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***SRF3-bHLH* Crosstalk in *Arabidopsis* Iron Homeostasis: Receptor Kinase Signaling, Transcriptional Regulation and Genetic Epistasis**

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ABSTRACT

In this review, the available evidence that connects the *SRF3* receptor kinase with the *bHLH*-driven Fe-deficiency regulatory network in *Arabidopsis* plants is summarized. The data currently available suggests that *SRF3* and *bHLH*-mediated regulation are functionally convergent in the regulation of *IRT1*, but there is currently no evidence for direct genetic epistasis or physical interaction between these regulators. Three possible models are considered: *SRF3* could act upstream of *bHLH* regulators; *SRF3* could be active alongside the *bHLH* pathway; or *SRF3* could have indirect effects on *bHLH* activity such as by phosphorylation, protein stability or subcellular localization. Based on these models, genetic interaction experiments between *SRF3* and important *bHLH* mutants, as well as transcriptional, protein-level and phosphoproteomic experiments, are suggested to determine the pathway hierarchy and molecular connectivity. Further research is also needed to uncover the role of *SRF3-bHLH* crosstalk in coordinating iron homeostasis with plant immunity and how insights can be used to develop strategies for enhancing iron acquisition and nutritional quality in crops. Overall, the *SRF3-bHLH* relationship is a hypothesis to be tested, not a pathway.

Keywords: *Arabidopsis*, Iron homeostasis; *bHLH* transcription factors; *SRF3*; receptor kinase signaling; genetic epistasis; plant immunity

INTRODUCTION

Iron (Fe) is a micronutrient element essential for several processes in plants such as photosynthetic electron transport, respiration, biosynthesis of chlorophyll and many other redox reactions (Bhat *et al.*, 2024). Although usually a problem in most soils, Fe can be unavailable to plants because it is poorly soluble when in the Fe³ form, especially when under neutral to alkaline soil conditions (Molnár *et al.*, 2023). Meanwhile, high levels of Fe can lead to the production of Reactive Oxygen Species (ROS) and cell damage. Plants must therefore have well-regulated systems to ensure that Fe acquisition and utilization balance with Fe toxicity (Jiang *et al.*, 2024). Recent studies have revealed that plant Fe homeostasis is regulated by multiple, interconnected, transcriptional, post-translational, metabolic and systemic signaling pathways, and not by a single uptake pathway (Li *et al.*, 2026). This is a multi-layered regulation that gives a physiological context to comprehend the plant response to Fe availability and

adapt Fe uptake in relation to the plant developmental and environmental needs (Shafi *et al.*, 2025).

For non-graminaceous plants including *Arabidopsis thaliana*, Fe acquisition is mainly mediated by a reduction-based strategy, where rhizosphere acidification, Fe reduction, and Fe uptake in the ferrous state converge to enhance Fe availability to the roots (Lurthy *et al.*, 2021). Ferric-chelate reductase FERRIC REDUCTION OXIDASE 2 (*FRO2*) and high-affinity Fe transporter IRON-REGULATED TRANSPORTER 1 (*IRT1*) play key roles in this response (Zhang *et al.*, 2019). The ability to acquire Fe is not, however, limited to such constitutive activation of these transport processes as uncontrolled Fe uptake can result in Fe toxicity and in a disturbance of other mineral nutrients (Zaid *et al.*, 2020). More recently, it has been shown that *Arabidopsis* can also utilize Fe mobilization with coumarin and that the old paradigm of the various Fe-acquisition strategies in plants is not as rigid as it may seem (Robe *et al.*, 2025). Therefore, the ability of the plant to control Fe uptake is not only dependent on the Fe

in the rhizosphere, but also on intracellular signaling networks that coordinate Fe uptake with whole-plant Fe demand (Birte Schwarz & Petra Bauer, 2020).

One of the key factors in this regulatory network is the basic helix-loop-helix (*bHLH*) family of transcription factors. In *Arabidopsis*, *bHLH* proteins are involved in hierarchical regulatory network controlling Fe deficiency responses, Fe acquisition, Fe translocation and storage (Trofimov *et al.*, 2025). The downstream FIT-Ib module comprises of fer-like iron deficiency-induced transcription factor (FIT/*bHLH29*) and the subgroup Ib proteins, *bHLH38*, *bHLH39*, *bHLH100* and *bHLH101* (Gao & Dubos, 2024). These factors create transcriptional complexes that induce the expression of Fe acquisition genes, such as *FRO2* and *IRT1*. Upstream *bHLH* regulators, such as *bHLH34*, *bHLH104*, *bHLH105/ILR3*, *bHLH115* and *bHLH121/URI* coordinate Fe-deficiency signaling and control downstream components of the transcriptional network (Li *et al.*, 2022). Recent research has focused on the fact that the *bHLH* proteins are not individual regulators, but rather elements of a multiplayer network, where the activity of transcription, stability of the protein, localization and protein phosphorylation play dynamic roles in the control of Fe homeostasis. Thus, knowledge of the upstream signals that control this *bHLH* hierarchy is critical for elucidating how Fe status is converted into proper transcriptional reactions (Gao *et al.*, 2019).

The upstream components are represented by *bHLH121/URI*, which has proven to be an important regulatory node between Fe status and downstream transcriptional responses (Kumar *et al.*, 2022). The activity of *bHLH121* is regulated by Fe-dependent post-translational mechanisms and involves the interaction with members of the IVc *bHLH* subgroup, which are involved in the activation of Fe-deficiency-responsive genes (Liang, 2022). Recent genetic evidence also suggests that interactions at the different levels of the *bHLH* hierarchy are important, as *bHLH121* and clade IVc *bHLH* factors can work in synergy to control Fe homeostasis (Lei *et al.*, 2020). Moreover, Fe-responsive proteins like Fe-deficiency regulator BRUTUS (*BTS*) and *BTS*-LIKE proteins help regulate the turnover of positive regulators of Fe uptake and prevent excessive activation of Fe uptake when Fe is available (Baby *et al.*, 2025). The results in this study suggest that the *bHLH* network is integrated into a larger signaling system, where both transcriptional and post-translational regulation play a role. This will lead to an interesting question: what is the links between upstream environmental/cell surface signals and the intracellular *bHLH* machinery?(Cappellari, 2012).

