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Review

# Iron sensors and signals in response to iron deficiency

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ABSTRACT

The transcription of genes involved in iron acquisition in plants is induced under iron deficiency, but our understanding of iron sensors and signals remains limited. Iron Deficiency-responsive Element-binding Factor 1 (IDEF1) and Hemerythrin motif-containing Really Interesting New Gene- and Zinc-finger proteins (HRZs)/BRUTUS (BTS) have recently emerged as candidate iron sensors because of their functions as potent regulators of iron deficiency responses and their iron-binding properties. IDEF1 is a central transcriptional regulator of graminaceous genes involved in iron uptake and utilization, predominantly during the early stages of iron deficiency. HRZs/BTS are E3 ubiquitin ligases and negative regulators of iron deficiency responses in both graminaceous and non-graminaceous plants. Rice OsHRZ1 and OsHRZ2 are also potent regulators of iron accumulation. Characterizing these putative iron sensors also provides clues to understanding the nature of iron signals, which may involve ionized iron itself, other metals, oxygen, redox status, heme and iron-sulfur clusters, in addition to metabolites affected by iron deficiency. Systemic iron responses may also be regulated by phloem-mobile iron and its chelators such as nicotianamine. Iron sensors and signals will be identified by demonstration of signal transmission by IDEF1, HRZs/BTS, or unknown factors.



**Contents**



## 1. Introduction

Fe is an essential element in virtually all living organisms. Fe is required in many metabolic processes, including photosynthesis and respiration. Despite its high abundance in the soil, Fe is only slightly soluble especially under alkaline and aerobic conditions. Plants grown under low Fe availability, such as in calcareous soils, often suffer from Fe deficiency, which reduces growth, crop yield and quality [1]. Thus, the development of crops tolerant to low Fe

*Abbreviations:* IRE, iron responsive element; IRP, iron regulatory protein.

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availability, or with high levels of Fe for human nutrition has long been pursued.

Living organisms have evolved elaborate systems that acquire adequate amounts of Fe from the environment and transport it throughout the body. Higher plants take up Fe from the rhizosphere using two major strategies: the reduction strategy (Strategy I) and a chelation strategy (Strategy II) [2]. Non-graminaceous plants utilize Strategy I, whereas graminaceous plants possess a specific ability to synthesize potent Fe(III) chelators designated mugineic acid family phytosiderophores and utilize Strategy II [2,3]. Molecular components of these strategies have been characterized [4–7]. These strategies have previously been considered mutually exclusive, but some exceptions have recently been reported in which Strategy II plants possess partial Strategy I uptake systems and *vice versa* [4,8,9].

Excessive Fe is also deleterious, as ionized $Fe^{2+}$ catalyzes the generation of reactive oxygen species in the Fenton reaction where $H_2O_2$ is converted to highly reactive hydroxyl radicals, promoting oxidative stress [1]. Because of this toxic nature of Fe, Fe uptake mechanisms are induced only under low Fe availability, and repressed under Fe sufficiency. Fe is thought to be chelated by various biomolecules in the plant body in both ferrous and ferric forms to keep solubility and prevent toxicity. Only a small portion of ionized $Fe^{2+}$ and $Fe^{3+}$ is thought to be dissociated from the chelating molecules by equilibrium reaction, and these free Fe ions, preferably $Fe^{2+}$, are thought to be incorporated into Fe proteins and other biomolecules. Fe overload induces expression of Fe-storage protein ferritin, which sequesters Fe in non-toxic form [4,10].

Genes involved in Fe acquisition strategies are transcriptionally upregulated in response to Fe deficiency [4–6]. This is in contrast to the animal system, in which the primary components of Fe acquisition are post-transcriptionally regulated [11,12]. Key transcription factors regulating Fe acquisition-related genes have been identified in both non-graminaceous and graminaceous plants [4,5]. However, signal substances and the sensors regulating this response have not been identified. In this review, we summarize recent findings which shed light on Fe sensors and signals. Although numbers of metabolites affected by Fe nutritional conditions could act as Fe signaling molecules, we mainly focus on possibilities of more direct Fe sensing which might be performed by binding Fe and other metals by Fe regulators.

## 2. Candidate iron sensors

### 2.1. Definition of iron sensors

Oxford dictionaries (http://oxforddictionaries.com/) define the word "sensor" (noun) as "a device which detects or measures a physical property and records, indicates, or otherwise responds to it". From a biochemical and physiological standpoint, we propose a definition of Fe sensor in a living system as a biomolecule that (i) binds Fe or an intimately related molecule(s) (input); (ii) thereby changes its function (transmission and conversion); and (iii) regulates Fe homeostasis (output).

