Iron (Fe) is an essential micronutrient for many key processes in plants. In response to iron deprivation, plants activate a well-known series of responses that mediate iron uptake from the surrounding rhizosphere. In most dicots these responses include acidification of the rhizosphere, reduction of ferric iron to ferrous iron, and subsequent uptake of ferrous iron into root epidermal cells. Following uptake, iron is bound to various chelators, transported through the vasculature, and translocated from the root to shoot and sink tissues. Two regulatory pathways controlled by bHLH transcription factor have been shown to mediate these responses in Arabidopsis thaliana. The bHLH transcription factor FIT controls the initialization of the iron deficiency response by directly and indirectly controlling the expression of genes involved in rhizosphere acidification, ferric reduction, and ferrous iron uptake. The bHLH transcription factor POPEYE (PYE) mediates control by directly regulating the expression of genes involved in intercellular transport, mitochondrial ferric reduction, and other processes. PYE physically interacts with PYE-like (PYEL) proteins including bHLH104, ILR3, and bHLH115; and several PYEL proteins directly target PYE transcriptionally. Similar to PYE, loss of PYEL expression leads to decreased tolerance to iron deficiency, suggesting that PYE and PYEL proteins may have somewhat redundant functions. While PYE and PYEL proteins physically interact, only PYEL proteins have been shown to interact with a third protein, BRUTUS (BTS). BTS, similar to PYE and PYEL proteins, is induced transcriptionally in response to iron deprivation. Unlike pye and pyel loss of function mutants, bts mutants that exhibit decreased induction of BTS expression under iron deficiency show increased tolerance to iron deprivation. Thus, BTS and PYE/PYEL proteins act in opposing manners to control the iron deficiency response.

BTS encodes a multi-domain protein with 3 hemerythrin (HHE) domains and a RING domain flanked by a CHY-type zinc finger and a zinc ribbon domain. The HHE domains are left-twisted 4-α-helical bundles that, in marine invertebrates and the mammalian iron homeostasis protein FBXL5, provide a hydrophobic pocket wherein diiron binds oxygen. Correspondingly, we found that the BTS HHE domains also bind both iron and zinc. We expressed recombinant GFP tagged BTS lacking all 3 of the HHE domains (BTSΔHHE) and driven by the native BTS promoter (BBΔHG) in bts-1 mutants and found that removal of the HHE domains does not affect the ability of the truncated protein to complement bts-1 phenotypes including root length and iron reductase activity. Moreover, while full-length recombinant BTS synthesized by in vitro translation using a wheat germ system exhibits decreased stability in the presence of increasing concentrations of soluble iron, recombinant BTSΔHHE protein maintains stability regardless of iron concentration. We also observed enhanced accumulation of BTSΔHHE protein in planta by analyzing transgenic bts-1 mutants transformed with either BBG (pBTS::BTS-GFP) or BBΔHG (pBTS:: BTSΔHHE-GFP) constructs. These findings suggest that the presence of the HHE domains confers protein instability upon binding of iron.

The presence of an E3 RING-H2 domain near the C-terminus of BTS suggested that BTS may act as an E3 ligase. E3 ligases catalyze the final step in the ubiquitination of an interacting E3 substrate. Ubiquitination targets proteins for degradation via the 26S proteasome. Initially, ubiquitin is activated by ATP and transferred via a ubiquitin-activating enzyme (E1) to a ubiquitin-conjugating enzyme (E2), generating E2 thioesterified with ubiquitin (E2-Ub). Ubiquitin-ligating
enzymes (E3), including members of the RING H2 family, bind both a targeting substrate and E2-Ub and facilitate the transfer of the ubiquitin from the E2-Ub to the substrate. RING domains alone purified from diverse enzymes have been shown to demonstrate intrinsic E3 ligase activity in the presence of a conjugating E1, E2, ubiquitin, and ATP.\(^\text{12,13}\)

We have shown that full length \textit{in vitro} translated and purified BTS protein exhibits E3 ligase capacity.\(^\text{4}\) We also showed that this E3 capacity is able to affect the stability of 2 BTS targets – the PYEL proteins ILR3 and bHLH115.\(^\text{4}\) Strikingly, deletion of the BTS HHE region confers apparent increased E3 ligase functionality \textit{in vitro} and enhanced ability to facilitate the degradation of PYEL proteins in cell free degradation assays due to increased protein stability.\(^\text{3}\) These findings indicate that the BTS HHE domains play a critical role in the stability of the protein, which increases the accumulation of BTS and therefore E3 activity \textit{in vitro}. However, the role of the E3 domain alone in mediating iron deficiency responses and protein stability has not yet been explored.