One possible point of connection between extracellular signals and intracellular regulation is receptor kinases. The leucine rich repeat receptors kinases (LRR-RKs) in plants respond and/or interact

outside and on the cell surface, and can trigger cellular responses downstream via phosphorylation, trafficking and transcriptional regulation (Escocard de Azevedo Manhães *et al.*, 2021). STRUBBELIG RECEPTOR KINASE 3 (*SRF3*) has been of specific interest in Fe homeostasis since genetic and molecular studies have shown connections between *SRF3* and root responses in Fe and immune signaling (Xie *et al.*, 2026). It is noteworthy that *SRF3* is necessary for proper activation of *IRT1* in Fe deficient conditions. It has also been discovered that several Fe-homeostasis-related genes are altered in *SRF3* mutants, suggesting that the effects of *SRF3* on Fe status are not limited to *IRT1*. Hence, an upstream receptor-mediated signaling component upstream of a known Fe-acquisition output is provided by *SRF3* (Matthieu Pierre Platre, Santosh B Satbhai, *et al.*, 2022).

Both *SRF3* and the *bHLH* network modulate the same Fe-acquisition pathway but at different levels; the relationship between them becomes significant. The FIT-Ib complex is a direct activator for *IRT1* transcription while *SRF3* is necessary for the correct Fe-dependent activation of *IRT1* (Pan *et al.*, 2024). However, this convergence does not prove that *SRF3* is upstream of FIT, *bHLH121* or some other *bHLH*. Actually, *SRF3* could act indirectly in the *bHLH* network, in parallel or as part of a downstream common regulatory step (de Martin *et al.*, 2021). Recent studies have strengthened the complexity of Fe signaling with evidence that Fe-regulatory transcription factors can be phosphorylated, ubiquitinated and therefore potentially subject to a number of other post-translational modifications that could be exploited by receptor-mediated signaling to alter the transcriptional activity of these proteins. It therefore appears that the shared regulation of *IRT1* is a functional convergence rather than a concrete demonstration of the *SRF3*–*bHLH* pathway (Knez *et al.*, 2017; Verma, 2025).

This distinction highlights one of the unanswered questions in the current knowledge of *Arabidopsis* Fe homeostasis: whether *SRF3* acts genetically upstream of the *bHLH* regulatory network, in parallel to the *bHLH*-dependent regulation, or whether it can modify the activity of network *bHLH*-dependent components via post-translational signaling? (Gao & Dubos, 2021). So far, direct genetic interaction between *SRF3* and important *bHLH* regulators has not been demonstrated and direct physical interactions among the signaling components associated with *SRF3* and the *bHLH* proteins are not known (Hao *et al.*, 2021). It is crucial to note that the differences between these models will affect predictions of the regulatory architecture and thus the nature of the experiments required. In combination with *bHLH121* genetic analysis of *SRF3*, fit or selected downstream mutants could help to define pathway relationships, while the analysis of the level of *bHLH*

activity following protein-level phosphorylation and/or interacting with other proteins could help to determine whether receptor signaling directly or indirectly affects *bHLH* activity (Qian *et al.*, 2021).

The present review, therefore, brings together the current knowledge on the *Arabidopsis bHLH*-centered Fe-regulatory network and *SRF3*-mediated signaling and, in particular, their potential functional overlap. We briefly recap the hierarchical organization of the *bHLH* network regulating the Fe acquisition and homeostasis, and then review the evidence that supports the involvement of *SRF3* in the regulation of Fe responses and its relation to immune signaling. Next, we consider three mechanistic models of *SRF3*–*bHLH* crosstalk and review the literature on genetic epistasis as a means to identify whether pathway hierarchy or parallel convergence is the case. Finally, experimental priorities are identified, such as the study of double mutants, of transcriptional profiling, characterization of proteins at protein level and phosphoproteomic approaches, and how such understanding of Fe regulatory networks could eventually be used for crop improvement and Fe biofortification. By distinguishing between known evidence and proposed mechanisms, this framework is designed to elucidate the current state of the knowledge gap and provide paths for experimental testing of how to address the link between receptor kinase signaling and Fe homeostasis *bHLH*-dependent transcriptional control.

The *bHLH* Regulatory Network in Iron Homeostasis

The FIT-Ib module: The terminal transcriptional response to Fe-deficiency is mostly regulated by FIT in concert with the subgroup Ib *bHLH* proteins *bHLH38*, *bHLH39*, *bHLH100* and *bHLH101* (Zhang *et al.*, 2023). These heterodimeric complexes are responsible for the activation of genes that help to acquire Fe, such as *FRO2* and *IRT1* (Yuan *et al.*, 2008). Molecular evidence confirms that FIT forms functional transcriptional complexes through direct interactions with *bHLH38* and *bHLH39* (Andriankaja *et al.* 2014; Gao *et al.* 2020). The genetic redundancy among the Ib sub-group should be considered when interpreting mutant phenotypes. Single mutants might exhibit milder phenotypes because other paralogs are able to compensate for the loss of the mutant factor (Maier *et al.*, 2005). Thus, the contribution of individual *bHLH* proteins to Fe-deficiency responses might require higher order mutant combinations.

