.676
AT4G21830	methionine sulfoxide reductase B7	0.676
AT1G54010	GDSSL-like Lipase/Acylhydrolase superfamily protein	0.676
AT5G17060	ADP-ribosylation factor B1B	0.676
AT1G55380	Cysteine/Histidine-rich C1 domain family protein	0.676
AT5G51160	Ankyrin repeat family protein	0.675
AT5G01830	ARM repeat superfamily protein	0.675
AT2G31030	OSBP(oxysterol binding protein)-related protein 1B	0.674
AT4G01700	Chitinase family protein	0.674
AT5G05640	nucleoprotein-related	0.673
AT1G79450	ALA-interacting subunit 5	0.673
AT5G13420	Aldolase-type TIM barrel family protein	0.673
AT4G32140	EamA-like transporter family	0.673
AT1G54890	Late embryogenesis abundant (LEA) protein-related	0.673
AT1G17060	cytochrome p450 72c1	0.673
AT3G17420	glyoxysomal protein kinase 1	0.672
AT4G21410	cysteine-rich RLK (RECEPTOR-like protein kinase) 29	0.672
AT1G67940	non-intrinsic ABC protein 3	0.672
AT3G52450	plant U-box 22	0.672
AT3G05990	Leucine-rich repeat (LRR) family protein	0.672
AT1G47600	beta glucosidase 34	0.672
AT5G66690	UDP-Glycosyltransferase superfamily protein	0.671
AT3G45650	nitrate excretion transporter1	0.671
AT5G57090	Auxin efflux carrier family protein	0.671
AT1G15670	Galactose oxidase/kelch repeat superfamily protein	0.671
AT5G45510	Leucine-rich repeat (LRR) family protein	0.671
AT2G45510	cytochrome P450, family 704, subfamily A, polypeptide 2	0.670
AT5G42010	Transducin/WD40 repeat-like superfamily protein	0.670
AT3G04060	NAC domain containing protein 46	0.669

AT1G14780	MAC/Perforin domain-containing protein	0.669
AT3G21230	4-coumarate:CoA ligase 5	0.669
AT2G47000	ATP binding cassette subfamily B4	0.669
AT1G24180	Thiamin diphosphate-binding fold (THDP-binding) superfamily protein	0.668
AT5G54670	kinesin 3	0.668
AT2G27510	ferredoxin 3	0.668
AT3G26470	Powdery mildew resistance protein, RPW8 domain	0.668
AT4G33360	NAD(P)-binding Rossmann-fold superfamily protein	0.668
AT5G16910	cellulose-synthase like D2	0.667
AT1G20900	Predicted AT-hook DNA-binding family protein	0.667
AT1G62790	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	0.667
AT1G05700	Leucine-rich repeat transmembrane protein kinase protein	0.667
AT2G16760	Calcium-dependent phosphotriesterase superfamily protein	0.666
AT1G30510	root FNR 2	0.666
AT5G60300	Concanavalin A-like lectin protein kinase family protein	0.666
AT2G05260	alpha/beta-Hydrolases superfamily protein	0.666
AT2G17130	isocitrate dehydrogenase subunit 2	0.665
AT1G31470	Major facilitator superfamily protein	0.664
AT5G49680	Golgi-body localisation protein domain ;RNA pol II promoter Fmp27 protein domain	0.663
AT5G11230	Nucleotide-sugar transporter family protein	0.663
AT5G12200	pyrimidine 2	0.662
AT5G44790	copper-exporting ATPase / responsive-to-antagonist 1 / copper-transporting ATPase (RAN1)	0.661
AT2G44790	uclacyanin 2	0.661
AT3G63010	alpha/beta-Hydrolases superfamily protein	0.661
AT1G70170	matrix metalloproteinase	0.660
AT2G24170	Endomembrane protein 70 protein family	0.660
AT3G46280	protein kinase-related	0.660
AT3G03520	non-specific phospholipase C3	0.660
AT2G35610	xyloglucanase 113	0.659
AT1G15110	phosphatidyl serine synthase family protein	0.659
AT5G13110	glucose-6-phosphate dehydrogenase 2	0.659
AT2G34020	Calcium-binding EF-hand family protein	0.658
AT3G12090	tetraspanin6	0.657
AT1G15380	Lactoylglutathione lyase / glyoxalase I family protein	0.657
AT5G39990	Core-2/l-branching beta-1,6-N-acetylglucosaminyltransferase family protein	0.656
AT1G05620	uridine-ribohydrolase 2	0.656

