Iron Uptake, Translocation, and Regulation in Higher Plants

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Abstract

Iron is essential for the survival and proliferation of all plants. Higher plants have developed two distinct strategies to acquire iron, which is only slightly soluble, from the rhizosphere: the reduction strategy of nongraminaceous plants and the chelation strategy of graminaceous plants. Key molecular components—including transporters, enzymes, and chelators—have been clarified for both strategies, and many of these components are now thought to also function inside the plant to facilitate internal iron transport. Transporters for intracellular iron trafficking are also being clarified. A majority of genes encoding these components are transcriptionally regulated in response to iron availability. Recent research has uncovered central transcription factors, *cis*-acting elements, and molecular mechanisms regulating these genes. Manipulation of these molecular components has produced transgenic crops with enhanced tolerance to iron deficiency or with increased iron content in the edible parts.

Contents

INTRODUCTION

Among the essential micronutrients in plants, iron (Fe) is required in the greatest abundance. It functions in various important processes, including photosynthesis, respiration, and chlorophyll biosynthesis, and is a component in heme, the Fe-sulfur cluster, and other Fe-binding sites. The chemical properties of Fe that make it suitable for redox reactions also make it prone to generating reactive oxygen species when it exists in a free state and in large quantities (69). To acquire enough Fe while avoiding toxicity, plants tightly control uptake, utilization, and storage in response to environmental availability. In addition to studies in classical plant physiology, biochemistry, agronomy, soil science, and breeding, recent advances in molecular biology, genetics, analytical chemistry, and bioinformatics have dramatically advanced the understanding of Fe homeostasis in plants at the molecular level. In this article, we review the present understanding of Fe uptake, translocation, subcellular translocation, and regulation in response to Fe shortage or excess in higher plants at the molecular level; **Table 1** lists the central genes responsible for these processes. Recent progress in Fe regulation has focused on *Arabidopsis thaliana* and rice, which generally represent nongraminaceous and graminaceous systems, respectively. We also review transgenic crops with increased tolerance to Fe deficiency and improved nutritional quality.

MAs: mugineic acid family phytosiderophores

IRON UPTAKE

Despite its abundance in the soil, Fe is only slightly soluble under aerobic conditions, especially in high-pH and calcareous soils (69). In the 1970s, Takagi (121) discovered the mugineic acid family phytosiderophores (MAs), which are Fe(III)-solubilizing molecules secreted from Fe-deficient graminaceous plants. Römheld & Marschner (104) reexamined the Fe acquisition mechanisms in various plant species and placed them into two categories: Strategy I in nongraminaceous plants and Strategy II in graminaceous plants. Since the end of the 1990s, genes involved in these strategies have been identified that almost perfectly fit the model scheme proposed by Römheld & Marschner (104) (**Figure 1**).

The two main processes in the Strategy I response, which is utilized by all higher plants except those in the Graminaceae family, are the reduction of ferric chelates at the root surface and the absorption of the generated ferrous ions across the root plasma membrane. The dominant genes responsible for these processes were first cloned from *Arabidopsis* in the 1990s—namely, the ferric-chelate reductase oxidase gene *FRO2* (102) and the iron-regulated transporter gene *IRT1* (22). Since then, homologs of *FRO* and *IRT* have been cloned from various plant species. Other processes involved in Strategy I include excretion of proton and phenolic compounds from the roots to the rhizosphere, which is thought to help increase the solubility of ferric ions or support the reducing capacity of ferric Fe on the root surface. Among the large number of H^+-ATPase (HA) genes, some are induced under Fe deficiency and are thought to function in Strategy I responses, as with cucumber *CsHA1* (107) and *Arabidopsis AHA2* (109). Genes involved in phenolic secretion have not been identified in nongraminaceous plants. However, recent identification of the involvement of rice protocatechuic acid effluxer PEZ1 (PHENOLICS EFFLUX ZERO 1) in Fe

Table 1 Central genes responsible for Fe homeostasis reviewed in this article

Name	Function	Fe deficiency response	Representative gene[a]
Strategy I Fe uptake			
FRO	Ferric-chelate reductase	Induced (strong)	At *FRO2* (102)
IRT	Ferrous ion transporter	Induced (strong)	At *IRT1* (22)
HA	Proton efflux transporter	Induced	*CsHA1* (107)
PEZ[b]?	Phenolics efflux transporter	?	—
Strategy II Fe uptake			
TOM1	MAs efflux transporter	Induced (strong)	Os *TOM1* (87)
YS1/YSL	Fe(III)-MAs transporter	Induced (strong)	Zm *YS1* (15)
NAS	NA synthase	Induced (strong)	*HvNAS1* (32)
NAAT	NA aminotransferase	Induced (strong)	*HvNAAT-A* (125)
DMAS	Deoxymugineic acid synthase	Induced (strong)	*OsDMAS1* (3)
IDS2	Putative epihydroxymugineic acid synthase	Induced (strong)	Hv *IDS2* (83, 93)
IDS3	Mugineic acid synthase	Induced (strong)	Hv *IDS3* (49, 82, 83)
SAMS/MAT	*S*-adenosyl-L-methionine synthetase	Induced (weak)	*OsSAMS2* (54)
MTN	Methylthioadenosine/*S*-adenosyl homocysteine nucleosidase	Induced	*OsMTN* (54, 105)
MTK	Methylthioribose kinase	Induced	*OsMTK1* (54, 110)
IDI2/MTI	Methylthioribose-1-phosphate isomerase	Induced	Hv *IDI2* (120, 146)
DEP	Methylthioribulose-1-phosphate dehydratase-enolase-phosphatase	Induced	Os *DEP* (54)
IDI1/ARD	Acireductone dioxygenase	Induced	Hv *IDI1* (145)
IDI4[b]*/AAT*[b]	Putative aminotransferase catalyzing the synthesis of methionine	Induced	*OsIDI4* (54)
FDH	Formate dehydrogenase	Induced	Hv *Fdh* (118)
APRT	Adenine phosphoribosyltransferase	Induced	*HvAPT1* (39)
Fe translocation			
FRD3/FRDL	Citrate efflux transporter	Induced/constitutive	At *FRD3* (20, 103)
PEZ	Phenolics efflux transporter	Induced (weak)	Os *PEZ1* (35)
FPN[b]*/IREG*[b]	Putative Fe efflux transporter	Induced	At *FPN1/IREG1* (76)
TOM[b]?	MAs efflux transporter	Induced (strong)	Os *TOM1* (87)
ENA[b]?	NA efflux transporter	Induced	Os *ENA1* (87)
YS1/YSL	Fe(III)-MAs/Fe(II)-NA transporter	Induced/repressed	*OsYSL2* (56)
FRO[b]*?*	Ferric-chelate reductase for Fe translocation	?	—
IRT[b] and/or *NRAMP*[b]?	Ferrous ion transporter for Fe translocation	?	—
Fe storage			
Ferritin	High-capacity Fe storage and sequestration	Repressed	*AtFer1* (95)
Fe compartmentalization			
PIC1	Chloroplast Fe transporter	Constitutive	At *PIC1* (21)
FRO	Ferric-chelate reductase for chloroplast Fe transport	Constitutive	*AtFRO7* (41, 143)
MIT	Mitochondrial Fe transporter	Repressed	Os *MIT* (4)
VIT1	Fe transporter into vacuole	Constitutive/repressed	At *VIT1* (46)