Upstream regulation by *bHLH121*/URI and the IVc subgroup: The IVc *bHLH* proteins *bHLH34*, *bHLH104*, *bHLH105*/ILR3 and *bHLH115* are upstream of the FIT-Ib module and stimulate the expression of downstream Fe-regulatory genes (Li *et al.*, 2016). *bHLH121*/URI is an important upstream integrator and regulated by Fe-dependent phosphorylation and proteasomal degradation (Spielmann *et al.*, 2023). In Fe deficiency, phosphorylated URI is involved in the activation of Fe-

deficiency response, while Fe restored causes its degradation (Sun A Kim *et al.*, 2019). Genetic analyses in this network serve as a useful pre-model for epistasis analysis. *BHLH121* and mutants of IVc subgroup mutants yielded additive effects when combined, as noted by the higher Fe-deficiency phenotypes than the single mutants (Gao *et al.*, 2024). This precedent is important for the proposed *SRF3*–*bHLH* relationship since it shows that genetic interaction analysis can uncover functional relationships between components of a network of proteins that have previously been assumed to be entirely redundant.

Negative regulation and post-translational control:

The Fe deficiency response needs to be carefully regulated to avoid Fe overload. *BTS* and *BTSL1*/*BTSL2* act as negative regulators that help in degradation of positive Fe-regulatory proteins (Mankotia *et al.*, 2023). Fe-responsive transcription is also repressed by other proteins associated with *bHLH*, such as *PYE*. Another way that *bHLH* activity can be regulated is by post-translational modifications that affect the signaling pathways (Li *et al.*, 2022). For instance, *CIPK11* phosphate regulates the localization, *bHLH39* interaction and activity of *FIT*. In addition, *FIT* can also interact with components of the ethylene signaling pathway, including *EIN3* and *EIL1*, showing that both hormonal and kinase-dependent pathways can influence the Fe-deficiency transcriptional response (Gratz *et al.*, 2019). Such mechanisms are applicable to *SRF3* as receptor kinases do not typically directly regulate transcription but rather relay extracellular signals via phosphorylation dependent signaling (Azam *et al.*, 2022). Thus, one possible pathway in which *SRF3* signaling may regulate the Fe-regulatory transcriptional machinery is through the post-translational regulation of a *bHLH* component.

IMA/FEP peptides and network fine-tuning: In addition to Iron Production Inducing Peptides (IRA), there are other peptides that are involved in the regulation of Fe homeostasis called IRON MAN/FE-UPTAKE-INDUCING PEPTIDES (IMA/FEP) (Chia & Vatamaniuk, 2024; Tabata, 2023). The peptides affect the stability of Fe-regulatory proteins by binding to *BTS* and *BTSL* proteins (Wang *et al.*, 2023). The involvement of their activity in Fe homeostasis demonstrates that Fe is not only regulated at the transcriptional level, but rather it is regulated by a system of interconnected transcriptional, post-translational and systemic signaling. In the case of the *SRF3*–*bHLH* model, these regulatory layers are relevant because they allow several potential intermediation points between receptor-mediated signaling and to the transcriptional output.

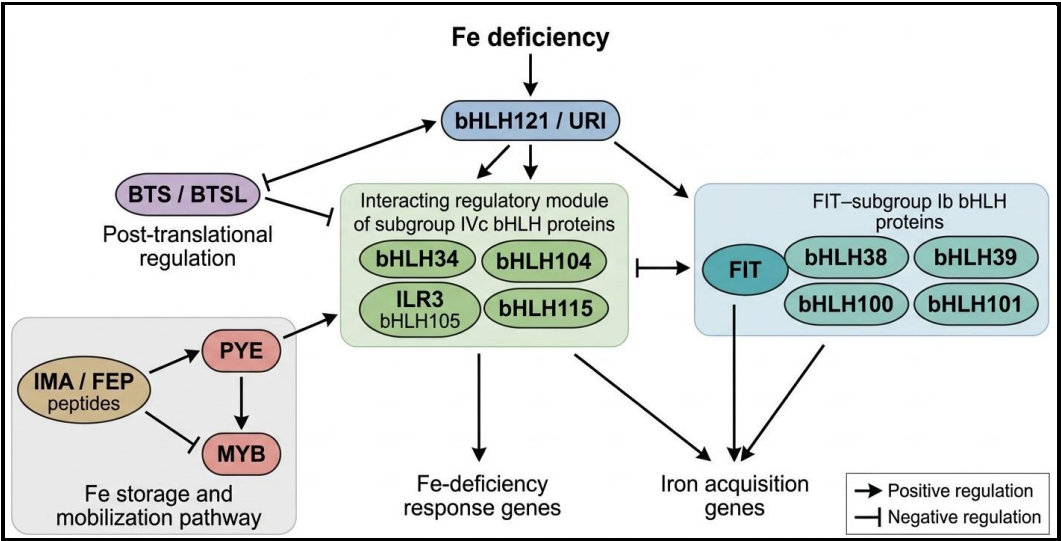


Figure 1. Core *bHLH* transcriptional network regulating iron homeostasis in *Arabidopsis*. Under Fe deficiency, *bHLH121/URI* and FIT interact with subgroup IVc and Ib *bHLH* proteins, respectively, to regulate Fe-deficiency responses and Fe acquisition. *BTS/BTSL* proteins contribute to post-translational regulation, while IMA/FEP peptides, PYE and MYB pathways contribute to Fe homeostasis. Arrows indicate positive regulation and blunt-ended lines indicate negative regulation.