Known Fe sensors conforming to these criteria include the bacterial ferric uptake regulation (Fur) protein [13] and the mammalian iron regulatory protein (IRP)/iron responsive element (IRE) system [11] (Fig. 1). Fur is a ferrous Fe-binding transcriptional repressor [criteria (i)]. When the Fur lacks Fe, it loses its DNA-binding activity [criteria (ii)]. Consequently, Fur is unable to repress Fe acquisition-related genes under Fe-deficient conditions [criteria (iii)] (Fig. 1A). On the other hand, IRP1 and IRP2 post-transcriptionally regulate mammalian Fe responses by binding to IRE, which is a stem-loop structure found in various mRNAs involved in Fe homeostasis. When bound to IRP, IRE located in the 5′-untranslated regions represses translation, while IRE in 3′-untranslated regions stabilizes its mRNA. Under Fe-replete conditions, IRP1 binds an Fe–sulfur (S) cluster [criteria (i)] and loses its ability to bind IRE [criteria (ii)], negating its IRE-mediated regulation [criteria (iii)]. IRE in the 5′-untranslated regions also binds ferrous Fe, changing its binding affinities with both IRP and translation initiation factors [14]. IRP2 is another IRE-binding protein lacking a Fe–S cluster. This protein loses its activity under Fe sufficiency because of Fe-dependent proteasomal degradation mediated by another Fe sensor, the F-box leucine rich repeat protein 5 (FBXL5) [15,16]. FBXL5 binds Fe *via* the hemerythrin domain [criteria (i)] and mediates ubiquitination of IRP2 [criteria (iii)]. Under low Fe conditions, FBXL5 itself is subjected to proteasomal degradation, which is associated with the absence of Fe in the hemerythrin domain [criteria (ii)] [15–17] (Fig. 1B).

**Table 1**
Comparison of IDEF1 and HRZs/BTS as candidate Fe sensors.

| Functions as a Fe sensor | IDEF1 | HRZs/BTS |
|---|---|---|
| **(i) Binding Fe or an intimately related molecule(s) (input)** | | |
| Binding Fe | Yes ($Fe^{2+}$) | Yes |
| Binding other metals | Yes ($Zn^{2+}$ *etc.*) | Yes (Zn) |
| Binding other molecules | ? | ? |
| **(ii) Thereby changing its function (transmission and conversion)** | | |
| DNA binding | No change?[a] | ? |
| Transactivation | Increased without metals?[a] | ? |
| Degradation/acumulation | ?[b] | No change?[c] |
| Modification | ? | ? |
| Binding co-regulators | ?[b] | ?[d] |
| Localization | ? | ? |
| Ubiquitination activity | – | ? |
| **(iii) Regulating Fe homeostasis (output)** | | |
| Fe deficiency response | Yes (positive) | Yes (negative)[e] |
| Fe deficiency tolerance | Yes (positive) | Yes (negative) |
| Fe accumulation | No? | Yes (negative)[c] |

[a] Based on *in vitro* and yeast results. In contrast, transgenic rice plants overexpressing *IDEF1* without metal-binding regions fail to induce target genes at an early stage of Fe deficiency [19], suggestive of a positive involvement of metal binding in DNA binding and/or transactivation *in planta*.
[b] An IDEF1-binding Bowman-Birk trypsin inhibitor IBP1 protects IDEF1 from protein degradation, and the *IBP1* transcript level is induced under Fe deficiency [25].
[c] Results reported only for rice HRZs but not for *Arabidopsis* BTS [20].
[d] BTS interacts with bHLH transcription factors involved in regulation of Fe homeostasis [21].
[e] Results reported precisely for rice HRZs but only preliminarily for *Arabidopsis* BTS [20,21].

Neither the Fur and IRP/IRE systems, nor a biomolecule conforming to all of these three criteria, have not been identified in plants. Nevertheless, our recent studies identified two types of regulatory proteins that conform to (i) and (iii) of the above-mentioned criteria; namely, Iron Deficiency-responsive Element-binding Factor 1 (IDEF1) [18,19] and Hemerythrin motif-containing Really Interesting New Gene (RING)- and Zinc-finger proteins (HRZs) [20] in rice (Table 1 and Fig. 2). HRZs are homologous to the previously identified protein BRUTUS (BTS) in *Arabidopsis thaliana* [21].

### 2.2. IDEF1

IDEF1 has been identified as a rice transcription factor that specifically binds the CATGC sequence within the Fe deficiency-responsive *cis*-acting element IDE1 [18], and it is a positive regulator of the majority of rice genes responsible for Fe uptake and utilization especially during early stages of Fe deficiency [18,22] (Fig. 2). IDEF1-binding sequence was also predicted as one of the most predominantly accumulated sequences within 500 bp-upstream regions of Fe deficiency-responsive genes in rice roots [23], suggesting that IDEF1 plays an important role in the response to Fe deficiency. *IDEF1* transcript levels do not change in response to Fe availability [18,22,24], in contrast to the majority of other