AT1G64530		Plant regulator RWP-RK family protein	0.656
AT2G24550		Unknown	0.656
AT5G01620		TRICHOME BIREFRINGENCE-LIKE 35	0.655
AT1G24320		Six-hairpin glycosidases superfamily protein	0.654
AT5G10740		Protein phosphatase 2C family protein	0.654
AT3G14470		NB-ARC domain-containing disease resistance protein	0.654
AT1G30650		WRKY DNA-binding protein 14	0.654
AT5G65020		annexin 2	0.654
AT5G66280		GDP-D-mannose 4,6-dehydratase 1	0.653
AT3G09805		Unknown	0.652
AT3G14570		glucan synthase-like 4	0.651
AT2G45330		RNA 2'-phosphotransferase, Tpt1 / KptA family	0.651
AT3G25290		Auxin-responsive family protein	0.651
AT2G39110		Protein kinase superfamily protein	0.651
AT5G42630		Homeodomain-like superfamily protein	0.651
AT2G23300		Leucine-rich repeat protein kinase family protein	0.651
AT2G42570		TRICHOME BIREFRINGENCE-LIKE 39	0.651
AT1G66450		Cysteine/Histidine-rich C1 domain family protein	0.650
AT4G31320		SAUR-like auxin-responsive protein family	0.650
AT3G14940	PPC3	phosphoenolpyruvate carboxylase 3	0.649
AT5G64370		beta-ureidopropionase	0.649
AT5G65530		Protein kinase superfamily protein	0.649
AT2G47550		Plant invertase/pectin methylesterase inhibitor superfamily	0.648
AT5G40760	G6PD6	glucose-6-phosphate dehydrogenase 6	0.648
AT5G07820		Plant calmodulin-binding protein-related	0.648
AT5G05880		UDP-Glycosyltransferase superfamily protein	0.648
AT2G16530		3-oxo-5-alpha-steroid 4-dehydrogenase family protein	0.648
AT5G59940		Cysteine/Histidine-rich C1 domain family protein	0.648
AT2G26650		K ⁺ transporter 1	0.647
AT1G61360		S-locus lectin protein kinase family protein	0.647
AT2G26480		UDP-glucosyl transferase 76D1	0.647
AT4G23010		UDP-galactose transporter 2	0.647
AT4G02180		DC1 domain-containing protein	0.646
AT1G29020		Calcium-binding EF-hand family protein	0.646
AT5G35580		Protein kinase superfamily protein	0.646
AT1G64060		respiratory burst oxidase protein F	0.645
AT3G54100		O-fucosyltransferase family protein	0.645
AT5G16510		Alpha-1,4-glucan-protein synthase family protein	0.645
AT4G28650		Leucine-rich repeat transmembrane protein kinase family protein	0.645
AT5G02420		Unknown	0.645
AT5G44380		FAD-binding Berberine family protein	0.645
AT5G62150		peptidoglycan-binding LysM domain-containing protein	0.644

