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Highlights: Within this review, current knowledge on the control of iron homeostasis in plants is presented and future prospects to improve our understanding of this complex mechanism are provided.

Abstract

Iron is one of the most important micronutrient for plant growth and development. It functions as the enzyme cofactor or component of electron transport chains in various vital metabolic processes, including photosynthesis, respiration and amino acid biosynthesis. For maintaining iron homeostasis, and therefore preventing any deficiency or excess that could be detrimental, plants have evolved complex transcriptional regulatory networks to tightly control iron uptake, translocation, assimilation and storage. Such regulatory networks are composed of various transcription factors among them members from the basic helix-loop-helix (bHLH) family play an essential role. Here, we first review recent advances in understanding the roles of bHLH transcription factors involved in the regulatory cascade controlling iron homeostasis in the model plant Arabidopsis, extending it to rice and other plant species. The importance of other classes of transcription factors will also be discussed. Secondly, we elaborate on the posttranslational mechanisms involved in the regulation of these regulatory networks. Finally, we give some perspectives on future research that should be conducted in order to precisely depict how plants control the homeostasis of this micronutrient.

Key words: *Arabidopsis thaliana*, basic helix-loop-helix, bHLH, iron homeostasis, rice, transcription factor.

Introduction

Iron (Fe) is an essential micronutrient for almost all living organisms. In humans, iron deficiency anemia is a major global health issue affecting about one billion people worldwide (Camaschella, 2015). Therefore, increasing the iron content in plants, especially in crops (biofortification), would have enormous benefits for human health. To achieve this goal, a critical question is to uncover the mechanisms controlling iron homeostasis in plants. Like in humans, iron is an essential microelement for plant growth and development. It functions in various vital metabolic processes (e.g. photosynthesis, respiration, amino acid biosynthesis) by acting as cofactor for several metalloproteins or component of electron transport chains (Hänsch and Mendel, 2009; Touraine *et al*., 2019). However, the excess of iron is deleterious to plants because of its capacity to interact with oxygen, generating reactive oxygen species (ROS) via the Fenton Reaction. Thus, the levels of iron in plant cells must be tightly regulated to avoid iron deficiency or iron excess, both of which severely affecting crops yield and the quality of their derived products (Briat *et al*., 2015).

Although iron is the fourth most-abundant element on earth, much of it is not readily available for plant use due to the poor solubility of its main (hydro)oxides, especially in neutral-to-alkaline soils (Guerinot and Yi, 1994; Colombo *et al*., 2018). To adapt to low iron conditions and acquire iron from soil, higher plants have evolved two different strategies (Marschner and Römheld, 1994) (Figure 1). Dicots and non-graminaceous monocots (non-grass species) employ the reduction strategy (named Strategy I). The graminaceous utilize the chelation strategy (named Strategy II). In strategy I, non-grass plants are able to acidify the rhizosphere to promote iron solubility through protons release and mobilize Fe^{3+} through coumarins (*i.e.* fraxetin and sideretin) or riboflavins secretion (Santi and Schmidt, 2009; Fourcroy *et al*., 2016; Robe *et al*., 2020). Following iron mobilization, Fe^{3+} is reduced into Fe^{2+} (a more soluble form of iron) that is subsequently transported into the root epidermal cells via high affinity iron transporters from the Zrt/Irt-like protein (ZIP) family.

In *Arabidopsis thaliana*, the release of protons into the rhizosphere is insured by the H^+-ATPASE 2 (AHA2; Santi and Schmidt, 2009) whereas the reduction of iron and its translocation into the roots are ensured by FERRIC REDUCTION OXIDASE 2 (FRO2) and IRON-REGULATED TRANSPORTER1 (IRT1), respectively (Brumbarova *et al*., 2015; Connorton *et al*., 2017). AHA2, FRO2 and IRT1 associate into a complex at the surface of the

root epidermal cells most likely allowing optimizing iron acquisition and therefore its uptake (Martín-Barranco *et al*., 2020). Coumarins secretion is ensure by the PLEIOTROPIC DRUG RESISTANCE 9/ATP-BINDING CASSETTE G37 (PDR9/ABCG37) transporter (Fourcroy *et al*., 2014; Fourcroy *et al*., 2016). In strategy II, plants biosynthesize and secrete phytosiderophores of the mugineic acid family (MAs) into the rhizosphere to chelate Fe^{3+} (Kobayashi *et al*., 2014). Fe^{3+}-MAs complexes are then transported into root cells by transporters of the YELLOW STRIPE 1 (YS1) and YELLOW STRIPE 1–like (YSL) family (Inoue *et al*., 2009; Murata *et al*., 2006). Interestingly, it was recently shown that some plant species might use both strategies. For instance, when grown in waterlogged soil condition, rice (*Oryza sativa*), a graminaceous species, acquires Fe^{2+} from the soil through the activity of two Fe^{2+} transporters, OsIRT1 and OsIRT2 (Ishimaru *et al*., 2006). The secretion of caffeic and protocatechuic acids via the PHENOLICS EFFLUX ZERO 2 (PEZ2) transporter participates to Fe^{3+} mobilization and reduction into Fe^{2+} (Bashir *et al*., 2011). If iron acquisition is the first step that participates to the maintenance of iron homeostasis in plants, it should be noted that it is not the sole mechanism involved in this process. Iron translocation, compartmentalization, assimilation and storage are also important processes required for the maintenance of iron homeostasis at the cellular and subcellular levels, throughout the whole plant body (Kobayashi and Nishizawa, 2012; Kobayashi *et al*., 2019).

Gene regulation is a crucial step for coping with iron fluctuations. For instance, *FRO2* and *IRT1* expression is induced when iron availability is low whereas the expression of the genes encoding the iron storage ferritin proteins (i.e. *FER1, 3* and *4*) is induced in response to iron excess (Tissot *et al*., 2019). How plants control iron homeostasis, by regulating the expression of genes involved in the various facets of this complex mechanism, was a critical question for the past three decades. To address this question, several studies mostly based on forward and reverse genetic approaches were conducted, leading to the identification and characterization of several transcription factors (TFs). These studies, mostly conducted in Arabidopsis and rice, allowed establishing regulatory networks controlling iron homeostasis in which basic helix-loop-helix (bHLH) TFs play a preponderant role (Gao *et al*., 2019; Gao *et al*., 2020a; Li *et al*., 2019) (Figure 2 and Table 1). bHLH proteins form one of the largest families of TFs (Heim *et al*., 2003) known to modulate several facets of plant growth and development, including cell differentiation, secondary metabolite biosynthesis, hormone signaling or the responses to environmental factors (Carretero-Paulet *et al*., 2010). Indeed,

additional TFs and protein regulating TFs activity were also identified (Yan *et al*., 2016; Palmer *et al*., 2013; Rodríguez-Celma *et al*., 2019a).

Within this review, current knowledge on the control of iron homeostasis by TFs, especially from the bHLH family, will be presented and discussed with a special emphasis on the latest findings. The transcriptional and posttranslational regulation of the iron homeostasis regulatory networks will also be documented. Last, some perspectives on future research to be conducted in order to improve our understanding of this complex mechanism will be provided.

The transcriptional regulation of iron homeostasis in strategy I plants, a preponderant role for bHLH TFs

The model plant Arabidopsis has allowed the identification of several factors involved in the regulation of iron homeostasis, notably by studying its response to iron deficiency. Such studies highlighted that the regulation of iron homeostasis was essentially occurring at the transcriptional level and was involving several TFs, in particular those of the bHLH family. This topic has also been covered in recent reviews by Gao *et al*., 2019; Kobayashi, 2019; Kobayashi *et al*., 2019; Wu and Ling, 2019; Schwarz and Bauer, 2020.