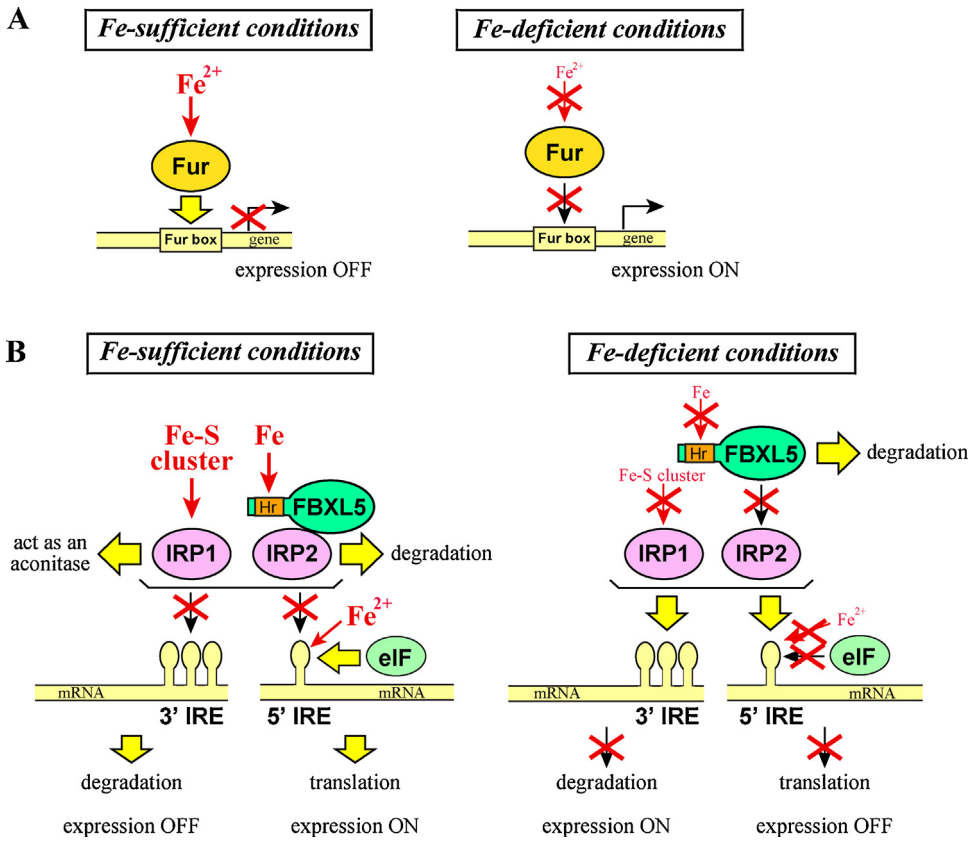


**Fig. 1.** Schematic representation of the Fur and IRP/IRE systems. (A) Regulation mediated by Fur. (B) Regulation mediated by IRP/IRE and its modulation by FBXL5. In both systems, direct binding of Fe or Fe-S cluster to the regulator proteins or RNA affect the affinity to their interacting partners, consequently regulating the expression of Fe homeostasis-related genes. Hr, hemerythrin domain; eIF, eukaryotic initiation factor.

transcription factors regulating response to Fe deficiency that are transcriptionally induced under Fe deficiency [4,5]. This suggests that IDEF1 is situated upstream of other factors in the Fe deficiency response cascade, and thus is a good candidate Fe sensor. Moreover, IDEF1 possesses characteristic histidine-asparagine repeat and proline-rich regions that bind $Fe^{2+}$ and other divalent metals, such as $Zn^{2+}$, $Cu^{2+}$ and $Ni^{2+}$, based on *in vitro* experiments using recombinant IDEF1 protein [19]. Thus, this factor conforms to criteria (i) and (iii) as an Fe sensor (Table 1).

Experimental evidence supporting criterion (ii) includes the results obtained using transgenic rice plants overexpressing *IDEF1* devoid of its metal-binding regions; these transgenic rice lines failed to induce target genes at an early stage of Fe deficiency, even though this phenomenon was less obvious at subsequent stages of Fe deficiency [19]. These results suggest that metal binding to IDEF1 is necessary for its function during an early stage of Fe deficiency, when the genes involved in Fe uptake and utilization are the most strongly regulated by IDEF1 [22]. However, electrophoretic mobility shift assay and yeast one-hybrid analysis showed that the metal-binding regions do not affect DNA binding but negatively affect transactivation activity in yeast [19]. Although it is expected that metal-binding status of IDEF1 would affect conformation of its protein structure and consequently its function, it is not clear whether the conformation change caused by deletion of metal-binding regions of IDEF1 is comparable to that caused by metal depletion from full-length IDEF1. It is possible that the metal-binding regions of IDEF1 are required for protein structure but not for the direct sensing of Fe *in vivo*. Thus, molecular evidence supporting criterion (ii) is required to demonstrate that IDEF1 is a real Fe sensor. Stereostructural analysis of IDEF1 protein, as well as detection of metal-binding status of IDEF1 *in vivo* using a technological innovation would greatly contribute to clarify the relationship between metal-binding status and IDEF1 function.

As candidate mechanisms of signal transmission and conversion by IDEF1, protein degradation, modification, localization and/or interaction with other regulatory proteins may be affected by metal-binding status (Table 1). Recently, we observed that the IDEF1 protein is degraded *via* the 26S proteasome system, and this degradation is inhibited by a Bowman-Birk trypsin inhibitor designated IDEF1-binding protein 1 (IBP1) identified as an IDEF1-interacting partner based on yeast two-hybrid and pull-down assays [25]. *IBP1* expression is transcriptionally induced under Fe deficiency in an IDEF1-dependent manner, suggesting that IBP1 might play a role in keeping the IDEF1 protein level enough for its function under Fe deficiency [25].

IDEF1 is thought to be conserved among graminaceous plants, but the length and sequence of its metal-binding regions are relatively less conserved among the species compared with the highly conserved DNA-binding region [19]. This fact raises the possibility that metal-binding property of IDEF1 is species-dependent. Although the presence of the IDEF1 system in non-graminaceous species has not been determined, several evidences support the presence of similar transcription factors in dicots. IDEF1-binding sequences are functional when introduced into tobacco, and they are enriched in the promoters of Fe deficiency-inducible genes in *Arabidopsis* [4]. IDEF1 belongs to ABI3/VP1 family transcription factors [22], which regulate responses to drought stress and abscisic acid in both graminaceous and non-graminaceous plants. Indeed, IDEF1 regulates some genes involved in drought tolerance