(*Continued*)

Table 1 (*Continued*)

Name	Function	Fe deficiency response	Representative gene[a]
FPN/IREG	Fe transporter into vacuole	Constitutive	At *FPN2/IREG2* (76, 112)
NRAMP	Fe transporter into cytosol from vacuole	Induced	*AtNRAMP3* (58, 131)
Gene regulation in Strategy I plants			
FER/FIT	Positive transcriptional regulator	Induced	Sl *FER* (63)
AtbHLH38, *AtbHLH39*	Positive transcriptional regulator	Induced	*AtbHLH38* (138, 139, 148)
PYE	Negative transcriptional regulator	Induced	At *PYE* (65)
BTS[b]	Putative transcriptional/posttranslational regulator	Induced	At *BTS* (65)
EIN3, *EIL1*	Ethylene signaling regulator	?	At *EIN3* (64)
TIC	Circadian clock regulator	?	At *TIC* (19)
Gene regulation in Strategy II plants			
IDEF1	Positive transcriptional regulator	Constitutive	Os *IDEF1* (53)
IDEF2	Positive transcriptional regulator	Constitutive	Os *IDEF2* (91)
IRO2	Positive transcriptional regulator	Induced (strong)	*OsIRO2* (89)
IRO3	Transcriptional regulator (putatively negative)	Induced	*OsIRO3* (150)

Abbreviations: MAs, mugineic acid family phytosiderophores; NA, nicotianamine. Species abbreviations: At, *Arabidopsis thaliana*; Cs, *Cucumis sativum* (cucumber); Hv, *Hordeum vulgare* (barley); Os, *Oryza sativa* (rice); Sl, *Solanum lycopersicum* (tomato; formerly described as Le for *Lycopersicon esculentum*); Zm, *Zea mays* (maize).

[a]Italic letters represent gene names; roman letters with a space in front of the gene name indicate the species of the respective gene in which the species prefix was not included in the original literature.

[b]Putative components whose involvement in processes has not been demonstrated at the molecular level.

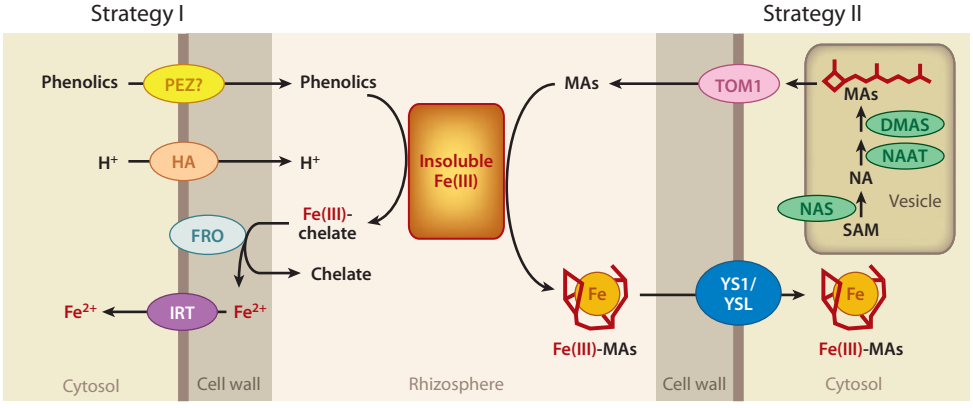

Figure 1

Fe acquisition strategies in higher plants: Strategy I in nongraminaceous plants (*left*) and Strategy II in graminaceous plants (*right*). Ovals represent the transporters and enzymes that play central roles in these strategies, all of which are induced in response to Fe deficiency. Abbreviations: DMAS, deoxymugineic acid synthase; FRO, ferric-chelate reductase oxidase; HA, H^+-ATPase; IRT, iron-regulated transporter; MAs, mugineic acid family phytosiderophores; NA, nicotianamine; NAAT, nicotianamine aminotransferase; NAS, nicotianamine synthase; PEZ, PHENOLICS EFFLUX ZERO; SAM, *S*-adenosyl-L-methionine; TOM1, transporter of mugineic acid family phytosiderophores 1; YS1/YSL, YELLOW STRIPE 1/YELLOW STRIPE 1–like.

solubilization in the stele (35) raises the possibility that its nongraminaceous counterparts may be responsible for the Strategy I response. Induction of these molecular components under low Fe availability is accompanied by morphological changes in root architecture, such as the formation of transfer cells and extra root hairs (69, 108). The *Arabidopsis* ubiquitin-conjugating enzymes UBC13A and UBC13B, which were recently found in a cucumber proteomic analysis of Fe deficiency–induced proteins in root tips, regulate root hair formation (62).

The Strategy II response relies on biosynthesis and secretion of MAs, which are specific to graminaceous plants. Nine types of MAs have been identified to date, all of which are synthesized through a conserved pathway from *S*-adenosyl-L-methionine (3, 68, 75, 115, 135). This pathway includes three sequential enzymatic reactions mediated by nicotianamine synthase (NAS), nicotianamine aminotransferase (NAAT), and deoxymugineic acid synthase (DMAS) (3, 32, 125), generating 2′-deoxymugineic acid (DMA), the precursor of all other MAs. In restricted species, such as barley and rye, DMA is further hydroxylated to other MAs by dioxygenases, such as iron deficiency–specific clone 2 (IDS2) and IDS3 (49, 83). Fe deficiency strongly induces the expression of genes encoding these biosynthetic enzymes. To supply methionine for the successive production of MAs, a set of recycling reactions called the methionine cycle or Yang cycle is employed (67). Genes encoding the enzymes involved in this cycle were deduced from sequence comparison with corresponding genes in bacteria and yeast (54, 120). All of these candidate genes were inducible in response to Fe deficiency (54, 120), and the enzyme activities of most of the encoded proteins have recently been demonstrated (98, 105, 110, 111).