Table 1. Core Regulatory Components Linking Fe Deficiency Signaling to Iron Acquisition

Regulatory component	Molecular class	Position/function	Major target/process	Relevance to <i>SRF3</i> – <i>bHLH</i> model	References
<i>bHLH121/URI</i>	<i>bHLH</i> transcription factor	Fe-deficiency regulator	Fe-acquisition program	Candidate for <i>SRF3</i> -dependent regulation	Li, Han and Lan 2022
IVc <i>bHLH</i> subgroup	<i>bHLH</i> transcription factors	Fe-responsive regulators	FIT and Fe-acquisition machinery	Intermediate regulatory layer	Esparza-Reynoso, Ayala-Rodríguez and López-Bucio 2024
FIT	<i>bHLH</i> transcription factor	Central Fe-uptake regulator	<i>IRT1</i> , <i>FRO2</i>	Connects <i>bHLH</i> regulation with <i>SRF3</i> -associated outputs	Cui, Chen et al. 2018
Ib <i>bHLH</i> subgroup	<i>bHLH</i> transcription factors	FIT partners	<i>IRT1</i> , <i>FRO2</i>	Terminal transcriptional module	Zhang, Cheng et al. 2023
<i>BTS/BTSL</i> proteins	E3 ubiquitin ligase-related regulators	Negative Fe-deficiency regulators	Fe-regulatory protein stability	Potential Fe-immune interface	Pullin, Rodríguez-Celma et al. 2025
IMA/FEP peptides	Signaling peptides	Systemic Fe regulators	<i>BTS/BTSL</i> -associated regulation	Non-transcriptional Fe regulation	Lichtblau, Schwarz et al. 2022
<i>SRF3</i>	LRR receptor kinase	Cell-surface Fe signaling	<i>IRT1</i> , callose and immune responses	Candidate link between receptor signaling and <i>bHLH</i> network	Platre et al. 2022
BAK1/SERK-related signaling	Receptor kinase signaling	Receptor-mediated signaling	MAPK, ROS and callose responses	Potential signaling route to downstream regulators	Greeff, Roux et al. 2012

SRF3 Signaling and Its Connection to Iron Homeostasis

SRF3 as a cell-surface regulator: *SRF3* is a leucine-rich-repeat receptor kinase of the "STRUBBELIG

receptor family". The gene was discovered in a genome-wide association study that looked at the natural diversity in *Arabidopsis* root growth under Fe deficiency (Pfister, 2008). *SRF3* is localized at the plasma membrane and

plasmodesmata, and plays a role in regulation of root growth, Fe responses and cell-to-cell communication. In considering the role of *SRF3*, it is important to remember that it belongs to the wider STRUBBELIG receptor family (Receptor-Like Kinase–membrane-anchored, 2021). It is not possible to directly extrapolate evidence for the biochemical activity of the founding member STRUBBELIG to *SRF3* without experiments (Tang *et al.*, 2017). Therefore, the role of atypical kinase activity in the family should be regarded as a hypothesis, not a fact about *SRF3*.

***SRF3* and *IRT1* regulation:** That there is a link between *SRF3* and Fe homeostasis is best illustrated by *IRT1*. In the presence of Fe deficiency, *SRF3* plays a role in the activation of the *IRT1* promoter (Berger *et al.*, 2024). *SRF3* is also involved in the regulation of callose deposition, meaning that the connectivity of plasmodesma and cell-to-cell signaling could be involved in this response (De Storme & Geelen, 2014). Inhibition of callose synthesis alone however does not fully recapitulate the *SRF3* phenotype, suggesting that this is not the only mechanism by which callose is involved in regulating the activation of *IRT1* by *SRF3* (Wang *et al.*, 2021). This distinction is a very important one. The FIT-Ib *bHLH* complex is a direct transcriptional activator of *IRT1*, whereas the activation of *IRT1* under Fe deficiency requires the presence of *SRF3* (H. Wu & H.-Q. Ling, 2019). Consequently, *IRT1* is a point of convergence that is supported by experiment, but is not a point of epistasis.

***SRF3*, *BAK1* and immune signaling:** *SRF3* has also been implicated in plant immunity and Fe-dependent signaling in *Arabidopsis* (Vélez-Bermúdez and Schmidt 2023). Evidence from rice further suggests a potential connection between *SRF3*-related receptor signaling and immune responses, as OsBAK1 has been reported to phosphorylate OsSRF3 and promote pathogen-responsive signaling, including MAP kinase activation and associated ROS and callose accumulation (Duan *et al.* 2024). These findings provide comparative evidence for a possible link between *SRF3*-associated signaling and immune responses, although its relevance to the *SRF3*–*bHLH* Fe-regulatory network in *Arabidopsis* remains to be established. Such observations bring together the concept of receptor kinase signaling, Fe homeostasis and immunity. The mechanism of phosphorylation of OsBAK1-OsSRF3 should not be extrapolated to *Arabidopsis*, however. The effect on *SRF3* in *Arabidopsis* is an intriguing experimental question.

Mechanistic Basis for *SRF3*–*bHLH* Crosstalk: The available evidence supports a functional intersection between *SRF3* signaling and the *bHLH* Fe-regulatory network, but it does not yet establish the mechanism. Three models can be considered:

Model 1: *SRF3* acts upstream of the *bHLH* network:

In this model, *SRF3*-dependent signaling would influence the expression of one or more *bHLH* components leading to a change in the transcription of *IRT1* and other Fe-responsive genes. *bHLH121/URI* is a potential candidate for analysis as it is known to be regulated by the Fe-deficiency network and is known to be regulated by phosphorylation (Shikha *et al.*, 2026).

Model 2: *SRF3* and *bHLH* factors act in parallel: In this model, the role of *SRF3* and *bHLH* factors in the process of gene activation is considered to be independent of each other. The movement of *SRF3* and the FIT-Ib network can take two different paths, which lead to *IRT1*. In this scenario, *SRF3* would not be required to strictly control activity of *bHLH*. Instead, both pathways would act independently and promote a different transcriptional or regulatory state essential for activation of *IRT1* (Song *et al.*, 2025).