AT1G30270		CBL-interacting protein kinase 23	0.644
AT3G15430		Regulator of chromosome condensation (RCC1) family protein	0.643
AT3G49210		O-acyltransferase (WSD1-like) family protein	0.643
AT1G34750		Protein phosphatase 2C family protein	0.643
AT2G01140		Aldolase superfamily protein	0.643
AT5G07390	RBOHA	respiratory burst oxidase homolog A	0.642
AT3G44320		nitrilase 3	0.642
AT5G43910		pfkB-like carbohydrate kinase family protein	0.642
AT2G37280		pleiotropic drug resistance 5	0.642
AT3G26440		Protein of unknown function (DUF707)	0.642
AT2G44010		Unknown	0.642
AT1G80360		Pyridoxal phosphate (PLP)-dependent transferases superfamily protein	0.641
AT1G54320		LEM3 (ligand-effect modulator 3) family protein / CDC50 family protein	0.641
AT2G47650		UDP-xylose synthase 4	0.640
AT1G01640		BTB/POZ domain-containing protein	0.639
AT2G14510		Leucine-rich repeat protein kinase family protein	0.639
AT3G14610		cytochrome P450, family 72, subfamily A, polypeptide 7	0.639
AT2G04100		MATE efflux family protein	0.639
AT5G35370		S-locus lectin protein kinase family protein	0.639
AT2G44350		Citrate synthase family protein	0.638
AT1G71697		choline kinase 1	0.638
AT5G16900		Leucine-rich repeat protein kinase family protein	0.638
AT1G07260		UDP-glucosyl transferase 71C3	0.638
AT5G20400		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.638
AT5G58375		Methyltransferase-related protein	0.637
AT1G80460		Actin-like ATPase superfamily protein	0.637
AT5G43390		Uncharacterised conserved protein UCP015417, vWA	0.637
AT5G15730		Protein kinase superfamily protein	0.637
AT1G02810		Plant invertase/pectin methylesterase inhibitor superfamily	0.636
AT2G17370		3-hydroxy-3-methylglutaryl-CoA reductase 2	0.636
AT1G01340		cyclic nucleotide gated channel 10	0.635
AT5G11970		Protein of unknown function (DUF3511)	0.635
AT5G14690		Unknown	0.635
AT3G03050		cellulose synthase-like D3	0.635
AT4G22130		STRUBBELIG-receptor family 8	0.635
AT5G26595		Unknown	0.634
AT1G14210		Ribonuclease T2 family protein	0.633
AT1G51890		Leucine-rich repeat protein kinase family protein	0.633
AT3G16340		pleiotropic drug resistance 1	0.633
AT3G47780		ABC2 homolog 6	0.633

AT3G51460		Phosphoinositide phosphatase family protein	0.633
AT3G15240		Serine/threonine-protein kinase WNK (With No Lysine)-related	0.633
AT1G53920		GDSL-motif lipase 5	0.632
AT4G36990	HSFB1	heat shock factor 4	0.632
AT5G11870		Alkaline phytoceramidase (aPHC)	0.631
AT4G23480		Unknown	0.631
AT1G78000		sulfate transporter 1;2	0.631
AT5G01320		Thiamine pyrophosphate dependent pyruvate decarboxylase family protein	0.631
AT1G26440		ureide permease 5	0.631
AT1G74210		PLC-like phosphodiesterases superfamily protein	0.630
AT5G60760		P-loop containing nucleoside triphosphate hydrolases superfamily protein	0.630
AT1G74500		activation-tagged BRI1(brassinosteroid-insensitive 1)-suppressor 1	0.630
AT1G07000		exocyst subunit exo70 family protein B2	0.630
AT1G02690		importin alpha isoform 6	0.630
AT3G13772		transmembrane nine 7	0.629
AT2G33630		NAD(P)-binding Rossmann-fold superfamily protein	0.629
AT4G37010		centrin 2	0.629
AT3G27000		actin related protein 2	0.629
AT3G21240		4-coumarate:CoA ligase 2	0.628
AT5G40590		Cysteine/Histidine-rich C1 domain family protein	0.628
AT5G65790		myb domain protein 68	0.628
AT3G07690		6-phosphogluconate dehydrogenase family protein	0.627
AT2G18450		succinate dehydrogenase 1-2	0.626
AT2G44500		O-fucosyltransferase family protein	0.626
AT3G59660		C2 domain-containing protein / GRAM domain-containing protein	0.626
AT3G50960		phosducin-like protein 3 homolog	0.626
AT1G63440		heavy metal atpase 5	0.626
AT5G10520		ROP binding protein kinases 1	0.625
AT5G58750		NAD(P)-binding Rossmann-fold superfamily protein	0.625
AT1G18270		ketose-bisphosphate aldolase class-II family protein	0.625
AT3G51350		Eukaryotic aspartyl protease family protein	0.625
AT5G63660		Scorpion toxin-like knottin superfamily protein	0.625
AT5G53320		Leucine-rich repeat protein kinase family protein	0.624
AT5G12340		Unknown	0.624
AT3G51360		Eukaryotic aspartyl protease family protein	0.623
AT4G19185		nodulin MtN21 /EamA-like transporter family protein	0.622