The secretion of MAs follows a diurnal pattern with a steep peak in the morning (122). Certain vesicles observed in Fe-deficient root cells are swollen in the early morning but shrink by the evening (84, 85). The NAS enzyme is localized on the membrane of these vesicles, whereas NAAT is present within the vesicles (86; S. Nagasaka, T. Kobayashi, M. Takahashi, S. Mori, H. Nakanishi & N.K. Nishizawa, unpublished results), suggesting that these vesicles are the site of MA biosynthesis. The genes responsible for MA secretion were the missing piece in the Strategy II mechanism. Just recently, Nozoye et al. (87) identified the transporter of mugineic acid family phytosiderophores 1 (TOM1) from rice and HvTOM1 from barley, revealing the final piece in the mechanism. The MAs secreted into the rhizosphere solubilize Fe(III), and the resulting Fe(III)-MA complexes are taken up into root cells by the YELLOW STRIPE 1 (YS1) and YELLOW STRIPE 1–like (YSL) transporters (15, 33, 59, 78).

Rice, despite being a Strategy II plant, possesses a ferrous transporter, OsIRT1, that allows this crop to absorb Fe^{2+} (38) in addition to its Strategy II–based Fe(III)-DMA uptake by the OsYSL15 transporter (33, 59). In contrast to Strategy I plants, however, rice has very low ferric-chelate reductase activity on the root surface (38), suggesting that it has adapted to directly take up Fe^{2+}, which is abundant in submerged and anaerobic conditions.

There are many reports on other divalent metal transporters in both nongraminaceous and graminaceous plants. The ZIP (zinc-regulated transporter, iron-regulated transporter–like protein) family was first discovered as a homolog of *Arabidopsis IRT1*; it transports various divalent metals, including Fe^{2+}, zinc $(Zn)^{2+}$, manganese $(Mn)^{2+}$, cadmium $(Cd)^{2+}$, nickel $(Ni)^{2+}$, and cobalt $(Co)^{2+}$ (30, 57, 81, 94). Another family of transporters, NRAMP (natural resistance-associated macrophage protein), also transports similar divalent metals. IRT transporters generally localize to the plasma membrane, whereas NRAMP transporters localize to either intracellular vesicles or the plasma membrane depending on the species of the protein (8, 58, 126, 130). Members of these transporter families in the plasma membrane of the root epidermis/exodermis are thought to be responsible for the uptake of essential metal elements from the rhizosphere

YS1: YELLOW STRIPE 1

YSL: YELLOW STRIPE 1–like

ZIP: zinc-regulated transporter, iron-regulated transporter–like protein

NRAMP: natural resistance-associated macrophage protein

NA: nicotianamine

Efflux transporters (exporters): proteins that transport particular substances across the limiting membrane from the cytosol out of the cell or into intracellular compartments

Influx transporters (importers): proteins that transport particular substances across the limiting membrane from extracellular or intracellular compartments into the cytosol

(8, 94). Fe deficiency–induced expression of these members, such as *Arabidopsis IRT1* and rice *OsIRT1*, is thought to be a major route for accumulation of harmful metals, including Cd under Fe deficiency (76, 81, 112).

In graminaceous plants, MAs are also involved in the chelation and uptake of non-Fe metals, including Zn as a form of Zn(II)-MAs (120). In contrast, chelate formation of Cd(II)-DMA is reportedly much weaker than that of Fe(III)-DMA and Zn(II)-DMA, and thus DMA should not function in Cd uptake (74).

IRON TRANSLOCATION

Because of the poor solubility and high reactivity of Fe, its translocation inside the plant body must be associated with suitable chelating molecules and proper control of redox states between the ferrous and ferric forms (31, 69). Fe translocation in plants involves various steps, including radial transport across the root tissues, which must include symplastic transport to pass through the Casparian strip; xylem loading, transport, and unloading; xylem-to-phloem transfer; phloem loading, transport, and unloading; symplastic movement toward the site of demand; and retranslocation from source or senescing tissue (45). Physiological and molecular studies have indicated some principal chelators inside the plant body, such as citrate (6, 132), nicotianamine (NA) (31, 124), and MAs (1, 43). Recent progress in identifying transporters has greatly improved our understanding of the components involved in the metal translocation process, but it is often difficult to determine the precise contribution of each component in the metal movement flux at each step of translocation. **Figure 2** represents our present understanding and deduction of the molecules involved in xylem and phloem loading. Because the xylem and phloem consist of dead and living cells, respectively, xylem loading is assumed to require efflux transporters, whereas phloem loading would require influx transporters.

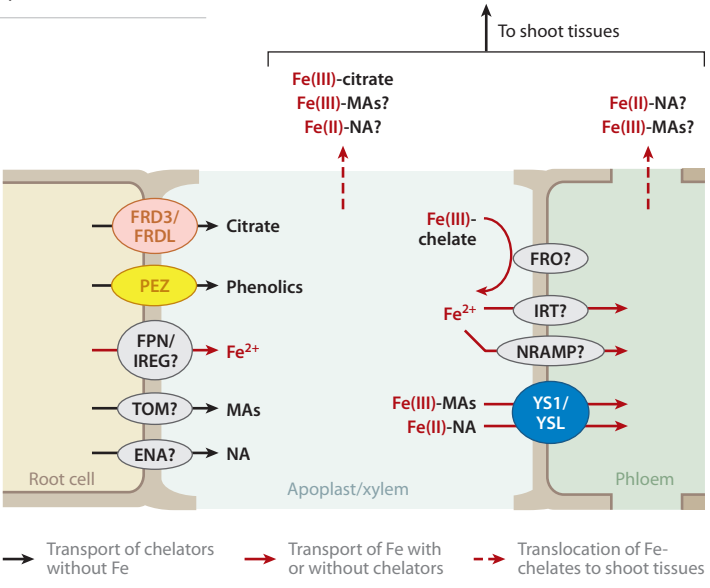

Figure 2

Molecules involved in xylem and phloem Fe loading. Only a few components have been proven to be responsible for a specific step in transport; the involvement of others (indicated by question marks) has been suggested by physiological, genetic, or biochemical studies. Abbreviations: ENA, efflux transporter of nicotianamine; FPN/IREG, ferroportin/iron regulated; FRD3/FRDL, FERRIC REDUCTASE DEFECTIVE 3/FERRIC REDUCTASE DEFECTIVE 3–like; FRO, ferric-chelate reductase oxidase; IRT, iron-regulated transporter; MAs, mugineic acid family phytosiderophores; NA, nicotianamine; NRAMP, natural resistance-associated macrophage protein; PEZ, PHENOLICS EFFLUX ZERO; TOM1, transporter of mugineic acid family phytosiderophores 1; YS1/YSL, YELLOW STRIPE 1/YELLOW STRIPE 1–like.