To date, at least six bHLH TF subfamilies (Heim *et al*., 2003), encoding 17 different proteins, are known to participate to the maintenance of iron homeostasis in Arabidopsis. These bHLH TFs form an intricate regulatory network composed of two interconnected regulatory modules (Figure 3).

The first module relies on the activity of FIT/bHLH29 (FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR), a clade IIIa bHLH TF (Colangelo and Guerinot, 2004; Jakoby *et al*., 2004; Yuan *et al*., 2005). FIT/bHLH29 is a direct regulator of *IRT1* and *FRO2* expression, highlighting its central role for the regulation of the iron uptake machinery (Wang *et al*., 2013b). FIT/bHLH29 activity relies on its interaction with the four members of the Ib bHLH clade (i.e. bHLH38, bHLH39, bHLH100 and bHLH101), forming heterodimer complexes displaying partial redundant activities (Colangelo and Guerinot, 2004; Maurer *et al*., 2014; Yuan *et al*., 2008; Wang *et al*., 2013b) (Figure 3). Recently, the members of the IVa bHLH clade (*i.e.* bHLH18, bHLH19, bHLH20 and bHLH25) were identified as FIT/bHLH29 interacting proteins (Cui *et al*., 2018). These interactions were shown to

promote the degradation of FIT/bHLH29 via the 26S proteasome pathway, in a jasmonic acid-dependent manner (Cui *et al*., 2018). It is noteworthy that the Ib bHLH and the IVa bHLH TFs antagonize the activity of each other in regulating FIT/bHLH29 protein accumulation to tightly regulate the iron uptake machinery in response to different environmental stimuli (Cui *et al*., 2018) (Figure 3).

The second module acts upstream from FIT/bHLH29. It involves the four members of the IVc bHLH clade, namely ILR3/bHLH105 (IAA-LEUCINE RESISTANT 3), IDT1/bHLH34 (IRON DEFICIENCY TOLERANT 1), bHLH104 and bHLH115. These four TFs play additive roles in the iron deficiency responses and their activity is thought to rely, at least in part, on their ability to form homo- or heterodimers (Li *et al*., 2016; Zhang *et al*., 2015; Liang *et al*., 2017) (Figure 3). In response to iron deficiency, these TFs directly activate the expression of clade Ib bHLH and indirectly the one of *FIT/bHLH29* (Li *et al*., 2016; Zhang *et al*., 2015; Liang *et al*., 2017).

Clade IVb bHLH TFs (*i.e.* PYE/bHLH47, bHLH11 and URI/bHLH121), also participate to the regulation of iron homeostasis in Arabidopsis. PYE/bHLH47 is a negative regulator (Long *et al*., 2010), which contains an EAR motif at its C-terminal region (*i.e.* DLNxxP; Kagale and Rozwadowski, 2011) that directly represses the expression of genes participating to the maintenance of iron homeostasis. Interaction studies highlighted that PYE/bHLH47 could heterodimerize with ILR3/bHLH105 and bHLH115 (Long *et al*., 2010; Zhang *et al*., 2015; Tissot *et al*., 2019). However, the role of these interactions was still a matter of debate until recently. In a recent study, ILR3/bHLH105 was found to play a central role in the regulation of iron homeostasis where it acts as both transcriptional activator and repressor of the plant responses to iron deficiency and excess, respectively (Kroh and Pilon, 2019; Tissot *et al*., 2019). In this study, the authors showed that the repressive activity of ILR3/bHLH105 was conferred by its dimerization with PYE/bHLH47 (Figure 3). The authors also highlighted that ILR3-PYE heterodimers might repress the expression of *PYE/bHLH47* when iron availability is not limiting *via* a negative feedback regulatory loop (Figure 3). bHLH11 is another transcriptional repressor also containing an EAR motif (*i.e.* LxLxL) in its C-terminal domain (Tanabe *et al*., 2019). Overexpression studies suggest that bHLH11 inhibits the plant tolerance to iron deficiency and the expression of *IRT1* and *FRO2*, most probably by indirectly repressing the expression of *FIT/bHLH29* (Tanabe *et al*., 2019) (Figure 3). In contrast to PYE/bHLH47 and bHLH11, URI/ bHLH121 (UPSTREAM REGULATOR OF

IRT1) has been recently identified and characterized by three different groups as a positive regulator of the plant responses to iron deficiency (Lei *et al*., 2020; Lockhart, 2020; Gao *et al*., 2020a; Kim *et al*., 2019). URI/bHLH121 can form heterodimers with clade IVc bHLH TFs (Lei *et al*., 2020; Gao *et al*., 2020a; Kim *et al*., 2019) (Figure 3). These interactions participate to the relocation of URI/bHLH121 from the cytosol into the nucleus (Lei *et al*., 2020). It also suggests that the transcriptional activation of clade IVc bHLH target genes requires, at least in part, URI/bHLH121(Lei *et al*., 2020; Gao *et al*., 2020a; Kim *et al*., 2019). In support of this assertion, it was demonstrated that several genes directly targeted by URI/bHLH121 are identical to that of clade IVc bHLH TFs (Figure 3). The expression of *FIT/bHLH29* also relies on URI/bHLH121 activity, most probably *via* an indirect mechanism (Lei *et al*., 2020; Gao *et al*., 2020a; Kim *et al*., 2019). It is noteworthy that under iron deficiency condition, URI/bHLH121 accumulates in its phosphorylated form (Figure 3) that increases its binding capacity to the promoter region of its target genes such as the clade Ib bHLH TFs (Kim *et al*., 2019). Interestingly, both URI/bHLH121 transcript and protein accumulate constitutively regardless of iron status (Gao *et al*., 2020a; Kim *et al*., 2019). In contrast, URI/bHLH121 cellular localization in roots differs depending on iron availability (Gao *et al*., 2020a). When iron is not limiting, URI/bHLH121 mainly localizes in the stele and the endodermis, whereas under iron deficiency condition, URI/bHLH121 is primarily observed in the cortex and the epidermis cells, where it promotes iron uptake (Gao *et al*., 2020a). The thorough characterization of URI/bHLH121 indicates that it plays a key role in the control of plant iron homeostasis mainly because it directly or indirectly regulates the expression of most of the known genes involved in this regulatory network (Gao *et al*., 2020a; Kim *et al*., 2019) (Figure 3). Interestingly, it was recently reported that URI/bHLH121 directly activates *FER1*, *FER3* and *FER4* expression when iron availability is not in excess, indicating that URI/bHLH121 positively regulates the transient storage of iron in addition to the iron deficiency response (Gao *et al*., 2020b).

Functional homologs of most of the above-described Arabidopsis bHLH TFs have been characterized in several dicots (Figure 2, Table 1) indicating that this regulatory mechanism is most likely conserved within strategy I plants.

bHLH TFs involved in the regulation of iron homeostasis in strategy II plants

As stated above, plants have developed two different strategies to take up iron from the soil, distinguishing the non-graminaceous (Strategy I, reduction strategy) and graminaceous (Strategy II, chelation strategy) species (Figure 1). However, the in depth study of the responses to iron deficiency in rice has highlighted that the regulation of iron homeostasis in strategy II plants involves the activity of several bHLH TFs belonging to the same clades than those identified in strategy I plant species (Figures 2 and 4, Table 1; Kobayashi, 2019; Kobayashi *et al*., 2019).