AT1G10050		glycosyl hydrolase family 10 protein / carbohydrate-binding domain-containing protein	0.622
AT1G25580		NAC (No Apical Meristem) domain transcriptional regulator superfamily protein	0.622
AT5G40690		Unknown	0.621
AT2G46700		CDPK-related kinase 3	0.621
AT1G76550		Phosphofructokinase family protein	0.621
AT2G43080		P4H isoform 1	0.621
AT2G29330		tropinone reductase	0.621
AT5G03290		isocitrate dehydrogenase V	0.621
AT5G40230		nodulin MtN21 /EamA-like transporter family protein	0.621
AT1G30720		FAD-binding Berberine family protein	0.621
AT1G78050		phosphoglycerate/bisphosphoglycerate mutase	0.620
AT1G79030		Chaperone DnaJ-domain superfamily protein	0.620
AT1G17430		alpha/beta-Hydrolases superfamily protein	0.620
AT1G48930		glycosyl hydrolase 9C1	0.620
AT3G58600		Adaptin ear-binding coat-associated protein 1 NECAP-1	0.619
AT5G23720		dual specificity protein phosphatase family protein	0.619
AT5G01490	CAX4	cation exchanger 4	0.619
AT3G07600		Heavy metal transport/detoxification superfamily protein	0.619
AT3G51160		NAD(P)-binding Rossmann-fold superfamily protein	0.619
AT5G62480	GSTU9	glutathione S-transferase tau 9	0.619
AT5G56610		Phosphotyrosine protein phosphatases superfamily protein	0.618
AT3G25585		aminoalcoholphosphotransferase	0.618
AT1G67800		Copine (Calcium-dependent phospholipid-binding protein) family	0.618
AT2G41660	MIZ1	Protein of unknown function, DUF617	0.618
AT1G11580		methylesterase PCR A	0.618
AT3G49390		CTC-interacting domain 10	0.618
AT1G26970		Protein kinase superfamily protein	0.618
AT3G02360		6-phosphogluconate dehydrogenase family protein	0.617
AT3G57330		autoinhibited Ca ²⁺ -ATPase 11	0.617
AT2G42490		Copper amine oxidase family protein	0.616
AT5G56630		phosphofructokinase 7	0.616
AT4G02730		Transducin/WD40 repeat-like superfamily protein	0.616
AT5G44460		calmodulin like 43	0.616
AT5G11920		6-&1-fructan exohydrolase	0.615
AT3G04780		Protein of unknown function (DUF1000)	0.615
AT1G12290		Disease resistance protein (CC-NBS-LRR class) family	0.615
AT5G35200		ENTH/ANTH/VHS superfamily protein	0.615
AT5G41040		HXXXD-type acyl-transferase family protein	0.615