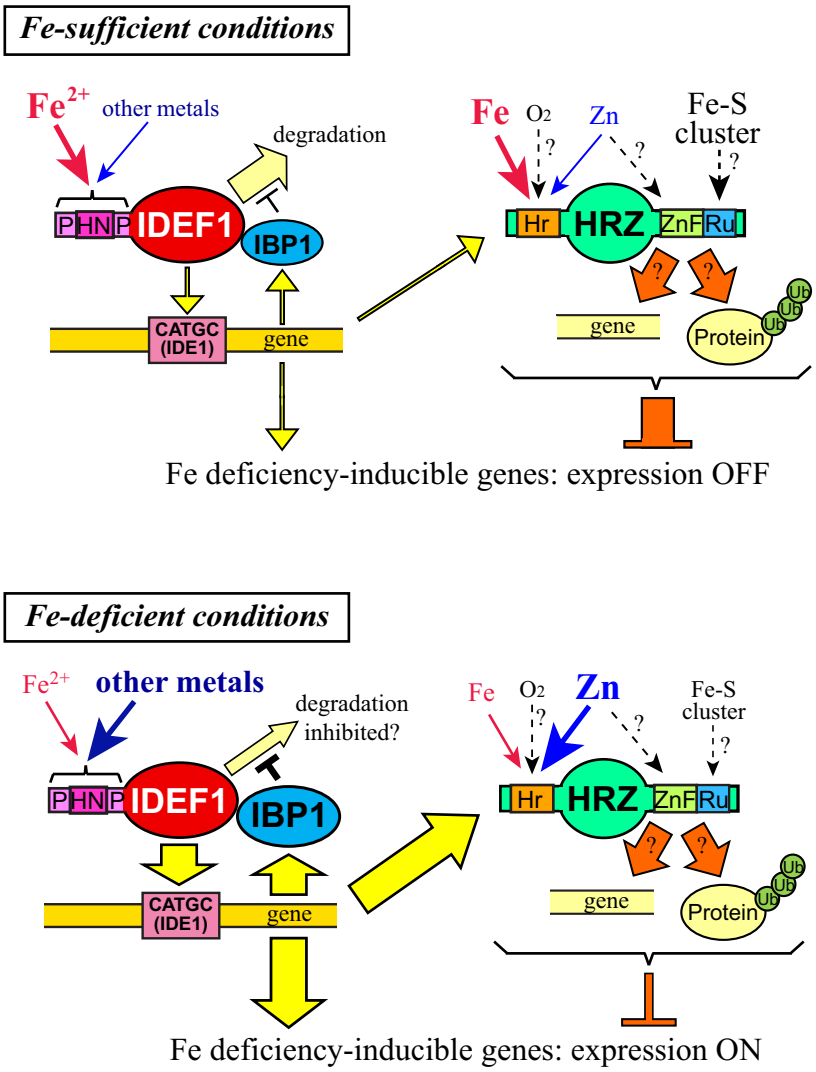


**Fig. 2.** Schematic representation of IDEF1 and HRZ functions. Regulations mediated by IDEF1 and HRZs are depicted by yellow and orange arrows, respectively. IDEF1 binds $Fe^{2+}$ and other divalent metals *via* histidine-asparagine repeat (HN) and proline-rich (P) regions [19]. IDEF1-regulated genes include *IBP1*, which encodes a Bowman-Birk trypsin inhibitor that binds IDEF1 and prevents its degradation [25], and *OsHRZ1* and *OsHRZ2*, which encode ubiquitin ligases [20]. HRZs bind Fe and Zn *via* hemerythrin (Hr) domains. This domain may also bind dioxygen. HRZs also contain RING-, CHY- and CTCHY-Zn-fingers (ZnF) that potentially bind Zn and mediate protein ubiquitination (Ub) and possibly transcriptional or post-transcriptional gene regulation, but these targets have not been identified. HRZs also contain rubredoxin-type fold (Ru), which may bind the Fe-S cluster. HRZs negatively regulate expression of a majority of Fe deficiency-inducible genes involved in Fe uptake and utilization predominantly under Fe sufficiency, and they regulate Fe deficiency tolerance and accumulation [20]. Fe and related molecules bound to IDEF1 and/or HRZs may function as Fe signals. Especially, cellular concentration ratio between Fe and other metals may be sensed by HN and P regions of IDEF1 and Hr domains of HRZs. Under Fe-sufficient conditions, positive regulation by IDEF1 is weak and negative regulation by HRZs is strong, keeping the expression of Fe deficiency-inducible genes at low levels. Under Fe-deficient conditions, IDEF1 enhances positive regulation especially at early stages of Fe deficiency, whereas HRZ function is mitigated, inducing the expression of Fe deficiency-inducible genes.

which are induced under Fe deficiency [22]. Substantial overlap of drought-induced and Fe deficiency-induced genes is also observed in *Arabidopsis* [26]. However, metal-binding domains of IDEF1 are not found in non-graminaceous proteins.