OsFIT/OsbHLH156 was recently identified as a positive regulator of the iron deficiency responses (Liang *et al*., 2020; Wang *et al*., 2020). Loss-of-function of *OsFIT/OsbHLH156* resulted in strong iron deficiency symptoms under upland condition, whereas no iron deficiency symptoms were observed when plants were grown in waterlogged soil (Wang *et al*., 2020). These results imply that the strategy II iron uptake system was impaired in the *Osfit* mutant, an hypothesis that is supported by the disruption of the expression of strategy II iron uptake related genes. It includes genes encoding enzyme involved in MAs biosynthesis (*e.g. NAS1* and *NAS2*, *NICOTIANAMINE SYNTHASE 1* and *2*) or Fe^{3+}-MA transport (*e.g. OsYSL15*, *YELLOW STRIPE-LIKE 15*) (Figure 1) (Liang *et al*., 2020; Wang *et al*., 2020). Interestingly, it was demonstrated that OsFIT/OsbHLH156 regulates the expression of *OsIRT1* and therefore participates also to the regulation of strategy I iron uptake mechanism (Liang *et al*., 2020) (Figure 1). OsFIT/OsbHLH156 interacts with OsIRO2/OsbHLH56, a clade Ib bHLH, and promotes its nuclear accumulation (Figure 4) (Liang *et al*., 2020; Wang *et al*., 2020). OsIRO2/OsbHLH56 was the first characterized bHLH TF involved in the control of iron homeostasis in rice, where it acts as a positive regulator of the iron deficiency response (Ogo *et al*., 2006; Ogo *et al*., 2007). Expression analysis indicated that the regulation of *OsIRT1* expression by OsFIT/OsbHLH156 might be different to that of Arabidopsis or that it might involve other Ib bHLH TFs than OsIRO2/OsbHLH56.

Three out of the four clade IVc bHLH TFs (*i.e.* OsPRI1/OsbHLH060, OsPRI2/bHLH058, OsPRI3/OsbHLH059; POSITIVE REGULATOR OF IRON HOMEOSTASIS 1, 2 and 3) have been identified in rice as playing a positive role in the iron deficiency responses (Zhang *et al*., 2017; Kobayashi *et al*., 2019; Zhang *et al*., 2020b). The characterization of loss-of-function mutants suggested that these three TFs directly regulate the expression of

OsIRO2/OsbHLH56, and indirectly the expression of *OsNAS1*, *OsNAS2*, and *OsYSL15 via* OsIRO2/OsbHLH56 (Figure 4) (Zhang *et al*., 2020b; Zhang *et al*., 2017). Among the potential direct targets of OsPRI1/OsbHLH060, OsPRI2/bHLH058 and OsPRI3/OsbHLH059, there is also *OsIRO3*/*OsbHLH63* (Figure 4) (Zhang *et al*., 2020b; Zhang *et al*., 2017). *OsIRO3*/*OsbHLH63* is a member of the IVb clade and the functional homolog of PYE/bHLH47 (Zheng *et al*., 2010). Like PYE/bHLH47, OsIRO3/OsbHLH63 functions as a negative regulator of the iron deficiency responses (Zheng *et al*., 2010). It is likely that OsIRO3/OsbHLH63 activity might antagonize OsIRO2/OsbHLH56 to tightly regulate iron uptake and avoid iron overload (Zhang *et al*., 2012; Zhang *et al*., 2020b; Zhang *et al*., 2017; Zheng *et al*., 2010). To date, there is no information on the role of OsbHLH057, the fourth member of the rice clade IVc bHLH. Indeed, based on the information gathered in Arabidopsis, it is likely that OsbHLH057 participates to the control of iron homeostasis in rice. Whether OsbHLH057 exerts a minor role in specific cell types or in specific environmental conditions remains to be determined.

OsbHLH133 functions as a negative regulator of iron translocation from roots to shoots (Wang *et al*., 2013a). It should be noted that OsbHLH133 is the only one member of the clade VIIIc reported to date as involved in the regulation of iron homeostasis in plants. The other members of this clade in Arabidopsis play a positive role in root hair development but have never been associated with the maintenance of iron homeostasis (Bruex *et al*., 2012). Whether these bHLHs have a similar and conserved role, as that of OsbHLH133 in the regulation of iron homeostasis in strategy II plants, is to be investigated.

The transcriptional regulation of iron homeostasis is not restricted to the activity of bHLH TFs

TFs from other families are involved in regulatory networks acting for iron homeostasis in both strategy I and strategy II plants (Figures 3 and 4, Table 2).

Several R2R3-MYB TFs from different plant species have been shown to be involved in the regulation of iron deficiency responses. In Arabidopsis, MYB10 and MYB72 are two iron deficiency inducible TFs required for proper iron uptake and whose expression is partially dependent on FIT/bHLH29 and URI/bHLH121 activities (Figure 3) (Gao *et al*., 2020a; Palmer *et al*., 2013; Zamioudis *et al*., 2014). Furthermore, MYB72 has been identified as a

transcriptional activator of genes associated with the biosynthesis and secretion of iron-mobilizing coumarins, highlighting its role in strategy I iron uptake system (Stringlis *et al*., 2018; Zamioudis *et al*., 2014) (Figure 1). Recently, it was shown that the Arabidopsis MYB28 and MYB29 are at the interface of the plant sensitivity to ammonium stress and the modulation of iron homeostasis (Coleto *et al*., 2020). Notably, the ammonium-dependent decrease of *MYB28* and *MYB29* expression (or the loss-of-function of both genes) leads to defects in iron translocation from roots to shoots and to the induction of the expression of *FIT/bHLH29*, clade Ib bHLHs, *MYB72* as well as *IMA1/FEP3* and *IMA3/FEP1* in roots. MdMYB58, a close homolog of MYB72, was recently characterized as a positive regulator of iron uptake and translocation in apple (Wang *et al*., 2018). Further investigation showed that MdMYB58 transcriptional activity is inhibited by its heterodimerization with MdSAT1/MdbHLH18, a IVa clade bHLH TF (Wang *et al*., 2018). In contrast to the above-mentioned MYB TFs, MxMYB1 may function as a negative regulator of iron uptake and storage (Shen *et al*., 2008).

In Arabidopsis, WRKY46 plays a role in iron translocation between root and shoot by directly regulating the expression of *VITL1* (*VACUOLAR IRON TRANSPORTER-LIKE 1*), a potential iron transporter involved in iron sequestration into the vacuoles (Yan *et al*., 2016; Gollhofer *et al*., 2014). HAP5A/NF-YC1 is also involved in iron translocation between root and shoot by regulating the expression of *NAS1* (Zhu *et al*., 2020). ERF4 and ERF72 are two Arabidopsis TFs belonging to AP2/ERF family that have been reported as potential negative regulators of iron deficiency responses by repressing the expression of genes involved in iron uptake such as *IRT1* (Liu *et al*., 2017a; Liu *et al*., 2017b). Similarly, MbERF4 and MbERF72 from *Malus baccata* as well as MxERF4 from *Malus xiaojinensis* act as negative regulators of the iron deficiency responses in these two apple species (Liu *et al*., 2018; Zhang *et al*., 2020a). In contrast, ERF95 was recently proposed to promote iron storage in Arabidopsis seeds (Sun *et al*., 2020). However, how iron distribution is controlled in seeds is not clearly established even if it was recently proposed that B3 TFs, which are involved in the regulation of embryo development and seed maturation, might be good candidates (Roschzttardtz *et al*., 2020).

IDEF1 and IDEF2 (IRON DEFICIENCY-RESPONSIVE ELEMENT FACTOR 1 and 2) are two rice TFs belonging to two different families, the ABI3/VP1 and NAC, respectively (Figure 4) (Kobayashi *et al*., 2007; Ogo *et al*., 2008). These two TFs participate to the regulation of iron homeostasis in rice. IDEF1 is required for the coordinated activation of

genes related to iron uptake and translocation, including *OsIRT1*, *OsNAS1*, *OsNAS2* and *OsYSL15* (Kobayashi *et al*., 2009; Kobayashi *et al*., 2007). In addition, IDEF1 positively regulates the expression of *OsIRO2*, indicating that IDEF1 functions upstream of OsIRO2 in the iron deficiency regulatory network (Figure 4) (Kobayashi *et al*., 2009; Kobayashi *et al*., 2007). Interestingly, it was shown that IDEF1 could bind to iron and zinc atoms and that this capacity was necessary for its activity (Kobayashi *et al*., 2012). It was therefore proposed that IDEF1 could sense the cellular metal ion balance caused by changes in iron availability, suggesting that IDEF1 could be a cellular iron sensor allowing the tight regulation of the iron deficiency responses (Kobayashi *et al*., 2012). Similarly to IDEF1, IDEF2 plays a positive role in the plant response to iron deficiency (Ogo *et al*., 2008).