AT1G06840	Leucine-rich repeat protein kinase family protein	0.614
AT5G08240	Unknown	0.614
AT1G52050	Mannose-binding lectin superfamily protein	0.614
AT1G48510	Surfeit locus 1 cytochrome c oxidase biogenesis protein	0.614
AT2G34300	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	0.614
AT5G25820	Exostosin family protein	0.614
AT3G59100	glucan synthase-like 11	0.614
AT5G19930	Protein of unknown function DUF92, transmembrane	0.614
AT5G50120	Transducin/WD40 repeat-like superfamily protein	0.613
AT5G51590	AT hook motif DNA-binding family protein	0.613
AT3G05150	Major facilitator superfamily protein	0.613
AT5G60530	late embryogenesis abundant protein-related / LEA protein-related	0.612
AT5G05440	Polyketide cyclase/dehydrase and lipid transport superfamily protein	0.612
AT1G27170	transmembrane receptors;ATP binding	0.612
AT2G37110	PLAC8 family protein	0.612
AT1G23140	Calcium-dependent lipid-binding (CaLB domain) family protein	0.612
AT3G07000	Cysteine/Histidine-rich C1 domain family protein	0.611
AT3G52400	syntaxin of plants 122	0.611
AT1G18980	RmlC-like cupins superfamily protein	0.611
AT3G50620	P-loop containing nucleoside triphosphate hydrolases superfamily protein	0.611
AT4G05200	cysteine-rich RLK (RECEPTOR-like protein kinase) 25	0.611
AT5G57480	P-loop containing nucleoside triphosphate hydrolases superfamily protein	0.611
AT2G26240	Transmembrane proteins 14C	0.611
AT5G35730	EXS (ERD1/XPR1/SYG1) family protein	0.611
AT5G09890	Protein kinase family protein	0.610
AT4G08780	Peroxidase superfamily protein	0.610
AT2G18160	basic leucine-zipper 2	0.610
AT5G45490	P-loop containing nucleoside triphosphate hydrolases superfamily protein	0.610
AT2G28760	UDP-XYL synthase 6	0.610
AT5G20960	aldehyde oxidase 1	0.610
AT5G01450	RING/U-box superfamily protein	0.610
AT4G10390	Protein kinase superfamily protein	0.610
AT1G29820	Magnesium transporter CorA-like family protein	0.609
AT5G06740	Concanavalin A-like lectin protein kinase family protein	0.609
AT2G30490	REF3 cinnamate-4-hydroxylase	0.609
AT2G41800	Protein of unknown function, DUF642	0.609
AT5G62165	AGAMOUS-like 42	0.609

AT5G54650		formin homology5	0.609
AT1G50630		Protein of unknown function (DUF3537)	0.609
AT2G20650		RING/U-box superfamily protein	0.609
AT1G19230		Riboflavin synthase-like superfamily protein	0.609
AT4G28540		casein kinase I-like 6	0.609
AT1G62800		aspartate aminotransferase 4	0.609
AT2G43040		tetratricopeptide repeat (TPR)-containing protein	0.609
AT5G04740		ACT domain-containing protein	0.609
AT4G26970	ACO2	aconitase 2	0.608
AT4G19030		NOD26-like major intrinsic protein 1	0.608
AT4G25320		AT hook motif DNA-binding family protein	0.608
AT1G76500		Predicted AT-hook DNA-binding family protein	0.607
AT5G58690		phosphatidylinositol-speciwc phospholipase C5	0.607
AT5G38550		Mannose-binding lectin superfamily protein	0.607
AT5G66250		kinectin-related	0.607
AT4G28370		RING/U-box superfamily protein	0.606
AT4G29690		Alkaline-phosphatase-like family protein	0.606
AT1G65840	PAO4	polyamine oxidase 4	0.606
AT4G01480		pyrophosphorylase 5	0.606
AT1G18860		WRKY DNA-binding protein 61	0.606
AT2G22470		arabinogalactan protein 2	0.605
AT1G26640		Amino acid kinase family protein	0.605
AT3G02230		reversibly glycosylated polypeptide 1	0.605
AT1G51420		sucrose-phosphatase 1	0.604
AT3G02140		AFP2 (ABI five-binding protein 2) family protein	0.604
AT2G19590		ACC oxidase 1	0.604
AT5G07360		Amidase family protein	0.604
AT3G06420		Ubiquitin-like superfamily protein	0.604
AT4G30640		RNI-like superfamily protein	0.604
AT2G45920		U-box domain-containing protein	0.603
AT3G50300		HXXXD-type acyl-transferase family protein	0.603
AT3G23800		selenium-binding protein 3	0.603
AT4G24400		CBL-interacting protein kinase 8	0.603
AT1G12000		Phosphofructokinase family protein	0.602
AT1G79380		Ca(2)-dependent phospholipid-binding protein (Copine) family	0.602
AT1G56120		Leucine-rich repeat transmembrane protein kinase	0.602
AT3G10640		SNF7 family protein	0.602
AT1G53180		Unknown	0.602
AT5G38340		Disease resistance protein (TIR-NBS-LRR class) family	0.602
AT5G45500		RNI-like superfamily protein	0.602
AT3G52430		alpha/beta-Hydrolases superfamily protein	0.602
AT5G37600	GSR 1	glutamine synthase clone R1	0.602
AT1G18390		Protein kinase superfamily protein	0.602
AT4G35985		Senescence/dehydration-associated protein-related	0.602