### 2.3. HRZs/BTS

HRZs and BTS have been identified in rice and *Arabidopsis*, respectively, as Fe deficiency-inducible genes [20,21]. These proteins contain several conserved domain structures: hemerythrin, CHY-, CTCHY- and RING-Zn-fingers, and rubredoxin-type fold [20]. The hemerythrin domain binds Fe in animals and bacteria, and is also present in the human Fe sensor FBXL5 [15,16,27]. RING-Zn-finger is known as a component of E3 ligases that ubiquitinate proteins for 26S proteasome-mediated degradation or modification [28]. CHY- and CTCHY-Zn-fingers may mediate transcriptional, post-transcriptional or post-translational gene regulation [29]. Rubredoxin-type fold is known to bind Fe–S clusters [30]. Thus, HRZs/BTS may be multifunctional proteins. Fe deficiency-inducible genes carrying these domain structures are well conserved among plant species, and even in chlorophyte alga such as *Chlamydomonas* [20,31]. We demonstrated that recombinant OsHRZ1, OsHRZ2 and BTS proteins bind Fe and Zn and possess ubiquitination activity *in vitro* [20]. Hemerythrin domains accounted for the majority, but not all, of Fe and Zn bound to OsHRZs, while this domain was not necessary for ubiquitination activity. Although it has not been determined whether ferrous or ferric Fe is bound to HRZs/BTS, the canonical hemerythrin in invertebrates binds ferrous Fe, and a portion of Fe is then oxidized to ferric Fe [27].

HRZs/BTS contain domain combinations similar to those of the human Fe sensor FBXL5. They all contain Fe-binding hemerythrin domain(s) and constituents of E3 ubiquitin ligases; FBXL5

possesses an F-box domain responsible for IRP2 degradation [15,16]. Hemerythrin is a major oxygen-transporting protein in marine invertebrates and has also been proposed to be an oxygen sensor in bacteria [27]. However, its presence in higher plants and animals was only recently characterized. Conceivably, higher plants and animals have evolutionally acquired their independent Fe sensors utilizing this common domain by combining with E3 ligase constituents. The molecular linkage of sensors with constituents of E3 ubiquitin ligases occurs in receptors of various plant hormones [28], which is suggestive of a plant signal sensing strategy in which protein degradation is a direct output. Regulation of gene expression by protein degradation might be advantageous for rapid response to environmental changes. Substrates of ubiquitination mediated by HRZs/BTS have not been identified. Candidate substrates might include IDEF1, the *Arabidopsis* transcription factor FIT, and an uncharacterized repressor of ferritin synthesis, because all of these proteins are susceptible to ubiquitination-mediated degradation and are involved in Fe homeostasis [4,5,10,25]. The *Arabidopsis* $Fe^{2+}$ transporter IRT1 is also regulated by ubiquitination-mediated modification and degradation [4]. IRT1 degradation is mediated by an E3-ubiquitin ligase IRT1 degradation factor 1 (IDF1), which is distinct from HRZs/BTS [32]. Thus, multiple E3 ligases including HRZs/BTS and IDF1 appear to be involved in Fe responses at the protein level.

OsHRZs and BTS negatively regulate responses to Fe deficiency. Knockdown plants of these genes in rice and *Arabidopsis* show tolerance to Fe deficiency [20,21]. A more precise investigation of *OsHRZ*-knockdown rice demonstrated enhanced Fe accumulation in shoots and seeds irrespective of culture conditions. This was accompanied by enhanced expression of virtually all known Fe deficiency-inducible genes involved in Fe uptake or translocation, especially under Fe-sufficient conditions [20]. These results indicated that OsHRZs are potent regulators of both Fe efficiency and accumulation. HRZs/BTS appear to limit Fe uptake and translocation under Fe-sufficient conditions to prevent cellular Fe toxicity. Thus, HRZs/BTS again conform to (i) and (iii) of the criteria for Fe sensors, while criterion (ii) has not been confirmed (Table 1). OsHRZ1 and OsHRZ2 are thought to be actively degraded by the 26S proteasome in rice roots irrespective of Fe status [20]. BTS interacts with basic helix-loop-helix transcription factors which are involved in Fe homeostasis [21]. However, signal transmission by HRZs/BTS has not been characterized. *OsHRZ1* and *OsHRZ2* are also positively regulated by IDEF1 [20], possibly forming a negative feedback loop of the IDEF1 pathway (Fig. 2). Physiological significance of Fe deficiency-induced expression of HRZs/BTS is unclear, but this might be involved in fine-tuning the response to fluctuating Fe availability.

## 3. Candidate iron signals

### 3.1. Ionized iron and other metals

Although the phrase "Fe signals" can include both upstream and downstream Fe sensors, we focus on upstream signals that can be perceived by the sensors. Identification of IDEF1 and HRZs/BTS as Fe-binding regulators suggested that ionized Fe itself is an Fe signal. Notably, both IDEF1 and HRZs/BTS bind not only Fe, but also other divalent metals, such as Zn. Because many Fe transporters and chelators have affinity to other metals, such as Zn, Mn and Cu, Fe-deficient conditions in plants result in progressive accumulation of these metals [4]. Expression of the *Arabidopsis* $Fe^{2+}$ transporter IRT1 and ferric-chelate reductase FRO2 is Fe deficiency-inducible but also regulated by Zn concentrations [33]. Tobacco plants subjected to combined deficiency of Fe and other microelements such as Zn, Mn and Cu exhibit mitigated Fe deficiency symptoms and attenuated activity of Fe deficiency-inducible *IDS2* gene promoter, compared with sole Fe deficiency [34]. Based on these observations, we proposed that plant Fe sensors detect the cellular concentration ratio between Fe and other metals rather than the absolute Fe concentration [19,20]. This hypothesis is especially consistent with the property of IDEF1 whose target genes are partially altered during the progression of Fe deficiency; this property may be better tuned to detection of metal balances [19]. Expression of IDEF1-target genes involved in Fe uptake and translocation is rapidly induced at an early stage of Fe deficiency [22], which could be triggered by an early signal of Fe deficiency. At subsequent stages of Fe deficiency, IDEF1 preferentially regulate another subset of genes related to drought stress [22]. This response might be caused by a subsequent Fe signal. Histidine residues are thought to be responsible for reversible binding of IDEF1 to Fe and other divalent metals, based on *in vitro* results of immobilized metal ion affinity chromatography [19].