Citrate has long been thought to play a dominant role in the chelating and trafficking of Fe in xylem sap (6). Recently, Rellán-Álvarez et al. (101) identified an actual form of Fe-citrate in tomato xylem sap as a tri-Fe(III) tri-citrate complex, modeled as having an oxo-bridged tri-Fe core. FERRIC REDUCTASE DEFECTIVE 3 (FRD3), an *Arabidopsis* transporter of the multidrug and toxin efflux (MATE) family, facilitates citrate efflux into the xylem (20, 103). *FRD3* was cloned as the causative gene of the *frd3* mutant, which exhibits leaf Fe deficiency–associated chlorosis and constitutive activation of Strategy I, leading to abnormal distribution and accumulation of various metals; the *frd3* mutant is allelic to *manganese accumulator 1* (*man1*) (16, 103). Mutation of the FRD3 transporter results in Fe localization to the central vascular cylinder

of the roots and failure to transport this metal to aerial parts, suggesting that citrate plays a dominant role in xylem Fe transport (29). An *FRD3*-like gene in rice, *OsFRDL1*—which is specifically expressed in root pericycle cells, similar to *Arabidopsis FRD3* (29, 34)—also encodes a citrate effluxer required for efficient Fe translocation (147). However, *OsFRDL1* knockout plants show only mild defects in Fe homeostasis compared with the severe phenotype of *Arabidopsis frd3* mutants, suggesting that rice possesses alternative chelators for xylem Fe transport.

Another novel effluxer, PEZ1, was named after its mutant phenotype, which has dramatically lower amounts of protocatechuic acid and caffeic acid in the xylem sap (35). PEZ1 is thought to be responsible for xylem loading of these phenolics, facilitating remobilization of precipitated apoplasmic Fe inside the plant body.

Because FRD3, FRDL1, and PEZ1 are thought to efflux Fe-chelating molecules in their free forms (without Fe), Fe must also be effluxed into the xylem by one or more other transporters. To date, the most promising candidate for this function is *Arabidopsis* ferroportin 1/iron regulated 1 (AtFPN1/AtIREG1) (76), which is a homolog of the mammal Fe efflux transporter that functions in Fe absorption in the intestines and Fe recycling in macrophages. Although transport activity for AtFPN1 has not been reported, it localizes to the plasma membrane, its promoter activity is dominant in the stele, and its loss-of-function mutants possess less chlorophyll in both Fe-sufficient and Fe-deficient media, suggesting its essential role in Fe transport to shoots (76).

Among the influx transporters, YSL family members are widely involved in Fe translocation. The founding member of the YSL family, maize YS1, which facilitates Fe(III)-DMA uptake from the rhizosphere, is induced in both roots and shoots in response to Fe deficiency (15), suggesting another role inside the plant. Moreover, YSL family members are also present in nongraminaceous plants, which do not synthesize MAs. It is generally thought that these nongraminaceous YSL transporters are involved in the translocation of metals chelated with NA (14). NA is a precursor and structural analog of MAs; it chelates various metals and is synthesized by all plants, in contrast to the Graminaceae-specific synthesis of MAs. AtYSL1 and AtYSL3 play partly redundant roles in the translocation of Fe and other metals (10, 14, 61, 140). AtYSL2 is also thought to be involved in the lateral movement of metals, although its transporting substrate is still controversial (14, 17, 113).

Rice, a Strategy II plant, possesses 18 YSL members (OsYSL1–18). Among them, OsYSL2 transports Fe(II)-NA and Mn(II)-NA but not Fe(III)-MAs (56). Research with transgenic plants has revealed that OsYSL2 is responsible for long-distance transport of NA-chelated Fe and Mn into sink tissues, including leaves and grains (37, 56). OsYSL15, which transports Fe(III)-DMA, is thought to be responsible for both the root absorption of Fe and the internal translocation of Fe for long-distance transport and seedling growth, because of its expression in vascular tissues and the Fe-inefficient phenotypes of its knockdown and knockout mutants (33, 59). Another Fe(III)-DMA transporter gene, *OsYSL18*, belongs to a graminaceous-specific branch of the YSL family (1). *OsYSL18* is expressed in restricted plant parts, including reproductive tissues (especially pollen and pollen tubes) and the phloem of laminar joints, suggesting a role in fertilization and phloem Fe transport. Analysis using a positron-emitting tracer imaging system revealed that Fe(III)-DMA supplied to barley roots is translocated mostly via the xylem to older leaves but mainly via the phloem to the youngest leaves (133), suggesting the importance of Fe transfer from xylem to phloem in laminar joints.

Similar to OsYSL15, the DMA effluxer TOM1 and the ferrous transporter OsIRT1 are also expressed in vascular tissues in rice (38, 87), suggesting the involvement of these transporters in not only Fe uptake but also Fe translocation inside the plant. Nozoye et al. (87) also identified the rice efflux transporter of nicotianamine 1 (ENA1) and ENA2. These

effluxers, as well as their nongraminaceous orthologs, might be involved in NA transport inside the plant body.

Some *FRO* genes are expressed in shoots or root vascular cells (38, 77, 143), raising the possibility of their function in Fe reduction in plant tissues, which might be coupled to Fe translocation by IRT, NRAMP, or other unknown ferrous transporters.

SUBCELLULAR TRANSPORT OF IRON

Once Fe enters a cell, it must be delivered to appropriate compartments for utilization in cellular function and to prevent it from accumulating in excess, which could lead to cytotoxicity. Researchers have just begun to identify the molecular components involved in Fe compartmentalization in plant cells.

The chloroplast is the largest pool of Fe in plant cells, accumulating approximately 80%–90% of cellular Fe (69). There is a high demand for Fe in the photosynthetic apparatus, and Fe deficiency hinders electron transfer between the two photosystems, leading to photooxidative damage. A recent study (106) credited the remodeling capacity of the light-harvesting antenna proteins as a factor in barley's superior tolerance to prolonged Fe deficiency. Another study (21) proposed that *Arabidopsis* permease in chloroplasts 1 (PIC1) transports Fe into the chloroplast, because it is localized in the inner envelope of the chloroplast and its expression in yeast complements metal uptake defects. PIC1 was originally reported as an essential component in protein translocation across the inner envelope in chloroplasts (129). However, precise reinvestigation of *pic1* knockout mutants, which are severely chlorotic and dwarfish with altered Fe homeostasis, suggested that PIC1 mainly regulates Fe compartmentalization and that possible involvement in protein translocation might be an indirect effect (21). *Arabidopsis* FRO7 ferric-chelate reductase is also localized to the chloroplast (41). *fro7* loss-of-function mutants show decreased Fe in the chloroplast, impaired photosynthesis, and severe chlorosis in alkaline soils, suggesting that ferric-chelator function is required for Fe import into the chloroplast.