A family of peptides named IMA/FEP (IRONMAN/FE-UPTAKE-INDUCING PEPTIDE) has been reported to play a positive role in iron deficiency responses in Arabidopsis, by regulating a set of deficiency-inducible genes including Ib bHLH TFs, a function that seems to be conserved across plant species (Grillet *et al*., 2018; Hirayama *et al*., 2018). Two recent studies showed that URI/bHLH121 is a direct positive regulator of *IMA1/FEP3* and *IMA2/FEP2* expression (Gao *et al*., 2020a; Kim *et al*., 2019). These results indicate that IMAs/FEPs are implicated in the bHLH-dependent regulatory network regulating iron homeostasis. However, the precise regulatory mechanisms by which IMAs/FEPs act are still unknown.

Post-translational regulation of the iron homeostasis regulatory networks

Protein-protein interactions as well as post-translational modifications (*i.e.* ubiquitination, sumoylation, phosphorylation) can significantly affect the regulatory activities of TFs. Such mechanisms were shown to play an important role in the maintenance of iron homeostasis in plants. This topic has also been covered in recent reviews by Kobayashi, 2019; Rodríguez-Celma *et al*., 2019a; Schwarz and Bauer, 2020; Spielmann and Vert, 2020; Wu and Ling, 2019.

As described earlier, the transcriptional activity of bHLH TFs involved in the control of iron homeostasis is extensively dependent on *in vivo* protein-protein interactions, in the form of homo- or heterodimers (Figures 3 and 4, Tables 1 and 2). For instance, FIT/bHLH29 heterodimerization with clade Ib bHLH TFs is required for its transcriptional activity and

stability, whereas its interaction with clade IVa members promotes its degradation *via* the 26S proteasome pathway (Cui *et al*., 2018). bHLH39 nuclear localization also depends on its interaction with FIT/bHLH29 since in the cells lacking FIT/bHLH29, bHLH39 localizes predominantly in the cytoplasm (Trofimov *et al*., 2019). Similarly, OsFIT/OsbHLH156 facilitates the nuclear localization of OsIRO2/OsbHLH156, the functional homolog of bHLH39 in rice, suggesting that this post-translational regulatory mechanism is conserved within the plant kingdom (Liang *et al*., 2020; Wang *et al*., 2020). FIT/bHLH29 activity and/or stability are also modulated by its interaction with several additional protein partners that do not belong to the bHLH family of TFs (Figures 3, Tables 1) (reviewed in Kobayashi, 2019; Schwarz and Bauer, 2020; Spielmann and Vert, 2020; Wu and Ling, 2019).

Several ubiquitin E3 ligases have been identified and characterized as negative regulators of iron uptake to avoid potential iron overload by targeting bHLH TFs for their degradation (for details, see Spielmann and Vert, 2020; Rodríguez-Celma *et al*., 2019a) (Figures 3 and 4). BTS (BRUTUS), whose expression is induced by iron deficiency in roots, is proposed to be a critical iron sensing E3 ubiquitin ligase in Arabidopsis (Long *et al*., 2010). BTS interacts with ILR3/bHLH105 and bHLH115 to facilitate their degradation via the 26S proteasome pathway, allowing the fine tuning of the expression of downstream iron deficiency response genes (Figure 3) (Selote *et al*., 2015; Long *et al*., 2010). Similarly, OsHRZ1 and OsHRZ2 (HAEMERYTHRIN MOTIF-CONTAINING REALLY INTERESTING NEW GENE (RING) AND ZINC-FINGER PROTEIN 1 and 2), two rice ubiquitin E3 ligases displaying strong sequence similarities with BTS, have been reported as potential iron sensors playing a negative role in iron acquisition under iron sufficient conditions (Kobayashi *et al*., 2013). It was shown that OsHRZ1 could interact with OsPRI1/OsbHLH60, OsPRI2/OsbHLH58 and OsPRI3/OsbHLH59 and mediate their degradation via the 26S proteasome (Figure 4) (Zhang *et al*., 2020b; Zhang *et al*., 2017). However, different results were reported in another study only validating the sole interactions between OsHRZ1 and OsHRZ2 with OsPRI1/OsbHLH60 and OsPRI2/OsbHLH58 (Kobayashi *et al*., 2019). Another *in vitro* ubiquitination study showed that neither OsPRI1/OsbHLH60, nor OsPRI2/OsbHLH58 or OsPRI3/OsbHLH59 were ubiquitinated by OsHRZ1 or by OsHRZ2 (Kobayashi *et al*., 2019). These discrepancies may be due to the different methodologies and materials used in these studies. Whether these interactions participate to the ubiquitination and degradation of OsPRI1/OsbHLH60, OsPRI2/OsbHLH58 and OsPRI3/OsbHLH59 remains to be further demonstrated and confirmed. IDEF1, whose activity is necessary for *HRZs* expression, is also

degraded via the 26S proteasome pathway through a yet uncharacterized mechanism (Zhang *et al*., 2014). However, IDEF1 degradation is inhibited by its interaction with the IBP1.1 (IDEF1-BINDING PROTEINS 1.1) Bowman–Birk trypsin inhibitor protein (Figure 4) (Zhang *et al*., 2014). BTSL1 and BTSL2, two Arabidopsis homologs of BTS, function redundantly as negative regulators of the iron deficiency response (Hindt *et al*., 2017). In addition, BTSL1 and BTSL2 could directly target FIT/bHLH29 and promote its ubiquitination and subsequent degradation via the 26S proteasome pathway, thus negatively regulating the expression of iron uptake related genes (Figure 3) (Rodríguez-Celma *et al*., 2019b; Rodríguez-Celma *et al*., 2019a). In apples, MdBT1 and MdBT2, two BTB-TAZ proteins, interact with MdbHLH104. MdBT proteins interact as well with MdCUL3 (CULLIN-RING UBIQUITIN LIGASE 3) to form $MdBT^{MdCUL3}$ complexes required for MdbHLH104 ubiquitination and degradation *via* the 26S proteasome pathway (Zhao *et al*., 2016a). In contrast, MdSIZ1, a SIZ/PIAS-type SUMO E3 ligase, directly sumoylates MdbHLH104, especially under iron deficiency conditions, to enhance its stability (Zhou *et al*., 2019). Recently, it was shown that the mutation of an alanine into valine within IDT1/bHLH34 enhances both its stability and nuclear localization (Sharma and Yeh, 2020). Since this amino acid is conserved among clade IVc bHLH TFs in both monocots and dicots (Sharma and Yeh, 2020), one might speculate that this amino acid plays an important role in regulating the stability of clade IVc bHLH and thus their degradation via the 26S proteasome.