AT4G27860	vacuolar iron transporter (VIT) family protein	0.602
AT3G04320	Kunitz family trypsin and protease inhibitor protein	0.601
AT1G33360	ATP-dependent Clp protease	0.601
AT4G01910	Cysteine/Histidine-rich C1 domain family protein	0.601
AT4G30060	Core-2/l-branching beta-1,6-N-acetylglucosaminyltransferase family protein	0.600
AT1G80240	Protein of unknown function, DUF642	0.600
AT5G48940	Leucine-rich repeat transmembrane protein kinase family protein	0.600
AT5G48230	acetoacetyl-CoA thiolase 2	0.600

Shoot co-expression network			Correlation to
ATG	Symbol	Description	BTS
<i>Genes co-expressed to BTS</i>			
AT5G53450	ORG1	OBP3-responsive gene 1	0.733984
AT1G23020	FRO3	ferric reduction oxidase 3	0.644513
AT5G05250		Unknown	0.643582
AT3G56360		Unknown	0.605387
AT3G47640	PYE	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	0.567715
AT1G29400		MEI2-like protein 5	0.533767
AT2G35050		Protein kinase superfamily protein with octicosapeptide/Phox/Bem1p domain	0.522358
AT1G48300		Unknown	0.509306
AT5G18190		Protein kinase family protein	0.501995

Supplementary Table S2. ATG numbers and gene description of genes appearing in the correlation networks shown in Figure 1.

<i>BTSL1 and BTSL2 root ferrome network</i>			<i>Correlation to</i>		
ATG	Symbol	Description	BTSL1	BTSL2	BTS
AT1G74770	BTSL1	BRUTUS-Like 1, zinc ion binding			
AT1G18910	BTSL2	BRUTUS-Like 2, zinc ion binding			
AT4G19690	IRT1	iron-regulated transporter 1	0.687		
AT3G56980	bHLH39	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	0.607		
AT3G07720	Kelch	Galactose oxidase/kelch repeat superfamily protein	0.600		
AT3G53480	PDR9	pleiotropic drug resistance 9		0.693	
AT5G35580		Unknown		0.646	
AT3G21240	AT4CL2	4-coumarate:CoA ligase 2		0.628	
AT5G03570	IREG2	iron regulated 2	0.765	0.711	
AT1G05700		Leucine-rich repeat transmembrane protein kinase protein	0.742	0.667	
AT3G13610	F6H1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.702	0.793	
AT3G60330	HA7	H(+)-ATPase 7	0.675	0.719	
AT3G58810	MTPA2	metal tolerance protein A2	0.664	0.643	
AT2G29750		Unknown	0.654	0.684	
AT3G61410		Unknown	0.649	0.632	
AT2G28160	FIT	FER-like regulator of iron uptake	0.649	0.748	
AT2G32270	ZIP3	zinc transporter 3 precursor	0.643	0.665	
AT3G18560		Unknown	0.626	0.659	
<i>BTS shoot ferrome network</i>			<i>Correlation to</i>		
ATG	Symbol	Description	BTSL1	BTSL2	BTS
AT3G18290	BTS	BRUTUS, zinc finger protein-related			
AT5G53450	ORG1	OBP3-responsive gene 1			0.734
AT1G23020	FRO3	ferric reduction oxidase 3			0.645
AT5G05250		Unknown			0.644
AT3G47640	PYE	basic helix-loop-helix (bHLH) DNA-binding superfamily protein			0.568