Measurements of metals in purified recombinant proteins revealed much higher amounts of Fe and Zn bound to HRZs/BTS compared with IDEF1 [19,20]. Because canonical hemerythrin domains form stable structures with diiron centers covered by α-helices [17,27], metal binding to HRZs/BTS is thought to be tighter and less reversible than that to IDEF1. Supporting this notion, the apo-form of human FBXL5 synthesized under Fe-deficient conditions does not readily bind Fe upon its resupply [34]. Nevertheless, high Fe concentrations during recombinant protein synthesis of OsHRZ1 resulted in reduced Zn bound to OsHRZ1 [20]. This is suggestive of competitive binding between Fe and Zn that could occur at or near the time of protein synthesis and folding where the metal species bound to a protein can be most affected [35]. Thus, HRZ proteins may also sense cellular concentration ratios of Fe and Zn. High levels of Zn binding to the hemerythrin domain appear to be specific to plants, although Zn binding to the hemerythrin domain has also been reported in a recombinant *Desulfovibrio vulgaris* protein as a result of Fe displacement during structural analysis [36].

### 3.2. Oxygen and redox status

Identification of HRZs/BTS also raised the possibility of oxygen as a Fe signal or its modulator. The canonical hemerythrin domain binds two Fe atoms and reversibly binds dioxygen at the second Fe site [27]. Although structural analysis of human FBXL5 does not support its apparent oxygen binding [17,35], FBXL5 is actively degraded not only under Fe deficiency but also under hypoxia [15–17], suggestive of a signaling linkage between Fe and oxygen.

In addition to direct oxygen binding, the redox status can modulate Fe sensing by affecting chemical properties of metals and metal-binding proteins. Fe solubility is affected by its redox status, since ferrous Fe is more soluble than ferric Fe. In addition, the ratio of binding Fe *versus* Zn in some metalloenzymes is affected by both the cytoplasmic concentrations of ionized Fe and Zn and the redox status [37,38]. Some Zn(II)-binding enzymes are thought to bind Fe(II) under anaerobic conditions, and the bound Fe(II) can be readily oxidized and replaced by Zn(II) when exposed to oxygen. This ability to switch metals is proposed to be a mechanism by which proteins sense environmental conditions [37]. Although advantage of this Fe-Zn switch as a sensing mechanism is unclear, this property of metal exchange might be applicable for all other metalloproteins, including putative sensors of Fe as well as other metals.

Another type of linkage between the Fe deficiency response and hypoxia has been observed for human hypoxia-inducible factor HIF-α, which regulates adaptive responses to low oxygen and Fe metabolism [12]. Under normal oxygen conditions, HIF-α activity is lost upon its degradation *via* the ubiquitin-proteasome system. This HIF-α degradation is regulated by its oxidization catalyzed by HIF

prolyl hydroxylases, which belong to 2-oxoglutarate-dependent dioxygenases and require Fe(II) and molecular oxygen for the enzymatic reaction. Interestingly, this enzymatic requirement of the dioxygenase determines the stability of HIF-α, and thus HIF prolyl hydroxylases are assumed to be sensors of both oxygen and Fe. Fe chaperones poly (rC) binding protein 1 (PCBP1) and PCBP2 activate the HIF prolyl hydroxylases by delivering cytosolic Fe to these enzymes [39]. In addition to this Fe-dependent HIF degradation, expression of HIF-2α is also regulated by IRP1 (but not IRP2) at protein synthesis [40]. Thus, Fe and hypoxia responses intimately affect each other in humans. By analogy, plant dioxygenases have also been proposed to be Fe sensors [41]. Plants do not have clear HIF homologs, but have prolyl hydroxylases, which might be involved in Fe sensing mechanism [42]. In this respect, all metalloenzymes can be candidate Fe sensors in a broad sense. However, there is no evidence supporting that plant metalloenzymes directly regulate Fe responses.

The link between Fe availability and redox status in plants has physiological implications. Fe deficiency forces the plants to spend energy to acquire Fe; conversely, it limits the activity of oxidative energy production, which requires Fe as a cofactor. Metabolic reprogramming occurs that addresses this problem [43]. Plants and alga induce the expression of monodehydroascorbate reductase gene and accumulate ascorbate under Fe deficiency [31,44]. Because ascorbate is a potent antioxidant, it might directly affect Fe signaling. Under prolonged Fe deficiency, physiological hypoxia occurs as a consequence of reduced metabolic activity. Hypoxia in plants also induces partial Fe deficiency responses [20]. On the other hand, Fe excess triggers oxidative stress and quick accumulation of nitric oxide in plastids, which mediates the expression of *Arabidopsis* ferritin gene *AtFER1* [10].