The mitochondrion is another dominant Fe-requiring site in the cell. Mitochondrial iron transporter (MIT) was recently identified through transfer DNA (T-DNA) mutant screening of rice under Fe deficiency (4). The homozygous knockout mutant of *MIT* is lethal, whereas *mit* heterozygous lines or *MIT* knockdown lines exhibit impaired growth with Fe accumulation in shoots but less Fe in mitochondria. MIT is homologous to yeast *Mrs3* and *Mrs4* and animal *Mitoferrin* genes known to be responsible for Fe transport into mitochondria. An *Arabidopsis* homolog of a zebrafish *Mitoferrin* gene, *AtMfl1*, is suggested to be localized in the chloroplast but not the mitochondrion, and its knockout mutants show inferior growth (127). Expression of both *MIT* and *AtMfl1* is induced by excess Fe and repressed under Fe deficiency (4, 127). Plastids and mitochondria are sites not only for Fe-mediated enzymatic reactions and electron transport but also for Fe-sulfur cluster biogenesis. Essential components of this vital process have been clarified, and they appear to be closely associated with the regulation of Fe homeostasis (97).

The vacuole generally functions as a metal pool to avoid toxicity. Double mutants of *Arabidopsis AtNRAMP3* and *AtNRAMP4* exhibit germination arrest and failure in retrieving Fe from the vacuolar globoids (58). Because AtNRAMP3 and AtNRAMP4 are metal influx transporters, they are thought to be responsible for Fe retrieval from the vacuole into the cytosol during germination. In contrast, *Arabidopsis* vacuolar iron transporter 1 (VIT1) is proposed to efflux Fe from the cytosol into the vacuole (46). *vit1* mutants show aberrant Fe localization in seeds and poor germination in alkaline soil. AtFPN2/AtIREG2, an Fe deficiency–induced tonoplast effluxer of Ni and Co into the vacuole in the detoxification in root epidermal cells (76, 112), might also transport Fe into the vacuole (76).

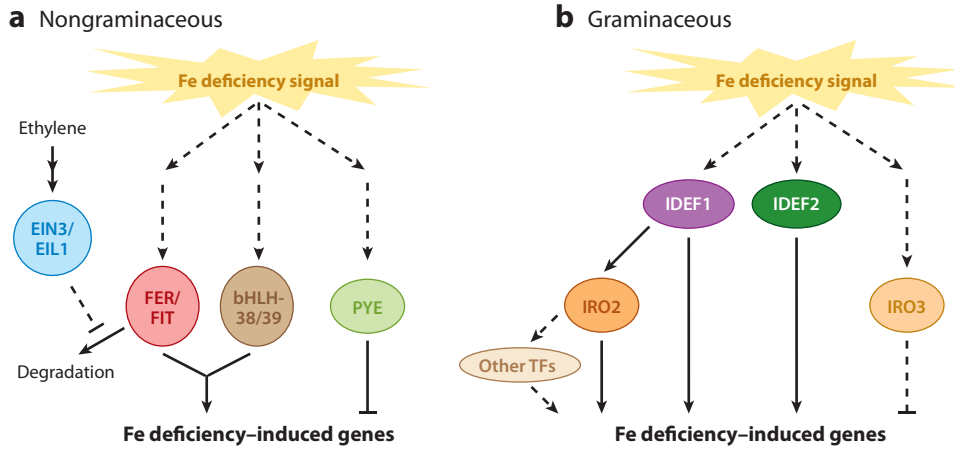

Figure 3

Regulation of Fe deficiency responses in (*a*) nongraminaceous and (*b*) graminaceous plants. Ovals indicate central transcription factors (TFs) involved in the regulation of Fe deficiency–induced genes. Dashed lines indicate putative or unverified pathways. Several other signaling molecules, including nitric oxide (NO), affect expression or activity of some of the depicted key factors through unknown mechanisms. On the other hand, the response to Fe overload is mediated through distinct pathways which are partly linked to the circadian clock and NO. Abbreviations: bHLH, basic helix-loop-helix; EIN3/EIL1, ETHYLENE INSENSITIVE 3/ETHYLENE INSENSITIVE 3–LIKE; FER/FIT, T3238FER/FER-like iron deficiency–induced transcription factor; IDEF, iron deficiency–responsive element-binding factor; IRO, iron-related transcription factor; PYE, POPEYE.

REGULATION OF IRON RESPONSES

Gene regulation is a crucial step for coping with fluctuating environments. Plants induce or repress various genes related to Fe homeostasis in response to Fe deficiency or excess. Induction of Fe acquisition–related genes under low Fe availability is especially pronounced in both nongraminaceous and graminaceous plants, and the central regulators of these genes have been clarified in this decade (**Figure 3**).

The key regulator in nongraminaceous plants was first identified from the tomato T3238*fer* mutant, which shows a defective Strategy I response. Map-based cloning of this mutant resulted in identification of a gene encoding a basic helix-loop-helix (bHLH) transcriptional regulator, *T3238FER* (*FER*) (63). Expression of *FER* is repressed under Fe sufficiency in roots mainly at the posttranscriptional level (7). The *Arabidopsis* ortholog of the *FER* gene, *FIT* (*FER-like iron deficiency–induced transcription factor*, formerly *FIT1/FRU/AtbHLH29*), also plays crucial roles in positively regulating various Fe deficiency–inducible genes, including *IRT1* and *FRO2* (5, 11, 40). *FIT* expression is transcriptionally induced by Fe deficiency in the roots. *FIT* loss-of-function mutants have chlorotic shoots and growth is severely retarded without excess Fe supply.

Constitutive *FIT* overexpression does not induce downstream genes under Fe sufficiency (11, 40), suggesting a requirement for interacting partners that are expressed or activated in response to Fe deficiency. Yuan et al. (148) demonstrated that constitutive coexpression of *FIT* with another bHLH gene, *AtbHLH38* or *AtbHLH39*, is effective in the induction of the target genes *IRT1* and *FRO2* even under Fe sufficiency, resulting in higher Fe content in shoots and enhanced tolerance in Fe-deficient media. AtbHLH38 and AtbHLH39 directly interact with FIT in plant cells, presumably forming heterodimers for essential function (148). *AtbHLH38* and *AtbHLH39* belong to

bHLH: basic helix-loop-helix

the subgroup Ib bHLH genes, together with *AtbHLH100* and *AtbHLH101* (139). These four Ib bHLH genes are strongly induced under Fe deficiency in both roots and leaves, presumably through signaling pathways different from those regulating *FIT*. Overexpression of *AtbHLH38* or *AtbHLH39* in tobacco enhances riboflavin secretion (138), which is a typical Fe deficiency response in some plant species.