Supplementary Table S3. List of primers.

RT-PCR			
ATG	Gene	Primer name	Sequence
AT4G34270	TIP41-like	AtTIP41F	GTGAAAACGTGTTGGAGAGAAGCAA ¹
AT4G34270	TIP41-like	AtTIP41R	TCAACTGGATACCCTTTTCGCA ¹
AT2G28390	SAND	AtSANDF	AACTCTATGCAGCATTGATCCACT ¹
AT2G28390	SAND	AtSANDR	TGATTGCATATCTTTATCGCCATC ¹
AT1G01580	AtFRO2	qS-FRO2F	TTACCCGATCGACCACAACAC
AT1G01580	AtFRO2	qA-FRO2R	CCGCACTACAAGTCGCCATTAT
AT4G19690	AtIRT1	qS-IRT1F	CACCATTTCGGAATAGCGTTAGG
AT4G19690	AtIRT1	qA-IRT1R	CCAGCGGAGCATGCATTTA
AT5G01600	AtFER1	AtFER1F	TCGTTGAGAGTGAATTTCTGG ²
AT5G01600	AtFER1	AtFER1R	ACCCCAACATTGGTCATCTG ²
AT1G74770	AtBTSL1	BTSL1Fnew	GGCAATGAAGATGGATTTGG
AT1G74770	AtBTSL1	BTSL1Rnew	TCATATGGAACCGTTGCTGA
AT1G18910	AtBTSL2	BTSL2Fnew	CGGGGCAGAATCCATCTTAT
AT1G18910	AtBTSL2	BTSL2Rnew	GTTGCAACAAGGAGCAAGAAG
AT2G28160	FIT	FIT1setAF	TCGCGGGTCTTGAAGCTTCT
AT2G28160	FIT	FIT1setAR	GCAGGAGGATTGATACCGCG
AT3G56970	bHLH38	bHLH38_QA	CTGACGAAACAGATACTCCCAAGCT
AT3G56970	bHLH38	bHLH38_QS	TTTCACAACTTCGGTTGGCC
Genotyping			
ATG	line	Primer name	Sequence
AT1G74770	<i>bts1-1</i>	bts1-1_LP	TGCTTGGCATAATCCTTCTTG
AT1G74770	<i>bts1-1</i>	bts1-1_RP	GAACCTCTCTTGCTTCCTGAAGC
AT1G74770	<i>bts1-1</i>	bts1-2_LP	GACTATCGTCAAGCAGCCAAG
AT1G74770	<i>bts1-1</i>	bts1-2_RP	GATGCTCCGGTTCTTTTCTTC
AT1G74770	<i>bts1-1</i>	bts1-3_LP	GGCTGACTCAGCTAATCTCCC
AT1G74770	<i>bts1-1</i>	bts1-3_RP	TGCAACACGAATTTAGACAAGG
AT1G18910	<i>bts2</i>	bts2_LP	TCGGTTATTTCAGGCAAAACAC
AT1G18910	<i>bts2</i>	bts2_RP	CCCTTTGTACTCATCAGCAGC
AT3G18290	<i>bts-1</i>	bts-1_LP	CCAAATGCGTTCGTAGGTAAG
AT3G18290	<i>bts-1</i>	bts-1_RP	TCAGATTTACACAAATTTGCAGC
Promoter-GUS			
ATG	Promoter	Primer name	Sequence
AT1G74770	BTSL1	BTSL1prom_G WF	GGGGACAAGTTTGTACAAAAAAGCAGGC TAGTATTGATTTTGTGAGCCCAATTAAC
AT1G74770	BTSL1	BTSL1prom_G WR	GGGGACCACTTTGTACAAGAAAGCTGGG TTCCACCGCAACAACAATAACGGTG
AT1G18910	BTSL2	BTSL2pro_GWF	GGGGACAAGTTTGTACAAAAAAGCAGGC TAAATTCATATAAGAATTGCTTTATAC
AT1G18910	BTSL2	BTSL2pro_GW R	GGGGACCACTTTGTACAAGAAAGCTGGG TCCACCATGAACAGGAGAGGAGGTG
BTSL-GFP			
ATG	Gene	Primer name	Sequence
AT1G74770	BTSL1	BTSL1_B1-F	GGGGACAAGTTTGTACAAAAAAGCAGGC TTGATGGGAGGCGGAAA
AT1G74770	BTSL1	BTSL1_B1-R	GGGGACCACTTTGTACAAGAAAGCTGGG TTAAGGAGCCTTGAGTT