### 3.3. Heme and iron-sulfur cluster

Fe is coordinated either as heme, Fe-S cluster or in other forms in functional Fe proteins [1]. Previous studies support that Fe is sensed not only as ionized form, but also as heme and Fe-S clusters [11,43,45–47]. Both heme and Fe-S clusters are synthesized in mitochondria in all eukaryotes and in plastids in plants. Dysfunction of these syntheses causes severe perturbation in Fe deficiency responses in various living systems, including yeasts, animals, and plants [43,45]. The major Fe deficiency response in budding yeast is regulated by the key transcription factor Aft1 [45]. Aft1 is a positive regulator in the nucleus but senses Fe nutritional conditions in mitochondria rather than the cytosol or nucleus. Dissociation of Aft1 from the target gene promoter under Fe-replete conditions is promoted by monothiol glutaredoxins Grx3/4, which are Fe-S cluster acceptors from mitochondria [46]. The Fe-S cluster in mammals is directly accepted by IRP1 [11], and heme is thought to be involved in IRP2 degradation [47], both of which result in abolishment of IRP-IRE binding and Fe deficiency responses. IRP1 acts as a cytosolic aconitase when bound to the Fe-S cluster [11], a common enzyme in the tricarboxylic acid cycle. IRP1-like proteins are conserved in all organisms including plants as aconitases. Single loss-of-function mutants of *Arabidopsis* aconitases do not show apparent alterations in Fe responses, suggesting that the IRP/IRE system might not be conserved in plants [48]. However, *Arabidopsis* aconitase genes appear to possess functional redundancy, and double mutants of *Arabidopsis* aconitase genes are lethal [48]. This enzyme is crucial for primary metabolism and Fe chelator synthesis, both of which severely affect Fe translocation and homeostasis. Thus, further study will be needed to identify the precise function of plant aconitases with respect to Fe signaling.

Mutants of plant Fe importers into either mitochondria or plastids, as well as those defective in Fe–S cluster synthesis, have disrupted regulation of nuclear-encoded genes responsive to Fe

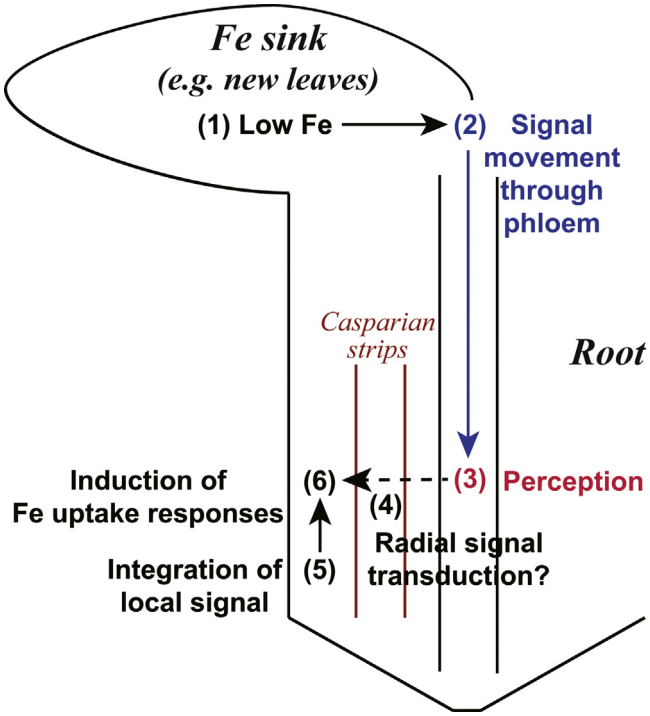


**Fig. 3.** Schematic representation of systemic Fe signal transmission. Fe deficiency signal in sink tissues such as new leaves is thought to be systemically transmitted and induce root Fe uptake responses through the following processes: (1) occurrence of low Fe concentration in sink cells; (2) phloem transport of systemic signals which might be ionized Fe, nicotianamine or Fe-nicotianamine complexes; (3) perception of phloem signal in root phloem or periphery cells which might be mediated by IDEF1 and/or HRZs/BTS; (4) radial signal transduction across the casparian strips toward the root surface; (5) integration of local signal from the root surface, and (6) induction of Fe uptake responses in root epidermis and exodermis.

availability [43]. Thus, the Fe deficiency response in the nucleus is thought to be regulated by mitochondrial and plastidic Fe signals, possibly in the form of heme and Fe-S clusters, as well as other intimately related compounds such as products of primary metabolism [43]. Interestingly, HRZs/BTS possess a putative binding site of Fe-S cluster called the rubredoxin-type fold [20]. It is possible that HRZs/BTS sense both free Fe and Fe-S clusters that integrate cytosolic and mitonchondrial/plastidic Fe signals. Coexistence of hemerythrin and rubredoxin in the same molecule is also observed in bacterial protein rubrerythrin [36], but there is no evidence supporting the function of rubredoxin as an Fe-sensing domain.

### 3.4. Phloem-mobile iron and nicotianamine

In addition to the above-mentioned cellular molecules involved in Fe sensing, multicellular organisms must coordinate systemic Fe homeostasis through long-distance Fe transport and signaling. The typical Fe deficiency response in roots of vascular plants is thought to be mediated by both shoot-derived long-distance and local Fe signals (Fig. 3), based on gene expression and morphological analysis in response to Fe deficiency or excess treatment at limited portions of the plant body [49,50]. The identity of the long-distance signal is suggested to be phloem-mobile Fe and/or its chelators.