Yeast two-hybrid screening revealed that FIT also interacts with the ETHYLENE INSENSITIVE 3 (EIN3) and ETHYLENE INSENSITIVE 3–LIKE1 (EIL1) transcription factors, which play central roles in ethylene signaling, revealing a molecular linkage between the Fe deficiency response and the ethylene pathway (64). Seedlings of *ein3 eil1* double mutants have a mitigated Fe deficiency response and reduced FIT protein accumulation, leading to the hypothesis that EIN3/EIL1 binds to FIT to prevent degradation. Indeed, examination of FIT protein levels in epitope-tagged FIT-overexpressing *Arabidopsis* plants revealed that proteasome-mediated FIT degradation is activated under Fe deficiency (116), in contrast to *FIT* transcript levels, which are upregulated under Fe deficiency. This posttranslational regulation of FIT is proposed to control FIT activity by supplying fresh activators to their target promoters (116).

Overexpression analyses also indicated that *Arabidopsis IRT1* and *FRO2* genes are subject to posttranscriptional regulation, which limits IRT1 protein accumulation and FRO2 enzyme activity under Fe sufficiency independent of transcriptional control (12, 13). Recently, Barberon et al. (2) reported contradictory results on this protein-level regulation of IRT1: Their *IRT1*-overexpressing *Arabidopsis* accumulated IRT1 protein and metals in both roots and leaves irrespective of the Fe nutritional status. They explained this discrepancy as an effect of the N-terminus-truncated IRT1 protein expressed by Connolly et al. (13), which might lead to misfolding and degradation, leaving only the Fe deficiency–specific accumulation of endogenous IRT1 protein. Barberon et al. (2) also reported that a majority of the IRT1 protein is localized to early endosomes and that monoubiquitination of IRT1 at two lysine residues controls subcellular localization, vacuolar sorting, and degradation. Regulation of plasma membrane protein degradation via ubiquitination and subcellular localization has also been shown in other systems, including the *Arabidopsis* borate transporter BOR1 (44).

In another approach to understanding Fe deficiency responses, Dinneny et al. (18) carried out cell type–specific microarray analyses in Fe-deficient *Arabidopsis* roots. They reported large variability in the genes responsive to Fe deficiency between cell layers: Genes involved in metal transport and chelation were induced mainly in the epidermis, whereas those involved in signaling and stress were enriched among the stele-induced genes. Long et al. (65) further searched for candidate regulators that are specifically induced in root pericycle cells under Fe deficiency. Among them, the bHLH transcription factor POPEYE (PYE) plays important roles in root growth under Fe deficiency; the *pye* mutant exhibits inferior growth in Fe-deficient media, which is associated with decreased elongation and swelling of root cells. Microarray and chromatin-immunoprecipitation-on-chip analyses revealed that PYE may negatively regulate Fe homeostasis-related genes. Long et al. (65) also reported another regulator similarly induced in Fe-deficient pericycle cells, BRUTUS (BTS). BTS possesses three putative domains: RING finger for E3 ligase activity, Zn finger for transcriptional regulation, and hemerythrin for Fe binding. In contrast to the *pye* mutant, the *bts* knockdown mutant showed superior growth on Fe-deficient media. Yeast two-hybrid analysis suggested an indirect interaction between PYE and BTS. The physiological relevance of the concomitant induction of PYE and BTS (which might act inversely on Fe deficiency tolerance) and the negative regulation of Fe deficiency–induced genes by PYE is not clear, but the presence of multiple signaling pathways and negative-feedback loops might be important for fine-tuning the Fe deficiency response under rapidly changing environments.

The key elements and factors of the Fe deficiency response in graminaceous plants have been clarified through another strategy. Stepwise promoter analysis of the barley *IDS2* gene in transgenic tobacco led to identification of the *cis*-acting iron deficiency–responsive element 1 (IDE1) and IDE2, which are the first identified elements related to micronutrient deficiency in plants (50). IDE1 and IDE2 synergistically induce Fe deficiency–responsive expression in tobacco roots as well as in rice roots and leaves (50, 51). Searches for factors that interact with IDEs resulted in the identification of two rice transcription factors, IDE-binding factor 1 (IDEF1) and IDEF2, which bind specifically to IDE1 and IDE2, respectively (53, 91). IDEF1 and IDEF2 belong to uncharacterized branches of the plant-specific transcription factor families ABI3/VP1 (ABSCISIC ACID INSENSITIVE 3/VIVIPAROUS 1) and NAC (NO APICAL MERISTEM, *Arabidopsis* transcription activation factor, and CUP-SHAPED COTYLEDON), respectively, and possess novel properties of sequence recognition: IDEF1 recognizes the CATGC sequence within IDE1, whereas IDEF2 recognizes CA(A/C)G(T/C)(T/C/A)(T/C/A) within IDE2 as its core binding site. In contrast to other identified factors involved in the Fe deficiency response, *IDEF1* and *IDEF2* are constitutively expressed in vegetative and reproductive tissues without induction by Fe deficiency (48, 52, 53, 91), suggesting their direct relationship to the perception of the Fe deficiency signal.

IDEF1 and IDEF2 regulate separate subsets of Fe deficiency–inducible genes with relatively little overlap (48, 52, 91). IDEF1 positively regulates the majority of known Fe uptake/utilization–related genes under Fe sufficiency and the early stages of Fe deficiency, whereas it partially changes the species of its downstream genes in the subsequent Fe deficiency stage. Among the IDEF1 target genes at this stage are Fe deficiency–inducible *late embryogenesis abundant* (*LEA*) genes, which are generally regulated by the RY element and thought to be involved in seed maturation and water stress. Microarray analysis combined with in silico promoter analysis suggested preferred recognition of the CATGC sequence by IDEF1 as well as increasing recognition of the RY element (CATGCA) in subsequent stages of Fe deficiency (48). In contrast, IDEF2 might not shift its target genes during Fe deficiency (52). This factor positively regulates the *OsYSL2* gene (91), enabling proper distribution of Fe in rice plants. Indeed, functional disruption of IDEF2 results in aberrant Fe distribution between the roots and shoots (91).

Microarray analysis has also revealed many regulators induced in Fe-deficient graminaceous roots and shoots (84, 89). Among these, the best characterized is the bHLH iron-related transcription factor gene *OsIRO2* in rice (89). *OsIRO2* expression is strongly induced under Fe deficiency (89) and is positively regulated by IDEF1 (48, 53). Biochemical analysis determined the core sequence for OsIRO2 binding to be CACGTGG (89). OsIRO2 positively regulates various genes related to Strategy II (including *OsNAS1*, *OsNAS2*, *OsNAAT1*, *OsDMAS1*, *TOM1*, and *OsYSL15*) and genes involved in the methionine cycle (88, 90). OsIRO2 also affects the expression of some Fe deficiency–inducible transcription factors, which might be involved in the indirect regulation of OsIRO2-downstream genes (90). Thus, a sequential link in the Fe deficiency response involving IDEF1, OsIRO2, and its downstream regulators has been proposed (53, 90) (**Figure 3*b***).