¹ Han, B., Yang, Z., Samma, M.K., Wang, R., and Shen, W. (2013). Systematic validation of candidate reference genes for qRT-PCR normalization under iron deficiency in Arabidopsis. *BioMetals* 26: 403–413.

² Bournier, M., Tissot, N., Mari, S., Boucherez, J., Lacombe, E., and Briat, J. (2013). Arabidopsis Ferritin 1 (AtFer1) Gene Regulation by the Phosphate Starvation Response 1 (AtPHR1) Transcription Factor Reveals a Direct Molecular Link between Iron and. 288: 22670–22680.

AT1G18910	BTSL2	BTSL2_B1-F	GGGGACAAGTTTGTACAAAAAAGCAGGC
			TTGATGGGAGTCGGAGA
AT1G18910	BTSL2	BTSL2_B1-R	GGGGACCACTTTGTACAAGAAAGCTGGG
			TTGAAAAGTCTGGTGTT

Recombinant protein production

ATG	Gene	Primer name	Sequence
AT2G28160	<i>FIT</i>	FITmyc_F	ACTCATATGGAAGGAAGAGTCAACGCTC
AT2G28160	<i>FIT</i>	FITmyc_R	CGCGGATCCCTACAGATCTTCTTCAGAAAT, AGTTTTTGTTCAGTAAATGACTTGATGAATT AAAACC
AT4G00120	<i>bHLH40</i>	40myc_F	ACTCATATGGAAAATGGTATGTATAAAAAGA AAGG
AT4G00120	<i>bHLH40</i>	40myc_R	CGCGGATCCCTACAGATCTTCTTCAGAAAT, AGTTTTTGTTCGGGTTGGGAGTTGTGGTAA AAC
AT1G74770	<i>BTSL1</i>	MBP-1CT_F	ACTCCATGGCTCAGAAGGTTAGCCAGTTTG
AT1G74770	<i>BTSL1</i>	MBP-1CT_R	TGAGTCGACTTATTTTTCGAACTGCGGGTG GCTCCAAAGGAGCCTTGAGTTGTAGGAG
AT1G18910	<i>BTSL2</i>	MBP-2CT_F	ACTCCATGGAATCTTTGATCCCTGATGAAC
AT1G18910	<i>BTSL2</i>	MBP-2CT_R	TGAGTCGACTTATTTTTCGAACTGCGGGTG GCTCCAGAAAAGTCTGGTGTTGTAGGAGGC