Phosphorylation plays also an important role in determining TF activity in the iron homeostasis networks (Figure 3). In Arabidopsis, FIT/bHLH29 phosphorylation by the calcium-dependent protein kinase CIPK11 (CBL-INTERACTING PROTEIN KINASE 11) positively regulates its activity by favoring its nuclear accumulation and dimerization with bHLH39 (Gratz *et al*., 2019). *CIPK11* is induced and activated *via* a CBL1/9-mediated Ca^{2+} sensing pathway under iron deficiency conditions. CIPK21 could also interact with FIT/bHLH29, indicating that this protein kinase might have a potential role in the regulation of the FIT/bHLH29-dependent iron deficiency responses (Gratz *et al*., 2019). Recently, the phosphorylation of URI/bHLH121 was also reported as a key mechanism that regulates its activity (Kim *et al*., 2019). It was shown that the phosphorylated form of URI/bHLH121 only accumulates in response to iron deficiency, whereas the turnover of phosphorylated URI/bHLH121 is dependent on BTS activity (Kim *et al*., 2019). Under iron deficiency, URI/bHLH121 showed enhanced heterodimerization capability with IVc bHLH TFs and increased binding ability to the promoter of its target genes, indicating that the

phosphorylation of URI/bHLH121 plays a positive role in the iron deficiency responses (Kim *et al*., 2019). Nevertheless, since URI/bHLH121 can activate the expression of *FER1*, *FER3* and *FER4* in the stele when iron availability is not limiting (Gao *et al*., 2020b), a growth condition where the phosphorylated form of bHLH121 is degraded (Kim *et al*., 2019), it is likely that URI/bHLH121 is transcriptionally active independently of its phosphorylation state. Interestingly, as stated earlier, the pattern of accumulation of URI/bHLH121 within the root cells is controlled by an iron-dependent mechanism. URI/bHLH121 is preferentially accumulated in the epidermis and the cortex when plants are grown under iron deficiency and in the stele when grown under iron sufficiency. From these observations, one might hypothesize that URI/bHLH121 cellular distribution, rather than its transcriptional activity, is regulated by its phosphorylation state. However, the precise regulatory mechanism leading to the phosphorylation of URI/bHLH121 remains to be characterized.

Conclusions and future prospects

In the past two decades, remarkable progresses have been made in decrypting the molecular mechanisms that regulate iron homeostasis in both strategy I and strategy II plants (Figure 1). They highlighted that iron homeostasis in plants is regulated at the transcriptional level and involves several bHLH TFs that function in intricate regulatory networks (Figures 2 to 4).

The function of most of these bHLH TFs (*i.e.* clades Ib, IIIa, IVb and IVc) is conserved among grass and non-grass species (Figure 2), in contrast to the downstream target genes of iron acquisition machinery, which are distinctive in strategy I and strategy II plants (Figure 1). How these bHLH functional homologs have evolved to target different genes is an intriguing question. bHLH from clade IVa are involved in the regulation of iron homeostasis in Arabidopsis but whether or not this clade of bHLH TFs has a similar role in other plant species needs to be investigated. Additional investigations will also be necessary to determine if the non-grass homologs of OsbHLH133 (clade VIIIc) play a role in the control of iron homeostasis. Other regulatory proteins are also conserved between plants from both strategies. It is for instance the case for MYB and ERF TFs (Table 2) or for haemerythrin E3-ubiquitin ligases. Interestingly, BTB-TAZ-CUL3 ubiquitin ligase complexes participate to the degradation of clade IVc bHLH in apples. To date, such mechanism has only been observed in apple, raising the question of the conservation of this regulatory mechanism in other plants species, from non-grass to grass and from perennials to annuals. Ubiquitination,

as well as phosphorylation and sumoylation, are crucial posttranslational modifications for the regulation of key bHLH TFs activities involved in the iron homeostasis networks, notably clade IVc bHLH FIT/bHLH29 and URI/bHLH121. Whether such modifications participate to the regulation of other TF activities within this network is still to be assessed. Determining the degree of conservation of this regulatory network between plants from strategy I and strategy II, and between annual and perennial species, is still on its own a question that deserves to be addressed.

How plants sense iron status and switch on or repress the downstream regulatory network to regulate iron uptake, translocation, storage or assimilation has remained elusive. To date, haemerythrin E3-ubiquitin ligases are the main candidates (Rodríguez-Celma *et al*., 2019a). These E3-ubiquitin ligases are induced in response to iron deficiency, participate to the degradation of key bHLH TFs (*i.e.* clade IVc and FIT/bHLH29) and are destabilized upon iron binding at their haemerythrin motifs. IDEF1, the rice iron binding TF mentioned earlier, was also proposed as a potential iron sensor (Kobayashi *et al*., 2012; Kobayashi *et al*., 2009; Kobayashi *et al*., 2007). The characterization of IDEF1 functional homologs in non-grass species would be an additional element in support of this later hypothesis. Another important question concerns the regulation of the most upstream bHLH TFs. For instance, the main challenges would be to determine how the expression of clade IVc bHLH is controlled and to identify which protein kinase modulates URI/bHLH121 activity.

Recently, epigenetic regulation has emerged as playing important role in the control of iron homeostasis, by regulating DNA accessibility to promoters, and thus the expression of both TFs and iron uptake genes. This is for instance the case in Arabidopsis with the iron-dependent deposition of repressive marks on H3 histone (i.e. H3K27m3) at the promoter *loci* of *FIT/bHLH29*, *IRT1* and *FRO2* (Park *et al*., 2019). This additional layer of regulation in the transcriptional networks controlling iron homeostasis raises questions on the importance and the significance of such regulatory mechanism in this process.

Within this review, it is possible to grasp the extent of the complexity of the regulatory networks that regulate iron homeostasis in plants. Nevertheless, another level of complexity still to be investigated concerns the apparent redundancies existing between several TFs, the localization of their target genes, and thus the physiological functions that are controlled. This will be achieved by the in depth study of the tissue and cellular localization of the

different TFs as well as the proteins involved in their regulation. Such knowledge is necessary to fully decrypt and understand the dynamics of this regulatory process. Unfortunately, such data are only available for a few of these proteins (Long *et al*., 2010; Gao *et al*., 2020a; Samira *et al*., 2018).

In conclusion, much work still lies ahead to fully comprehend the transcriptional regulatory network that regulate iron homeostasis in plants, which might offer novel opportunities for improving plant growth and health and for generating iron-fortified crops.

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Author Contribution

FG and CD wrote the manuscript

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Table 1. bHLH TFs involved in the regulation of iron homeostasis in plants.

Name	Clade	Species	Interacting proteins	Reference
AtbHLH38*	Ib	*A. thaliana*	FIT/AtbHLH29, DELLAs	Yuan *et al*., 2008; Wang *et al*., 2013
AtbHLH39*	Ib	*A. thaliana*	FIT/AtbHLH29, DELLAs	Yuan *et al*., 2008; Wang *et al*., 2013
AtbHLH100*	Ib	*A. thaliana*	FIT/AtbHLH29	Wang *et al*., 2013
AtbHLH101*	Ib	*A. thaliana*	FIT/AtbHLH29	Wang *et al*., 2013
FIT/AtbHLH29 *	IIIa	*A. thaliana*	AtbHLH38, AtbHLH39, AtbHLH100, AtbHLH101, AtbHLH18, AtbHLH19, AtbHLH20, AtbHLH25, BTSL1, BTSL2, CIPK11, DELLAs, EIL1, EIN3, MED16, ZAT12	Colangelo and Guerinot, 2004; Jakoby *et al*., 2004; Yuan *et al*., 2005
MYC2/AtbHLH6	IIIe	*A. thaliana*		Cui *et al*., 2018
AtbHLH18*	IVa	*A. thaliana*	FIT/AtbHLH29	Cui *et al*., 2018
AtbHLH19*	IVa	*A. thaliana*	FIT/AtbHLH29	Cui *et al*., 2018
AtbHLH20*	IVa	*A. thaliana*	FIT/AtbHLH29	Cui *et al*., 2018
AtbHLH25*	IVa	*A. thaliana*	FIT/AtbHLH29	Cui *et al*., 2018
AtbHLH11	IVb	*A. thaliana*	IDT1/AtbHLH34, AtbHLH104, ILR3/AtbHLH105, AtbHLH115	Tanabe *et al*., 2019
PYE/AtbHLH47	IVb	*A. thaliana*	ILR3/AtbHLH105, AtbHLH115	Long *et al*., 2010
URI/AtbHLH121	IVb	*A. thaliana*	IDT1/AtbHLH34, AtbHLH104, ILR3/AtbHLH105, AtbHLH115	Gao *et al*., 2020b; Kim *et al*., 2019; Lei *et al*., 2020
IDT1/AtbHLH34	IVc	*A. thaliana*	IDT1/AtbHLH34, AtbHLH104,	Li *et al*., 2016