Model 3: *SRF3* signaling modifies a *bHLH*-associated regulatory component: A third alternative is that the activity of the receptor kinase regulates the activity of *bHLH*'s indirectly by modifying their phosphorylation, stability, nuclear localization or by interacting with other proteins. This model is biologically plausible in light of existing evidence that FIT and other *bHLH* components are regulated post-translationally. These models need to be distinguished by experiments beyond the expression analysis. Thus, genetic interaction studies, protein-protein interaction assays, studies of protein phosphorylation, and promoter-level experiments are not necessarily alternative methods, but complementary (M. P. Platre *et al.*, 2022).

Epistasis as a Strategy to Test the *SRF3*–*bHLH* Relationship: Classical genetic epistasis can be used to tell if two genes are in the same pathway. For a simple linear pathway, the phenotype of a double mutant can be the same as either one of the single mutants in the pathway, leaving the epistatic gene in either place (Angeles-Albores *et al.*, 2018). This is a classical use, as opposed to statistical epistasis, which is used to refer to non-additive interaction between loci of quantitative traits. The *bHLH* Fe-regulatory network serves as a helpful model. Mutant combinations revealed functional relationships within this network as genetic combinations of *bHLH121* and IVc subgroup members resulted in non-additive phenotypes when grown under Fe deficiency (Riaz & Guerinot, 2021). For *SRF3*, the initial priority should be to test a few candidate *bHLH* components which are strategically located in the Fe-regulatory hierarchy, not to rush into creating a host of double mutants.

Table 2. Evidence status, knowledge gaps and experimental approaches for resolving the *SRF3*–*bHLH* relationship in *Arabidopsis* iron homeostasis

Regulatory question	Current evidence	Evidence status	Knowledge gap	Proposed approach	References
<i>SRF3</i> → <i>IRT1</i>	<i>SRF3</i> is associated with Fe-dependent <i>IRT1</i> regulation	Established/Supported	Molecular mechanism remains unclear	Reporter assays, qRT-PCR	(M. P. Platre <i>et al.</i> , 2022)
<i>SRF3</i> → <i>bHLH</i>	No direct genetic hierarchy established	Unknown	Upstream/downstream relationship unresolved	Double-mutant analysis	(Shikha <i>et al.</i> , 2026)
<i>SRF3</i> ↔ <i>bHLH</i>	Direct interaction not demonstrated	Unknown	Physical association unresolved	Co-IP, BiFC, split-LUC	(Bais <i>et al.</i> , 2023)
<i>SRF3</i> → <i>bHLH</i> phosphorylation	Hypothetical	Proposed	Relevant phosphosites unknown	Phosphoproteomics, kinase assays	(Shinde <i>et al.</i> , 2017)
<i>bHLH</i> → <i>IRT1</i>	Established transcriptional regulation	Established	Relationship with <i>SRF3</i> unresolved	Genetic interaction studies	(Ryu <i>et al.</i> , 2025)

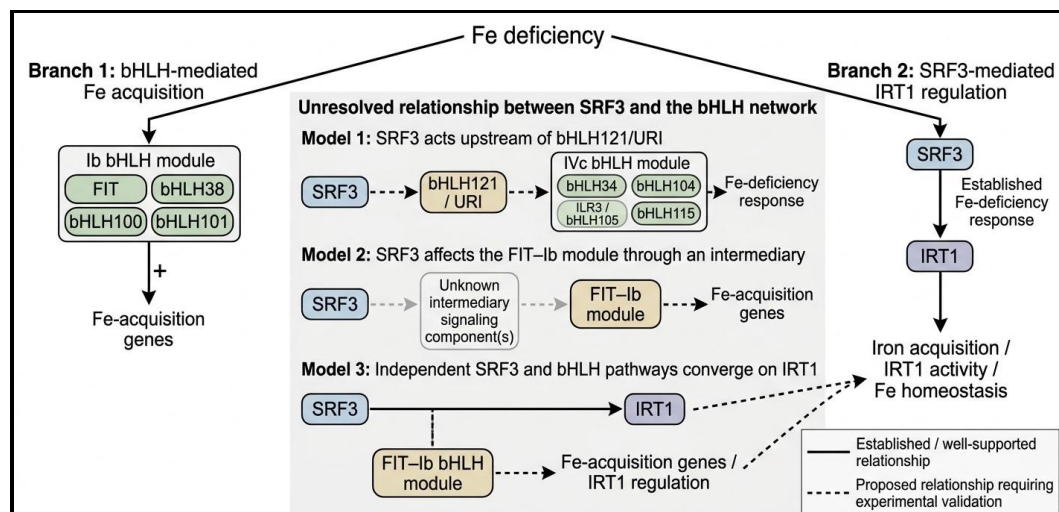


Figure 2. Proposed model of *SRF3*–*bHLH* signaling in Fe homeostasis. Established relationships include *SRF3*–dependent *IRT1* regulation and the Fe-deficiency-responsive Ib *bHLH* module. The *SRF3*–*bHLH* relationship remains unresolved and may involve upstream regulation, intermediary signaling or parallel convergence at *IRT1*. Dashed lines indicate proposed relationships, whereas solid lines indicate established or well-supported interactions.

Priority candidates

***SRF3* × *bHLH121/URI*:** The strongest initial candidate is *bHLH121/URI*, a network component that is upstream and phosphorylation regulated in the Fe-deficiency response. To assess the effect of loss of *SRF3* on this upstream *bHLH* regulator and/or whether the disruption of this *bHLH* affects the phenotype of a *SRF3* mutant, a double mutant should be generated (Pan *et al.*, 2024).