Here, to further explore the role of the BTS E3 domain, we expressed recombinant GFP tagged BTS lacking the E3 domain (BTS\(_{\Delta E3}\)) and driven by the native BTS promoter (BB\(_{\Delta E3}\)) in \textit{bts-1} mutants. We first identified several \textit{bts-1} mutant lines expressing BB\(_{\Delta E3}\) and chose to focus on line 6-1 with expression at levels most comparable to previously published lines expressing full length BTS (BBG 1-2) and BTS lacking the HHE domains (BB\(_{\Delta HG}\) 3-3) (Fig. 1A).\(^\text{4}\)

We used confocal analysis to confirm that structurally viable protein is produced and to identify any effects on stability. Accumulation of the BTS\(_{\Delta E3}\) protein is similar to that of full length BTS; both proteins accumulate minimally even under iron deficiency in contrast to the previously published more stable BTS\(_{\Delta HHE}\) protein (Fig. 1B). These findings indicate that while the HHE domains facilitate degradation of BTS protein, the E3 domain does not appear to play a critical role in BTS stability.

Next, we examined the capacity of the BB\(_{\Delta E3}\) construct to complement \textit{bts-1} mutant phenotypes. We found that, unlike full length BTS and BB\(_{\Delta HG}\), expression of BB\(_{\Delta E3}\) in a \textit{bts-1} mutant background results in root length (Fig. 2A, C) and iron reductase activity (Fig. 2B) not significantly different from that of the \textit{bts-1} mutant under iron deficiency, indicating that the BTS\(_{\Delta E3}\) protein is not functional in fulfilling BTS’ typical iron homeostasis roles. The presence of the BTS E3 RING domain is therefore critical for BTS’ ability to repress several classical responses to iron deficiency. Since loss of the E3 domain inhibits the ability of BTS to facilitate ubiquitin formation \textit{in vitro} and to interact with the target proteins ILR3 and bHLH115,\(^\text{4}\) the primary role of the BTS E3 domain is likely to facilitate the ubiquitination and subsequent degradation of target substrates independent of the function of the HHE domains. This provides further evidence that the overall physiological differences seen in the \textit{bts-1} mutant are indicative of its normal role in finely tuning the iron deficiency response, dependent on a functioning E3 ligase domain. Though some direct targets of BTS E3 ligase activity have been identified,\(^\text{4}\) the link between these targets and the phenotypic outputs of the \textit{bts-1} mutant is yet to be illuminated.

The BTS ortholog HRZ1 in \textit{Oryza sativa} has been shown to play a similar role to BTS in iron deficiency.\(^\text{14}\) Conserved domain architecture is likely to indicate both common functionality and evolutionary descent.\(^\text{15}\) We were therefore interested in exploring the taxonomic distribution of other proteins similar to BTS in an effort to infer both further functionality of the individual domains and potential evolutionary implications of their composition. For a broad overview, the Simple Modular Architecture Research Tool (SMART) was used to determine that the specific domain structure of BTS (3 HHE, one zinc finger CHY, one E3
RING, and one zinc ribbon domain) is present only in proteins from plant species.\textsuperscript{16,17} In order to confirm and expand on this observation, sequences with significant similarity to the full BTS amino acid sequence were analyzed based on domain structure (Fig. 3A). All BTS orthologs in species ranging from protozoa to animals contain RING, CHY, and zinc ribbon domains, or derivative sequences thereof. Indeed, the C-terminus of BTS aligns closely to the key cell cycle regulator Pirh2 (RCHY1) in Homo sapiens and its animal homologs (Fig. 3B).\textsuperscript{18,19} The combination of one or more HHE domains with the CHY-RING structure, however, only appears in photosynthetic organisms – mainly green algae and land plants but also in 3 species of red algae (Fig. 3A). It is clear that this combination is of evolutionary importance for plant species since it has appeared early and persisted throughout the evolution of plants. In fact, transcript coding for the BTS ortholog in Chlamydomonas reinhardtii (Cre05.g248550) is induced under iron deficiency, indicating a conserved role for BTS’ plant orthologs in iron deficiency.\textsuperscript{20} Iron deficiency is a significant issue for photosynthetic organisms in particular because of the requirement for iron in chlorophyll biosynthesis and the photosynthetic electron transport chain. BTS orthologs may have evolved as a multifunctional mechanism both to detect and respond to changes in iron availability through regulating the degradation of target proteins.