			ILR3/AtbHLH105, AtbHLH115, AtbHLH11, URI/bHLH121	
AtbHLH104	IVc	*A. thaliana*	IDT1/AtbHLH34, AtbHLH104, ILR3/AtbHLH105, AtbHLH115, AtbHLH11, URI/ bHLH121	Zhang *et al*., 2015
ILR3/AtbHLH105	IVc	*A. thaliana*	IDT1/AtbHLH34, AtbHLH104, ILR3/AtbHLH105, AtbHLH115, AtbHLH11, PYE/ bHLH47, URI/bHLH121, BTS	Zhang *et al*., 2015
AtbHLH115	IVc	*A. thaliana*	IDT1/AtbHLH34, AtbHLH104, ILR3/AtbHLH105, AtbHLH115, AtbHLH11, PYE/ bHLH47, URI/bHLH121, BTS	Liang *et al*., 2015
OsIRO2/OsbHLH56	Ib	*O. sativa*	OsFIT/OsbHLH156	Ogo *et al*., 2006
OsIRO3/OsbHLH63	IVb	*O. sativa*		Zheng et al., 2010
OsFIT/OsbHLH156	IIIa	*O. sativa*	OsIRO2/OsbHLH56	Liang *et al*., 2020; Wang *et al*., 2020
OsPRI1/OsbHLH60	IVc	*O. sativa*	OsHRZ1	Zhang *et al*., 2017
OsPRI2/OsbHLH58	IVc	*O. sativa*	OsHRZ1	Kobayashi *et al*., 2019
OsPRI3/OsbHLH59	IVc	*O. sativa*	OsHRZ1	Kobayashi *et al*., 2019
OsbHLH133	VIIIc	*O. sativa*		Wang *et al*., 2013
GmbHLH57	IIIa	*G. max*	GmbHLH300	Li *et al*., 2018
GmbHLH300	Ib	*G. max*	GmbHLH57	Li *et al*., 2018
FER	IIIa	*S. lycopersicum*	SlbHLH68	Ling *et al*., 2002
SlbHLH68	Ib	*S. lycopersicum*	FER	Du *et al*., 2015
MxIRO2	Ib	*M. xiaojinensis*		Yin *et al*., 2013
MxFIT	IIIa	*M. xiaojinensis*	MxERF4	Yin *et al*., 2014
PtFIT	IIIa	*P. tremula*		Huang and Dai, 2015
PtIRO	Ib	*P. tremula*		Huang and Dai, 2015
SAT1/MdbHLH18	IVa	*M. domestica*	MdMYB58	Wang *et al*., 2018

MdbHLH104	IVc	*M. domestica*	MdBT1, MdBT2, MdbHLH104, MdbHLH105, MdbHLH115, MdbHLH11, MdbHLH121, MdPYE	Zhao *et al*., 2016b
NtbHLH1	IVb	*N. tabacum*		Li *et al*., 2020
CmbHLH1	IVc	*C. morifolium*		Zhao *et al*., 2014
FEFE/CmbHLH38	Ib	*C. melo*	CmFIT	Ramamurthy and Waters, 2017
GmORG3	Ib	*G. max*		Xu *et al*., 2017

*: for details on posttranslational regulation of FIT activity, see Wu and Ling, 2020 and Schwarz and Bauer, 2020.

Table 2. TFs other than bHLHs involved in the regulation of iron homeostasis in plants.

TF family	Gene name	Species	Interacting proteins	Reference
ABI3/VP1	**IDEF1**	*O. sativa*	IBP1	Kobayashi *et al*., 2007
ARF	**OsARF12**	*O. sativa*		Qi *et al*., 2012
ARF	**OsARF16**	*O. sativa*		Shen *et al*., 2016
C2H2	**ZAT12***	*A. thaliana*	FIT/bHLH29	Le *et al*., 2016
EIL	**EIN3***	*A. thaliana*	FIT/bHLH29	Lingam *et al*., 2011
EIL	**EIL1***	*A. thaliana*	FIT/bHLH29	Lingam *et al*., 2011
ERF	**ERF4**	*A. thaliana*		Liu *et al*., 2017a
ERF	**ERF72**	*A. thaliana*		Liu *et al*., 2017b
ERF	**ERF95**	*A. thaliana*		Sun *et al*., 2020
ERF	**MxERF4**	*M. xiaojinensis*	MxFIT	Liu *et al*., 2018
ERF	**MbERF4**	*M. baccata*	MbERF72	Zhang *et al*., 2020a
ERF	**MbERF72**	*M. baccata*	MbERF4	Zhang *et al*., 2020a
MYB (R2R3)	**MYB10**	*A. thaliana*		Palmer *et al*., 2013
MYB (R2R3)	**MYB28**	*A. thaliana*		Coleto *et al*., 2020
MYB (R2R3)	**MYB29**	*A. thaliana*		Coleto *et al*., 2020
MYB (R2R3)	**MYB72**	*A. thaliana*		Palmer *et al*., 2013
MYB (R2R3)	**MdMYB58**	*M. domestica*	MdSAT1/MdbHLH18	Wang *et al*., 2018
MYB (R2R3)	**MxMYB1**	*M. xiaojinensis*		Shen *et al*., 2008
NAC	**IDEF2**	*O. sativa*		Ogo *et al*., 2008
NF-YC	**HAP5A/NF-YC1**	*A. thaliana*		Zhu *et al*., 2020
WRKY	**WRKY46**	*A. thaliana*		Yan *et al*., 2016

*: for details on posttranslational regulation of FIT activity, see Wu and Ling. 2020 and Schwarz and Bauer, 2020.