Another Fe deficiency–induced bHLH gene in rice, *OsIRO3*, appears to play a negative regulatory role on various Fe deficiency–induced genes (150). However, this negative regulation is based solely on overexpression lines, which might possess secondary effects. Our recent studies suggest more complicated regulation via OsIRO3 (R.N. Itai & N.K. Nishizawa, unpublished results).

Sequence comparisons have indicated that IRO2 is similar to AtbHLH38, -39, -100, and -101, whereas IRO3 is similar to PYE. In contrast, there appears to be no rice counterpart of FER/FIT, which is inducible

ABI3/VP1: ABSCISIC ACID INSENSITIVE 3/VIVIPAROUS 1

NAC: NO APICAL MERISTEM, *Arabidopsis* transcription activation factor, and CUP-SHAPED COTYLEDON

RY element: a *cis*-acting element containing CATGCA as the minimal sequence that is recognized by the ABI3/VP1 family of transcription factors and that regulates seed-specific expression of plant genes

by Fe deficiency (89). It is not known whether orthologs of IDEF1 or IDEF2 are present in nongraminaceous plants, although there are some nongraminaceous genes similar to these factors (53, 91). IDE elements are functional in tobacco plants (50, 53) and IDE1-like and RY elements are enriched in *Arabidopsis* Fe deficiency–induced genes (54, 80). Taken together, these studies suggest that the molecular components of Fe deficiency responses are only partially conserved between nongraminaceous and graminaceous plants.

Several common signaling molecules affect the Fe deficiency response. Nitric oxide (NO), a bioactive molecule involved in numerous physiological processes in animals and plants, improves Fe availability inside both graminaceous and nongraminaceous plants, such as maize and tomato (27, 28). NO accumulates in Fe-deficient tomato and *Arabidopsis* roots, and the scavenging of NO leads to defects in the induction of *FER/FIT* and their downstream *IRT* and *FRO* genes (9, 28). The plant hormone auxin also accumulates in Fe-deficient *Arabidopsis* roots, where it is postulated to act upstream of NO (9). Elevated carbon dioxide also leads to accumulation of NO and improvement in the Fe deficiency response in tomato (42). Ethylene is another plant hormone that accumulates under Fe deficiency in both nongraminaceous and graminaceous plants, including tomato, cucumber, *Arabidopsis*, and rice, where it acts positively on the induction of various Fe deficiency–induced genes (24, 66, 141, 144). In contrast, cytokinin and jasmonate negatively regulate *Arabidopsis IRT1*, *FRO2*, and *FIT* expression through distinct pathways (73, 114). The Fe deficiency response is also affected by an excess or insufficiency of other elements (e.g., 55, 149, 151), mainly owing to competition between Fe and other elements in various molecular processes, including transport, chelation, and protein binding.

Except for crosstalk among these common signaling molecules, Fe-specific signal substances and the sensors regulating the Fe deficiency response in plants have yet to be identified. Recently, we found that the rice IDEF1 protein directly binds to Fe and other divalent metals via a characteristic histidine-asparagine repeat and proline-rich regions (47), raising the possibility that IDEF1 itself might sense Fe status in plant cells. Fe deficiency responses in roots are proposed to be regulated by shoot-borne long-distance signals and local Fe at the rhizosphere or the root itself (23, 25, 136). Each Fe deficiency–induced gene appears to respond to one or both of these signals. The mechanism determining the tissue specificity of Fe-dependent gene expression is less well understood.

In response to Fe overload, plants induce a subset of Fe homeostasis–related genes, including those encoding ferritin, a ubiquitous protein for Fe storage (95). Through a precise promoter analysis and gel-retardation assay, Petit et al. (96) identified an iron-dependent regulatory sequence (IDRS), a *cis*-acting element that depresses the expression of the maize and *Arabidopsis* ferritin genes (*ZmFer1* and *AtFer1*, respectively) under Fe overload. NO is necessary to mediate transcriptional regulation by IDRS (79) and thus regulates the two opposite responses to Fe availability: Fe deficiency and Fe overload (25). IDRS also mediates *AtFer1* gene induction during dark-induced senescence, whereas *AtFer1* expression during age-dependent senescence or in young seedlings is regulated by other unknown *cis*-acting elements (128).

AtFer1 gene expression fluctuates daily with a peak at dawn, when light-induced oxidative stress in leaves is thought to increase (19). Knockout studies have suggested that the main function of *Arabidopsis* ferritin is protection from oxidative stress rather than simple Fe storage (100). Duc et al. (19) identified a nuclear regulator of the circadian clock, time for coffee (TIC), as an essential regulator of *AtFer1* promoter repression under low-Fe conditions that operates via a pathway that is distinct from the IDRS pathway (19). *tic* mutants are also defective in repressing other Fe overload–induced genes under low-Fe conditions, showing that central oscillator-dependent pathways are involved in the Fe overload response.

GENERATION OF TRANSGENIC CROPS WITH IMPROVED NUTRITIONAL TRAITS

Fe deficiency is a widespread agricultural problem that hinders plant growth and lowers crop yields and quality, especially in calcareous soils, which account for approximately 30% of the world's cultivated soils (69). Because plants are a primary food source for humans, the nutritional state of plants is also of central importance to human health.

Most crops worldwide belong to the Gramineae family; Fe deficiency tolerance differs among species in this family and is thought to depend mainly on the amount and types of MAs secreted. Rice secretes only small amounts of DMA among the MAs, and thus is much more susceptible to low Fe availability compared with species possessing a high capacity to produce MAs, such as barley. Introduction of barley genes encoding biosynthetic genes for MAs into rice was the first successful method for producing rice lines with enhanced tolerance to Fe deficiency (123). Introducing barley genome fragments containing *HvNAAT-A* plus *HvNAAT-B* into rice resulted in increased DMA secretion and substantial tolerance to calcareous soils. In a field experiment in calcareous soil under paddy conditions, substantial tolerance was observed in rice lines carrying a barley genome fragment of either *HvNAS1* or *IDS3* (119).

Another effective approach for improving Fe deficiency tolerance was introducing a ferric-chelate reductase gene into rice (36). Because rice can take up Fe^{2+} via the OsIRT1 transporter but has low ferric-chelate reductase activity, this study introduced a reconstructed and mutagenized derivative of the yeast ferric reductase gene *FRE1*, designated *refre1/372*. This gene was artificially evolved to exhibit enhanced enzymatic activity at a high pH for growth in calcareous soils (92); it is driven by the *OsIRT1* promoter to coexpress this reductase with the endogenous ferrous transporter. The resultant transgenic lines showed increased Fe uptake and enhanced tolerance to low Fe availability in both hydroponic culture and calcareous soils (36).