The evolutionary origin of plant HHE domains is difficult to trace beyond photosynthetic organisms. Other examples of HHE domains are found primarily in prokaryotes and invertebrates and in the mammalian F-box and leucine-rich repeat protein5 (FBXL5) family of proteins.\textsuperscript{16,17,21} BTS is commonly compared to FBXL5 due to key conserved amino acids and some functional similarity in iron regulation.\textsuperscript{4,14,22,23} FBXL5 acts with the E3 ligase RBXI as a part of an SCF complex to degrade the iron regulatory protein IRP2.\textsuperscript{9,10,24} BTS therefore effectively combines the activity of 2 mammalian proteins as domains in one protein. Despite these similarities, the HHE domains of BTS differ significantly from the FBXL5 HHE domain, sharing at most 20% identity. The iron-dependent regulation of FBXL5 and BTS is also different – FBXL5 is degraded in the absence of iron and BTS is degraded in the presence of iron.\textsuperscript{4,9} Additionally, the RING E3 domain of BTS is more similar to that of Pirh2 (44% identity) than to that of the SCF E3 ligase RBX1 (27%). The CHY domain of Pirh2 and BTS orthologs are also remarkably similar (46%), suggesting some unidentified but important functionality. In animals, the CHY domain is not required for binding or ubiquitinating the Pirh2 substrate p53, but may contribute to optimal activity.\textsuperscript{25} Interestingly, the Pirh2 amino acids that are predicted to interact with its target p53 (249-256)\textsuperscript{25} are almost completely different in BTS (Fig. 3B), indicating that BTS may interact with a different set of target proteins (including the known PYEL proteins).\textsuperscript{4} The high similarity of the C-terminus of BTS to the full amino acid sequence of Pirh2 grants more explanation to the unexpected complementation of bts-1 by BTS\textsubscript{AHHE},\textsuperscript{4} a truncated protein lacking the entire HHE region of BTS. This BTS\textsubscript{AHHE} protein resembles full Pirh2 in sequence and appears fully functional. Based on these analyses, BTS seems more structurally and perhaps functionally similar to Pirh2 than to FBXL5. The HHE domains are likely an evolutionary addition that grant more precise and adaptable control of a conserved regulatory mechanism.

Figure 2. Complementation of bts-1 mutant phenotypes with BTS and BTS deletion constructs. (A) Root length of 11 day old seedlings (4 d +Fe, 7 d +/-Fe). Error bars indicate ±SE (n = 32) and columns with different letters are significantly different from each other (ANOVA followed by Tukey-Kramer, p < 0.05). (B) Iron reductase activity of 10 day old seedlings (7 d +Fe, 3 d +/-Fe). Error bars indicate ±SE (n = 4) and columns with different letters are significantly different from each other (ANOVA followed by Tukey-Kramer, p < 0.05). (C) Image of root length of 14 day old seedlings (4 d +Fe, 10 d -Fe).
Further examination of the BTS CHY, RING, and zinc ribbon regions in relation to the well-studied Pirh2 protein may help to highlight commonalities and divergent functionality between Pirh2 and the BTS family of plant proteins.

**Materials and methods**

**Plant lines and growth conditions**

Mutant bts-1 plants and BBΔHG, BBΔEG lines as well as growth conditions, experimental setup, and statistics for all phenotypic assays were as previously described.3,4

**Expression analysis**

Total RNA was extracted from the root tissue of 7 day old seedlings (4 d +Fe, 3 d -Fe) using the GeneJET Plant RNA Purification Mini Kit (Thermo Scientific). cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Life Technologies). Quantitative Real-Time PCR was conducted using iTaq Universal SYBR Green Supermix (Bio-Rad) and the StepOnePlus Real-Time PCR System (Applied Biosystems). Relative expression was calculated using the comparative Ct method normalized to β-tubulin using the primers:

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**Figure 3.** The complete BTS protein domain architecture is observed only in photosynthetic organisms. (A) Phylogenetic tree is composed of species containing protein sequences that align significantly to BTS. Color indicates photosynthetic (green) and non-photosynthetic (gray) species. Symbols beside species name indicate domain composition of the BTS ortholog(s) in that species. The square(s) indicate presence of HHE domain(s) and the triangle indicates presence of CHY, RING, and zinc ribbon domains. Nodes are collapsed (e.g. Fungi) if the majority of contained species have the same protein structure. Domains were predicted using Pfam7 and SMART16,17 domain analysis; derivative structures not fitting algorithm criteria may be present. (B) Alignment of the C-terminus of BTS to full Pirh2 amino acid sequence. Conserved amino acids are colored black and domain regions are indicated with black labels. Zinc coordinating amino acids (all conserved) are colored red and Pirh2 amino acids that interact with p53 are underlined in red.
Confocal microscopy

Seven day old seedlings (4 d +Fe, 3 d -Fe) were imaged with a Zeiss LSM 710 microscope using 10 mM propidium iodide to visualize cell walls.

Phylogenetic tree

Sequences were selected using NCBI BLASTP 2.3.1+ with all non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects as of May 2016, using the BTs amino acid sequence and E value of less than 1e-20. Domains were predicted using Pfam and SMART. Domain classification for the closest Bts orthology per species was used to color the phylogenetic tree, assembled using NCBI Common Tree and visualized using EvolView.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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