***SRF3* × *BTS*:** *BTS* offers a second candidate because it is the link between Fe homeostasis and signaling in response to pathogens. The genetic interaction between *SRF3* and *BTS* would thus be used to determine if the receptor is involved in the cross talk between Fe and immune responses (Matthieu Pierre Platre, Santosh B. Satbhai, *et al.*, 2022).

SRF3 × FIT/IB subgroup: FIT and the Ib proteins are the direct link to the transcription of the *IRT3*. Due to the redundancy of these *bHLH*s (*bHLH38*, *bHLH39*, *bHLH100* and *bHLH101*), however, individual crosses can be difficult to interpret. Higher-order mutants may thus be needed (Cai *et al.*, 2021).

SRF3 × MYB72: *MYB72* is a candidate for studying the relationship between Fe mobilization and systemic resistance. This experiment is not directly related to the main *bHLH* pathway and is thus secondary (Zamioudis *et al.*, 2014).

Knowledge Gaps and Future Research Directions

Is SRF3 genetically epistatic to bHLH regulators: So far, no direct genetic studies have been performed to determine if *SRF3* and the Fe-regulatory *bHLH* network function in a single pathway. The remarkable absence of such evidence is the main shortcoming filled by the proposed *SRF3-bHLH* model (Ding *et al.*, 2009). A logical next step is the creation of logical double mutants in combination with the single mutants, and the comparison of their phenotypes with the single mutants grown under Fe-sufficient and Fe-deficient conditions. The following should be evaluated: root architecture, chlorosis, Fe accumulation, and expression of the canonical Fe-responsive genes.

How does SRF3 regulate IRT1: Although *SRF3* is required for *IRT1* activation, callose-dependent changes in cell-to-cell connectivity do not completely account for

the phenotype. Thus, the molecular connection between *SRF3* signaling and the *IRT1* promoter is still unclear (M. Platre *et al.*, 2022). Future studies should address the possibility that the changes in *IRT1* transcription, which result from altered *bHLH* activity, are different from those that result from independent *SRF3*-dependent signaling.

Does phosphorylation connect SRF3 to the bHLH network: The phosphorylation-dependent regulation of *bHLH121/URI* and FIT shows that phosphorylation may regulate key components of the Fe-deficiency pathway (Yu *et al.*, 2026). Moreover, the phosphorylation of OsSRF3, which is regulated by OsBAK1, is an example of receptor-mediated signaling in rice (Duan *et al.*, 2024). It would therefore be useful to conduct a phosphoproteomic analysis and immunoprecipitation and targeted validation of candidate phosphorylation sites in Fe-deficiency and immune-inducing conditions.

Does the SRF3–bHLH relationship extend to immunity: The *bHLH* network plays a role in immunity in a few ways: via *BTS* associated pathogen responses, *MYB72* dependent coumarin signaling and jasmonate associated regulation of FIT (Hao *et al.*, 2021). These pathways are candidate points of contact for *SRF3*-dependent signaling that may regulate Fe homeostasis and defense. These relationships should be experimentally separated from indirect effects due to general alterations in Fe status, however.

Table 3. Experimental Evidence and Knowledge Gaps in the SRF3–bHLH Regulatory Model

Question	Current evidence	Knowledge gap	Priority experiment	References
Does <i>SRF3</i> regulate <i>IRT1</i> ?	<i>SRF3</i> contributes to Fe-dependent <i>IRT1</i> regulation.	Mechanism unclear.	Promoter analysis; qRT-PCR	(Tsuchida <i>et al.</i> , 2026)
Does <i>SRF3</i> regulate <i>bHLH121/URI</i> ?	<i>bHLH121/URI</i> regulates Fe deficiency responses.	<i>SRF3–bHLH121</i> relationship unknown.	<i>SRF3 × bHLH121</i> ; phosphorylation analysis	(Berger <i>et al.</i> , 2023)
Does <i>SRF3</i> regulate FIT?	FIT regulates <i>IRT1</i> with Ib <i>bHLH</i> s.	Relationship with <i>SRF3</i> unknown.	FIT abundance; localization	(H. Wu & H.-Q. Ling, 2019)
Does <i>SRF3</i> interact with <i>bHLH</i> s?	Potential pathway convergence.	Physical interaction unestablished.	Y2H; BiFC; Co-IP	(Choi <i>et al.</i> , 2018)
Is phosphorylation involved?	<i>bHLH</i> factors undergo post-translational regulation.	<i>SRF3</i> -dependent phosphorylation unknown.	Phosphoproteomics; validation	(Radani <i>et al.</i> , 2023)
Are <i>SRF3</i> and <i>bHLH</i> pathways parallel?	Both affect Fe responses and <i>IRT1</i> .	Pathway hierarchy unknown.	Double-mutant epistasis	(Maurer <i>et al.</i> , 2014)
Is immunity involved?	<i>SRF3</i> has immune-related functions.	Effect on <i>bHLH</i> -mediated Fe regulation unclear.	Fe-deficiency/pathogen assays	(Shi <i>et al.</i> , 2026)
Does callose explain <i>SRF3</i> -dependent <i>IRT1</i> activation?	<i>SRF3</i> affects callose responses.	Causal role remains unclear.	Callose– <i>IRT1</i> genetic analysis	(Xie <i>et al.</i> , 2026)

Proposed Experimental Framework: A stepwise experimental approach would provide a systematic framework for testing the proposed *SRF3-bHLH* model:
Step 1: Genetic analysis. Produce *SRF3 bHLH121* and, as necessary, *SRF3 BTS* double mutants. Make a

comparison of single and double mutants in conditions with and without Fe (Zhang *et al.*, 2015).