Figure legends

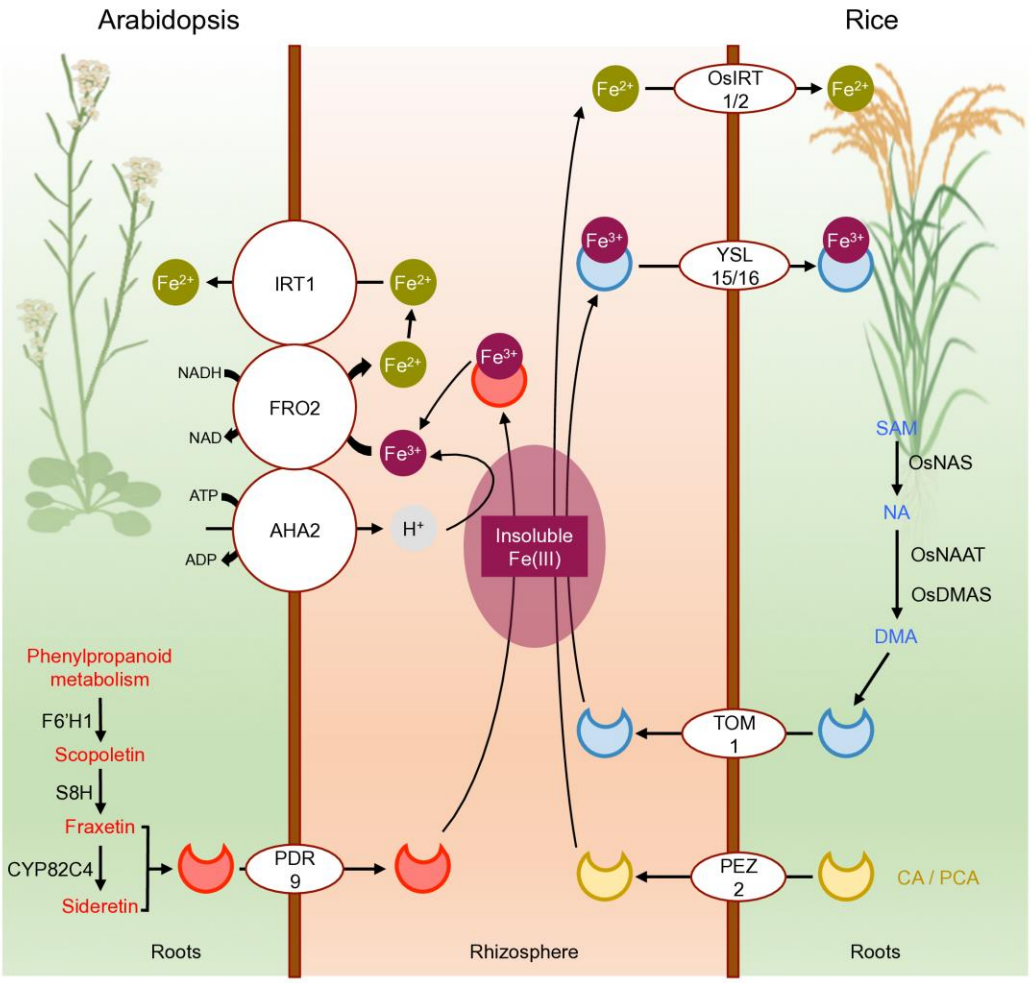
Figure 1. Schematic diagram of iron uptake strategies in Arabidopsis and rice. In Arabidopsis, AHA2 secretes protons into the rhizosphere to increase Fe^{3+} solubility; PDR9 secretes Fe-mobilizing coumarins (*i.e.* fraxetin and sideretin) to mobilize and chelate Fe^{3+}. Fe^{3+} is reduced into Fe^{2+} and is subsequently transported into the root epidermal cells by FRO2 and IRT1, respectively. AHA2, FRO2 and IRT1 form a protein complex optimizing Fe acquisition by creating a local environment with low pH and high Fe^{2+} concentration. Rice biosynthesizes and secretes DMA (2-deoxy-mugineic acid) to chelate Fe^{3+}. Fe^{3+}-DMA complexes are transported into root cells *via* YSL15 and YSL16. In addition, rice also uptakes Fe^{2+} from the soil through the activity of two Fe^{2+} transporters, OsIRT1 and OsIRT2 under waterlogged soil condition. The secretion of CA and PCA via the PEZ2 phenolics efflux transporter participates to Fe^{3+} mobilization and reduction into Fe^{2+}. AHA2: H^+-ATPase 2; PDR9: PLEIOTROPIC DRUG RESISTANCE 9/ATP-BINDING CASSETTE G37; FRO2: FERRIC REDUCTION OXIDASE 2; IRT1: IRON-REGULATED TRANSPORTER 1, F6'H1: FERULOYL CoA 6' HYDROXYLASE 1; S8H: SCOPOLETIN 8-HYDROXYLASE; CYP82C4: FRAXETIN 5-HYDROXYLASE; TOM1: TRANSPORTER OF MUGINEIC ACID 1; YSL15/16: YELLOW STRIPE-LIKE 15/16; OsIRT1/2: Rice IRON-REGULATED TRANSPORTER 1/2; PEZ2: PHENOLICS EFFLUX ZERO 2; OsNAS: NICOTIANAMINE SYNTHASE; OsNAAT: NICOTIANAMINE AMINOTRANSFERASE; OsDMAS: DEOXYMUGINEIC ACID SYNTHASE; SAM: S-adenosyl methionine; NA: nicotianamine; CA: caffeic acid; PCA: protocatechuic acid.

Figure 2. Phylogenetic tree of bHLH TFs involved in the regulation of iron homeostasis in plants. The tree was constructed by using the neighbor-joining method with the MEGA software (v10.0.5) using full-length bHLH amino acid sequences. The bootstrap analysis was carried out with 1000 replicates. Sequences were aligned prior to the construction of the phylogenetic tree using the ClustalX software (v2.0.11). The different bHLH clades are designated as previously reported (Heim *et al*., 2003). Species abbreviations: At, *Arabidopsis thaliana*; Md, *Malus domestica*; Nt, *Nicotiana tabacum*;

Os, *Oryza sativa*; Sl, *Solanum lycopersicum*; Gm, *Glycine max*; Mx, *Malus xiaojinensis*; Cm, (CmbHLH1), *Chrysanthemum morifolium*; Cm (CmbHLH38), *Cucumis melo*; Pt, *Populus tremula.*

Figure 3. bHLH TFs network that regulates iron homeostasis in Arabidopsis. Question marks indicate protein complexes for which transcriptional activities have not yet been clearly demonstrated. The colour code for the bHLH TFs refers to the clades described Figure 2. Non bHLH proteins are in grey. EIN3 and EIL1 favor FIT stability whereas MED16 and MED25 mediate the FIT/bHLH29-dependent iron deficiency responses (for additional details on posttranslational regulation of FIT, see Kobayashi, 2019; Schwarz and Bauer, 2020; Spielmann and Vert, 2020; Wu and Ling, 2019). EIN3: ETHYLENE INSENSITIVE 3; EIL1: ETHYLENE INSENSITIVE 3-LIKE1; FBP: FIT-BINDING PROTEIN; MED16 AND 25: MEDIATOR SUBUNIT 16 and 25; ZAT12: ZINC FINGER OF ARABIDOPSIS THALIANA 12.

Figure 4. bHLH TFs network that regulates iron homeostasis in rice. Question mark indicates the putative degradation mechanism for OsPRI1 and OsPRI2 via OsHRZ2. The colour code for the bHLH TFs refers to the clades described Figure 2. Non bHLH proteins are in grey. IBP1.1: IDEF1-BINDING PROTEINS 1.1.



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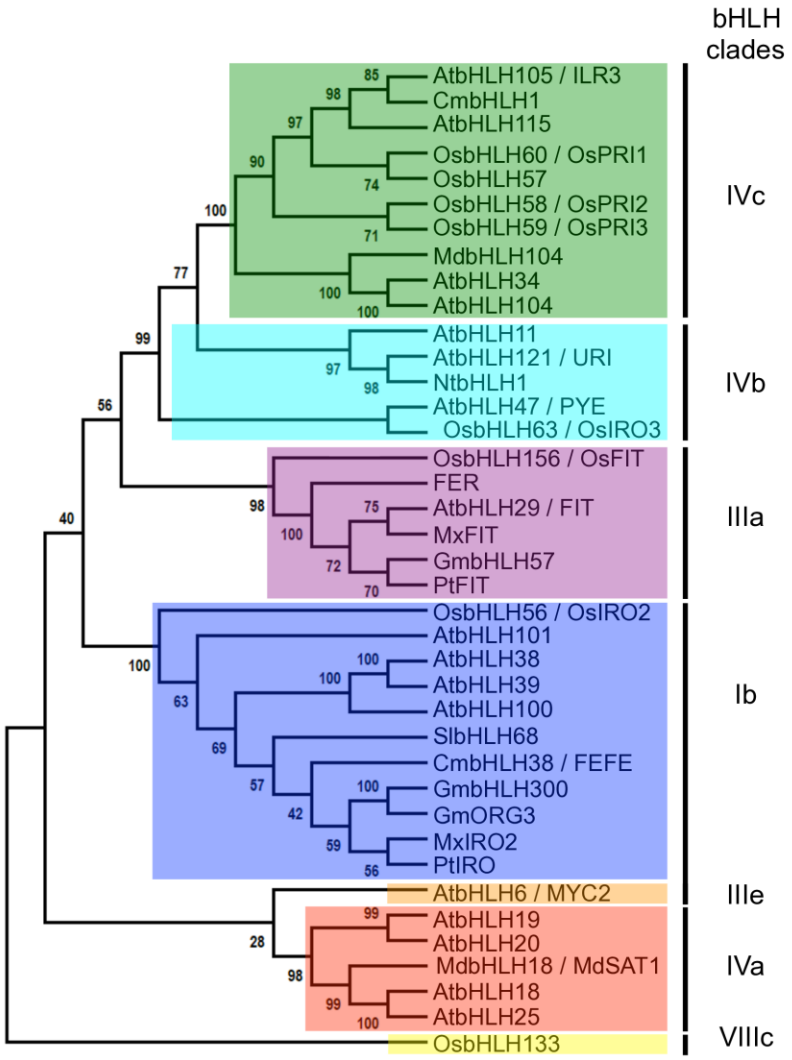