Fe is believed to be chelated by amino acid derivatives in the phloem, including nicotianamine and deoxymugineic acid [4,51,52]. These Fe chelators not only determine the mobility and availability of Fe in plant tissues by chelating it, but they are also thought to be possible signaling molecules. This notion is supported by several transgenic analyses, especially for nicotianamine. In transgenic rice lines that accumulate high nicotianamine, increased

expression is observed in many Fe deficiency-inducible genes involved in Fe uptake and utilization [53–55]. This phenomenon is more or less conserved in both mutants with a disrupted nicotianamine aminotransferase gene (*OsNAAT1*) that is defective in Strategy II-based Fe uptake [53], and in lines overexpressing nicotianamine synthase genes (*NASs*), which accumulate Fe without showing susceptibility to Fe deficiency [54,55]. In contrast, the *Arabidopsis ysl1 ysl3* double mutant carrying disruptions in metal-nicotianamine transporters does not have an enhanced Strategy-I Fe deficiency response in roots irrespective of compromised Fe translocation and severe symptoms of Fe deficiency [56]. These results suggest that the induction of Fe deficiency-responsive genes in roots is not simply regulated by shoot Fe nutritional status but also by the level of nicotianamine in the phloem. However, elevated root Fe deficiency responses are observed concomitant with severe Fe deficiency symptoms in tomato leaves having *chloronerva* mutant, defective in nicotianamine synthesis because of a mutation in the endogenous *NAS* gene [51]. This indicate that nicotianamine is dispensable for root Fe responses. Thus, it remains unknown whether Fe chelators in phloem are direct Fe signals or indirectly involved in Fe signaling by facilitating mobilization of Fe and other metals, or by rendering these metals unavailable to other essential molecules.

*Arabidopsis atopt3* mutants with repressed expression of a putative oligopeptide transporter have constitutively induced Fe deficiency responses in roots [57]. The shoots of this mutant accumulate Fe, suggestive of disruption of shoot-to-root Fe signaling. Although a yeast complementation assay indicated that AtOPT3 transports Fe [58], sequence homology of AtOPT3 with oligopeptide transporters suggests that it transports small peptides which chelate or sense Fe rather than ionized Fe itself [57]. Precise investigation of AtOPT3 transport activity using *Xenopus* oocytes, yeasts or other living cells will be needed to clearly demonstrate its precise substrates and functions.

Phloem-derived Fe signal may be perceived in root phloem or neighbor cells. Then, radial transmission of secondary signals may be required to trigger Fe acquisition responses in the epidermis and the exodermis, tissue outside the root cortex of many species, including rice, which also have casparian strips (Fig. 3). *IDEF1* expression in primary roots is mainly limited to phloem cells [24]. *BTS* is predominantly expressed in root pericycle cells [21], and *OsHRZ1* and *OsHRZ2* are preferentially expressed in the stele [20]. Thus, IDEF1 and HRZs/BTS might be responsible for perception of phloem-derived Fe signal. Identity of the radial transmission of secondary signals is still unknown, even though radial movement of regulatory proteins such as transcription factors might be responsible for this process [21,24].

## 4. Other implications and conclusions

The identification of IDEF1 and HRZs/BTS has paved the way for our understanding of Fe deficiency sensors and signals. Both IDEF1 and HRZs/BTS are susceptible to 26S proteasome-mediated degradation [20,25], and HRZs/BTS possess ubiquitination activity [20], suggestive of the importance of protein-level regulation in Fe deficiency responses. There has been no clear demonstration of IDEF1 or HRZs/BTS protein levels in plant cells, except in overexpressing lines detected by Western blot analysis or green fluorescent protein fusions [19,20,25]. This is possibly because of the low expression levels and unstable nature of these proteins. Production of highly specific antibodies and improvement of protein extraction methods might solve this problem. Regulation of Fe deficiency responses at the protein level has also been observed for two *Arabidopsis* proteins playing major roles in the Strategy I response; namely, $Fe^{2+}$ transporter IRT1 and transcription factor FIT [4,32,59]. Ubiquitination and degradation of IRT1 is thought to prevent excess uptake of $Fe^{2+}$ or other metals transported by IRT1. On the other hand, ubiquitination and degradation of FIT is thought to be essential for keeping the activity of this regulator by scavenging poorly functional older proteins from their target promoters [4,59]. Thus, plant responses to Fe deficiency appear to be mediated both by transcriptional upregulation and quality control by protein ubiquitination. It remains unclear how the latter step is regulated by Fe, except that FIT degradation is induced under Fe deficiency [59].

Plant responses to both Fe deficiency and excess are also regulated by complicated signaling cascades of the circadian clock, plant hormones, and small signaling molecules, such as NO [4,5,43,49,50,60–62]. In general, auxin, ethylene, abscisic acid, gibberellin and NO positively affects Fe deficiency responses, whereas cytokinin and jasmonic acid act negatively [4,5,50,61,62]. However, positive involvement of jasmonic acid in rice Fe deficiency responses has also been proposed [25]. Since these regulatory systems cover a wide variety of plant responses, characterizing the mechanisms conferring specificity to Fe responses is important.

It is possible that master regulators of Fe deficiency responses, such as IDEF1 and HRZs/BTS, directly bind free Fe and modulate their own activities. The concentrations and chemical forms of free Fe remain unknown in plant cells. However, dynamic imaging of cytosolic Zn has recently been achieved by FRET sensors [63]. Human Fe chaperones PCBP1 and PCBP2 deliver cytosolic Fe into Fe storage protein ferritin and HIF-oxidizing dioxygenases [39], although no plant counterparts of such Fe chaperones have been identified. The improvement and application of analytical methods that determine the form and amount of Fe in plant cells would increase our understanding of Fe signaling at the cellular level. Meanwhile, plant Fe sensors and signals can be identified by monitoring signal transmission and conversion [criteria (ii)] by IDEF1 and HRZs/BTS, as well as by alternative unknown sensors potentially identified using forward and reverse genetics approaches.

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