Step 2: Phenotypic characterization. Assess the primary-root growth and lateral-root development, the

Fe-deficiency phenotypes, and Fe accumulation in plant tissues (Rav & Satbhai, 2026).

Step 3: Molecular analysis. Use quantitative RT-PCR to measure the levels of transcript of the gene for *IRT1*, *FRO2*, *FIT* and some Fe-responsive genes. The crucial one is whether the transcriptional phenotype of the double mutant is additive, non-additive or similar to one of the single mutants (S. A. Kim et al., 2019).

Step 4: Protein-level analysis. Investigate the expression levels, localization and phosphorylation of candidate *bHLH* proteins. This would determine whether the activity of the receptors affects the post-translational regulation of *bHLH* activity (Huang et al., 2024).

Step 5: Interaction analysis. The use of protein-protein interaction techniques can help to identify if protein components of the *SRF3* signaling pathway physically associate with *bHLH* regulators. Genetic epistasis does not imply direct interaction (Pireyre & Burow, 2015).

Step 6: Promoter-level analysis. Identify whether *SRF3*-dependent signaling affects the regulation of the *IRT1* promoter by the *FIT*-Ib complex and/or other *bHLH* factors. Multiplex CRISPR methods can be helpful in creating higher-order mutants when phenotyping single-gene combinations is not interpretable, due to high *bHLH* network redundancy. Genome editing strategies are mentioned here as viable solution to this problem in the original literature (H. Wu & H. Q. Ling, 2019).

Table 4. Priority Genetic Tests for Investigating *SRF3*–*bHLH* Pathway Relationships

Genetic combination	Rationale	Key measurements	Interpretation	References
<i>SRF3</i> × <i>bHLH121/uri</i>	Tests <i>SRF3</i> – <i>bHLH121</i> interaction	Root growth; Fe; <i>IRT1</i> / <i>FRO2</i>	Non-additive phenotype supports pathway interaction	(B. Schwarz & P. Bauer, 2020)
<i>SRF3</i> × <i>BTS</i>	Tests Fe–immunity pathway intersection	Fe sensitivity; <i>IRT1</i> ; immunity	Genetic interaction suggests Fe–immune crosstalk	(Herlihy et al., 2020)
<i>SRF3</i> × <i>fit</i>	Tests relationship with <i>FIT</i>	<i>IRT1</i> / <i>FRO2</i> ; Fe-deficiency phenotype	Distinguishes hierarchy from parallel action	(McInturf, 2018)
<i>SRF3</i> × <i>Ib</i>	Tests direct <i>IRT1</i> regulators	Fe uptake; <i>IRT1</i>	Higher-order mutants may reveal redundancy	(Shin et al., 2013)
<i>SRF3</i> × <i>MYB72</i>	Tests Fe mobilization–ISR connection	Root exudates; Fe; defense	Supports broader Fe–immune crosstalk	(Stringlis et al., 2018)

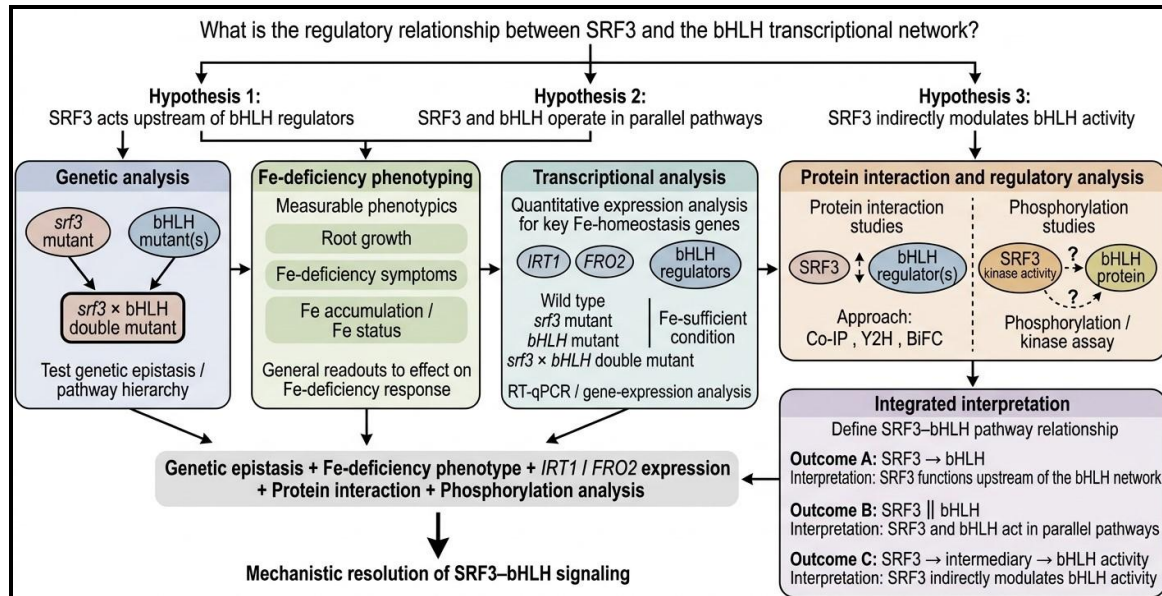


Figure 3. Experimental approach to addressing the regulatory relationship of *SRF3*–*bHLH*. Genetic, transcription and protein level studies can be combined to establish if *SRF3* acts upstream of the *bHLH* regulators, in parallel or indirectly modulates *bHLH* activity. Double-mutant analysis needs to be performed alongside Fe-phenotyping, *IRT1* and *FRO2* expression analysis, protein-interaction and phosphorylation studies to differentiate genetic pathway hierarchy from molecular association.