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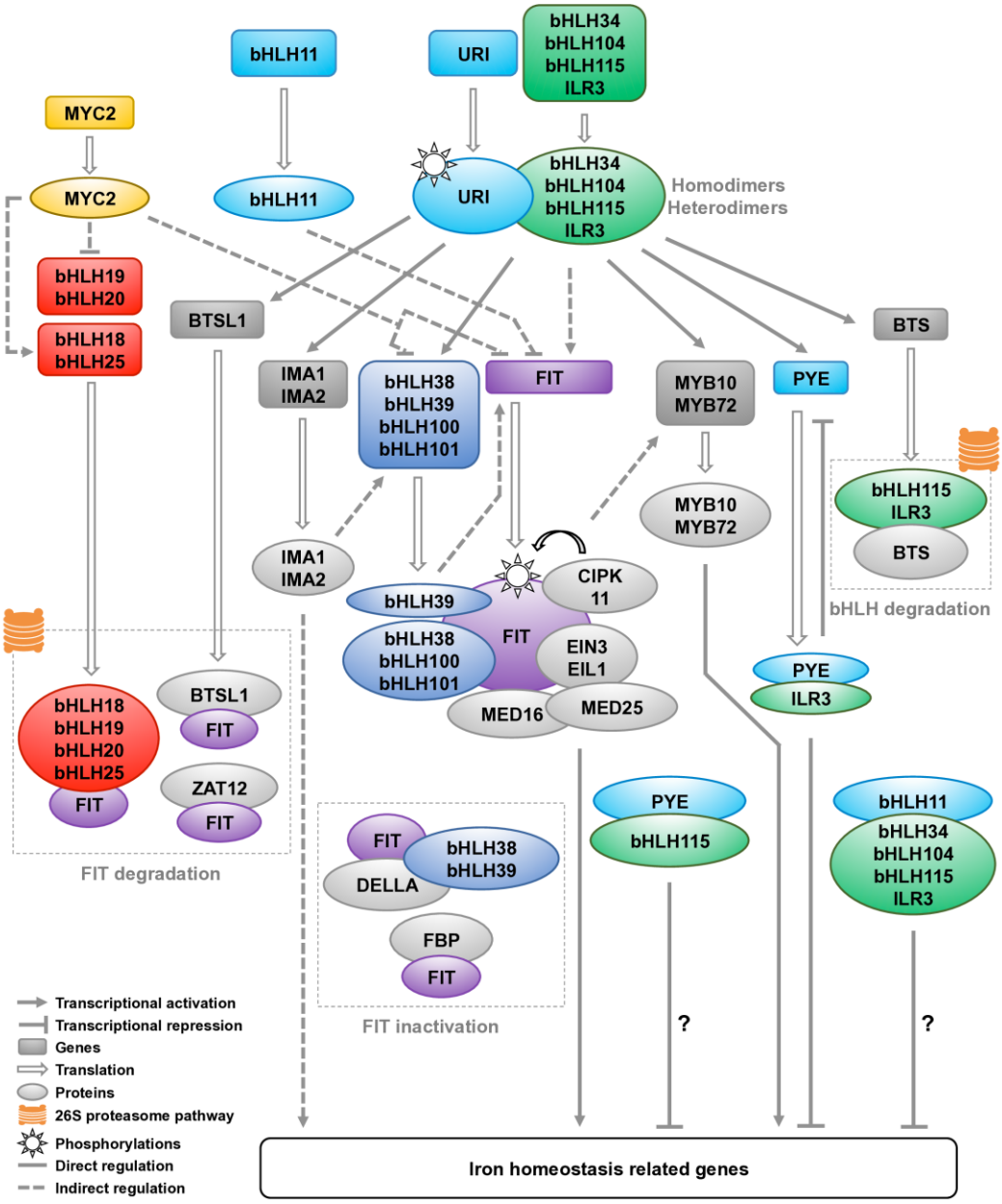

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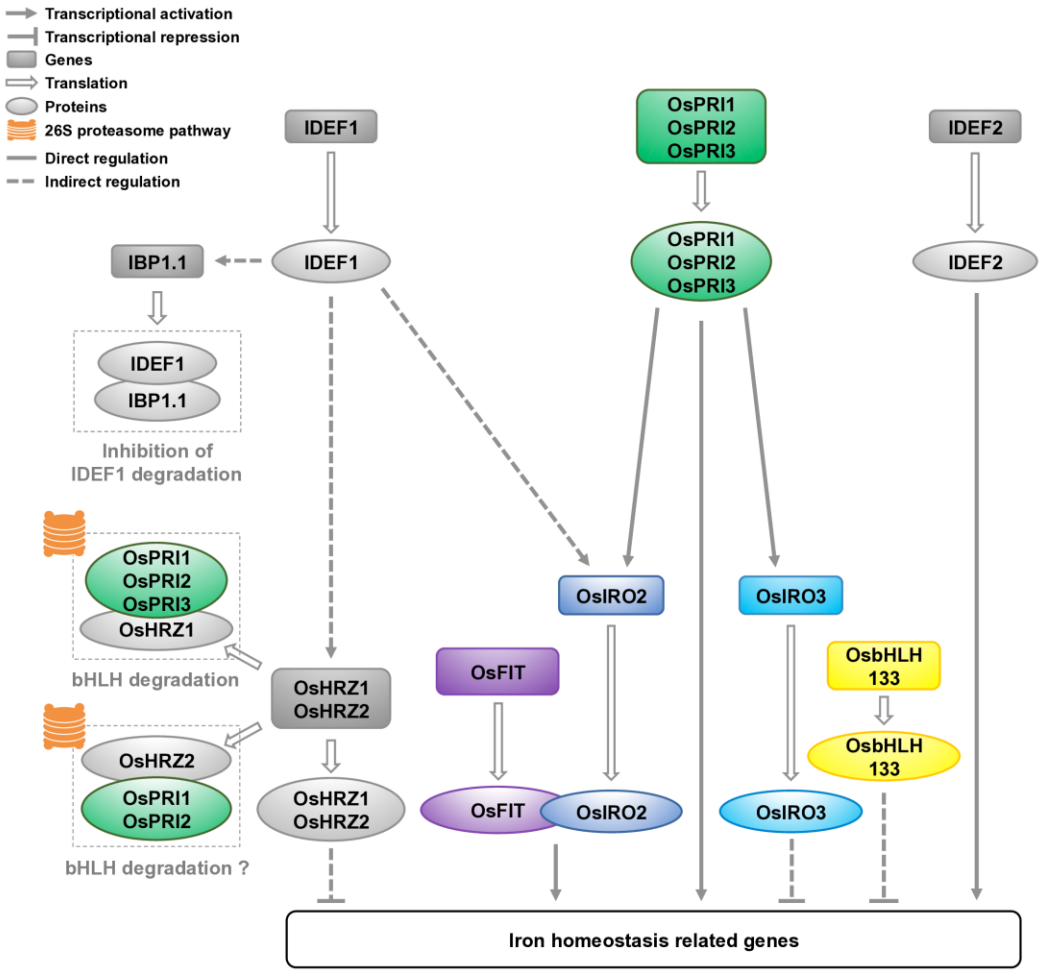

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