Manipulation of transcription factors regulating Fe homeostasis has also conferred Fe deficiency tolerance. Overexpression of *IDEF1* and *OsIRO2* in rice results in enhanced tolerance to Fe deficiency but in a different manner. *IDEF1* overexpression under the constitutive or Fe deficiency–inducible *IDS2* promoter confers Fe deficiency tolerance in early stages of deficiency (48, 53) but not necessarily under prolonged Fe deficiency (T. Kobayashi & N.K. Nishizawa, unpublished results). Constitutive *IDEF1* overexpression also leads to defects in germination and early seedling growth (47, 53). In contrast, constitutive overexpression of *OsIRO2* does not lead to any deleterious effects (88, 90) but does confer remarkable Fe deficiency tolerance, especially under long-term growth in calcareous soils (88). Such differences in the phenotypes of the overexpressors appear to be related to the distinct roles of these factors in the Fe deficiency response. In contrast to the positive regulation of Fe uptake/utilization–related genes by IDEF1 in the early stages, OsIRO2 is thought to be especially important for sustaining the induction of Fe uptake/utilization–related genes during prolonged Fe deficiency. Indeed, grain yield and seed Fe concentration in *OsIRO2* overexpression lines are remarkably higher compared with wild-type plants when grown in calcareous soil until maturation (88), providing a promising candidate for application in agricultural fields with problem soils. In contrast to these promising lines for rice, Fe research in *Arabidopsis* has not yet led to Fe deficiency–tolerant crops.

Rice grains are poor in Fe content, even though the majority of Asian people rely on this crop for Fe intake. Understanding Fe homeostasis has paved the way for fortifying the grains with both Fe and Zn. Generation of these mineral-fortified crops by a transgenic approach was first reported by Goto et al. (26), who expressed the soybean ferritin gene in rice grains through a grain-specific glutelin promoter. Qu et al. (99) further utilized the globulin promoter to achieve a 13-fold further

increase in soybean ferritin protein in rice grains. However, this ferritin hyperexpression led to only a 30% increase in Fe concentration in grains compared with the previous line (26), suggesting a requirement for concomitant fortification of Fe uptake and/or translocation (99).

Uauy et al. (134) reported that an NAC transcription factor gene, *NO APICAL MERISTEM B1* (*NAM-B1*), in wheat accelerates senescence and increases the remobilization of Fe and Zn from leaves to developing grains. Although rice counterparts of this gene have not been identified, another rice *NAC* gene, *OsNAC5*, is upregulated in flag leaves during grain filling and might be involved in the remobilization of Fe, Zn, and amino acids to grains (117).

Field trials have revealed that a rice line carrying a barley *IDS3* genome fragment exhibits both Fe deficiency tolerance in calcareous soils and moderately increased Fe and Zn concentration in its grains in both calcareous and Andosol paddy fields (71, 119). The *IDS3* gene encodes a dioxygenase that converts DMA to mugineic acid (49), which is more stable under mildly acidic conditions (137) and might be more favorable for internal mineral translocation. Also, overexpression of the NAS genes *HvNAS1* or *OsNAS3* in rice is effective at increasing Fe and Zn up to three- and twofold, respectively, in their grains (60, 72). In these plants, increased amounts of NA and DMA are thought to be involved in the efficient translocation of Fe and Zn into grains. Furthermore, increased Fe in grains became incorporated into the low molecular mass of a possible Fe-NA cluster and was effective in alleviating anemic symptoms in mice (60).

By introducing a combination of *Ferritin* and *NAS*, Wirth et al. (142) reported a greater than sixfold increase in Fe concentration in rice endosperm. In another approach, introduction of the *OsYSL2* gene under the control of a sucrose transporter (*OsSUT1*) promoter generated rice lines with up to 4.4-fold higher Fe concentrations in their polished seeds (37). Transgenic rice lines with introduced *Ferritin*, *NAS*, and *OsYSL2* in combination under various promoters have also been produced and are under examination in both greenhouses and fields (70).

Because Fe homeostasis is closely linked to that of other mineral elements, an understanding of this phenomenon will also serve as a basis for the production of crops with low concentrations of toxic metals (such as Cd) and transgenic plants for phytoremediation. As an agricultural practice, water management in rice paddy fields is effective in restricting Cd accumulation in grains. Theoretically, this management can be considered an application of Fe nutritional control, which affects Cd influx through Fe deficiency–induced transporters such as OsIRT1 (81).

SUMMARY POINTS

1. Fe acquisition in higher plants takes place via two distinct strategies: Strategy I in non-graminaceous plants and Strategy II in graminaceous plants. Some Strategy II plants also possess the partial machinery of Strategy I, such as the ferrous transporter OsIRT1 in rice.
2. Fe translocation inside plants is aided by various types of influx and efflux transporters as well as suitable chelators.
3. Subcellular Fe transport in plant cells has just begun to be understood and includes various transporters that are specific to each organelle.

4. Regulation of the Fe deficiency response is mediated by a combination of transcriptional and posttranscriptional control, the former being more pronounced among Fe acquisition–related genes. A network of transcription factors has been clarified, which is only partially conserved among nongraminaceous and graminaceous plants.
5. The response to Fe overload is mediated through different pathways than the response to Fe deficiency, but some signaling molecules, such as NO, link both responses.
6. Knowledge of Fe homeostasis has been successfully applied to generate valuable crops, especially rice, that are tolerant of Fe deficiency or whose edible parts are more nutritious.

FUTURE ISSUES

1. The conservation and divergence of the molecular components for Fe uptake and translocation among species will need to be clarified.
2. For each Fe translocation step, the speciation of Fe forms and chelators, the contribution of each type of transporter, and the precise Fe flux need to be clarified.
3. Subcellular Fe compartmentalization and its impact on Fe signaling have just begun to be clarified, and yet-unknown molecular components and mechanisms remain to be identified.
4. Signals and receptors regulating Fe nutritional status will need to be clarified.
5. Factors and mechanisms determining the tissue specificity of gene expression in Fe homeostasis will also need to be clarified.
6. Engineered crops that have improved tolerance to Fe deficiency or that are fortified with Fe, but that preserve desirable agricultural traits, need to be further produced and investigated in various species and cultivars for practical application.
7. Knowledge of Fe nutrition and other minerals needs to be comprehensively integrated both for an understanding of whole mineral nutrition and for phytoremediation or production of crops with lower contents of harmful metals.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Contents