Future Directions: Further studies on the molecular and physiological regulation of Fe sensing, uptake, transport

and storage in plants are needed to address how these processes are coordinated. In *Arabidopsis*, the

relationship between *SRF3* mediated signaling and the *bHLH* transcriptional network should be defined. Genetic interaction studies, transcriptomic and proteomic analysis, protein phosphorylation analysis and protein-interaction methods will identify the potential upstream, parallel or post-translational modulation of the activity of the *bHLH* regulators by *SRF3*. In addition to *Arabidopsis*, comparative research in major crops is required to understand if conserved Fe-regulatory mechanisms can be utilized for enhanced Fe-use efficiency and nutrition quality.

Enhanced knowledge of Fe homeostasis will also aid to modern breeding strategies. When used with molecular markers, genomic selection and other precision-breeding methods, speed breeding can help to reduce generation times and rapidly develop Fe-efficient or Fe-biofortified cultivars. Candidate genes/natural alleles associated to Fe uptake, transport, translocation and storage can be used in breeding programs, and genome-wide strategies can be used to find genomic regions linked to Fe accumulation and Fe-use efficiency. Thus, the use of marker assisted selection (MAS) or genomic selection in combination with fast generation advancement might enable the transfer and assessment of desirable Fe related traits to be achieved more quickly. There are several alternate methods that could be explored to reduce Fe uptake and enhance Fe nutrition of crops. Conventional breeding and marker assisted selection can take advantage of the natural variation in Fe acquisition and transport, while genome editing could be used to alter specific regulatory genes and/or transporters. Where soil Fe availability is a limiting factor for plant growth, agronomic biofortification, such as adequate Fe fertilization, can be used as an extra tool. In addition, manipulation of plant-microbe interaction could be a better way to enhance Fe mobilization and acquisition in the rhizosphere. However, just provision of Fe is not enough, as Fe uptake can lead to Fe toxicity and/or disrupt the homeostasis of other mineral nutrients. Future strategies for crop improvement should thus focus on optimizing Fe acquisition, transport, utilization and storage, rather than just on optimizing the uptake of Fe.

In summary, the utilization of molecular genetics, speed breeding, genomic selection, genome editing and agronomic management gives an outline for the development of crops with improved Fe acquisition, efficient Fe use and improved nutritional value. Understanding the molecular connection between receptor-mediated signaling and Fe regulation of transcription will be a crucial next step in the search for useful targets for these applications.

CONCLUSION

In *Arabidopsis*, iron homeostasis is regulated by a multi-layered network in which *bHLH* transcription factors regulate the responses to Fe-deficiency and the

expression of Fe-acquisition genes. The FIT-Ib module directly affects some of the targets including *IRT1* and *FRO2*; *bHLH121*/URI and the IVc subgroup work upstream of this regulatory hierarchy. *SRF3* is an extra receptor-kinase-mediated level of regulation, which is necessary for proper activation of *IRT1* under Fe-deficiency. Direct genetic epistasis or physical interactions between *SRF3* and the *bHLH* network have not been shown, however. The answer to the question of whether *SRF3* works upstream of the *bHLH* regulators, in parallel to the *bHLH* pathway, or by post-translational modification of the *bHLH* pathway is therefore not known. Genetic analysis of the mutants, *SRF3* × *bHLH121* and selected downstream mutants, and a combination of transcriptional, protein-localization and protein-phosphorylation studies will aid in discriminating these models. The understanding of this relationship can also help elucidate the plant immune signaling and Fe homeostasis. In addition to *Arabidopsis*, understanding of Fe uptake and transport has the potential to be applied to crop improvement and nutritional biofortification. The future research should involve the use of genetic variation, molecular breeding, genome editing, and plant-microbe interactions to enhance Fe acquisition and accumulation without increasing Fe toxicity and undesirable interactions with other mineral nutrients. Therefore, the *SRF3*–*bHLH* interaction is a potential model to explore the regulation of Fe homeostasis by receptors, but the hierarchy of the interaction needs to be tested.

ABBREVIATIONS

bHLH, basic helix-loop-helix; FIT, fer-like iron deficiency-induced transcription factor; *IRT1*, IRON-REGULATED TRANSPORTER 1; *FRO2*, FERRIC REDUCTION OXIDASE 2; *BTS*, BRUTUS; *BTSL1/2*, BRUTUS-LIKE 1/2; *ILR3*, IAA-LEUCINE RESISTANT3 (*bHLH105*); *URI*, UPSTREAM REGULATOR OF *IRT1* (*bHLH121*); *PYE*, POPEYE; *IMA/FEP*, IRON MAN/FE-UPTAKE-INDUCING PEPTIDE; *SRF3*, STRUBBELIG RECEPTOR KINASE 3; *LRR-RK*, leucine-rich-repeat receptor kinase; *BAK1/SERK3*, BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE3; *PTI*, pattern-triggered immunity; *flg22*, flagellin-derived elicitor peptide; *GSL*, GLUCAN SYNTHASE-LIKE; *CIPK*, CBL-INTERACTING PROTEIN KINASE; *EIN3/EIL1*, ETHYLENE INSENSITIVE3/ETHYLENE INSENSITIVE3-LIKE1; *ISR*, induced systemic resistance; *JA*, jasmonate.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

AUTHOR CONTRIBUTION

Z.H. contributed to the conceptualization, literature review, data curation and manuscript drafting. S.F.I. contributed to literature analysis, interpretation of the available evidence and manuscript revision. A.R. contributed to literature review, data interpretation and critical revision of the manuscript. A.L. contributed to conceptual development, literature analysis and critical revision. M.S. contributed to literature analysis, visualization and manuscript editing. M.A. contributed to conceptualization, methodology, literature synthesis, manuscript writing, critical revision and overall supervision of the work. All authors reviewed and approved the final version of the manuscript and agreed to be accountable for the content of the work.

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