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Iron Uptake and Loading into Rice Grains

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Abstract Iron (Fe) is an important micronutrient for living organisms. Fe deficiency severely impairs plant growth and is a widespread human dietary problem, with particularly high numbers of affected children and females. Rice (*Oryza sativa*) is a source of energy for more than half of the world's population. Thus, understanding the mechanisms of Fe uptake and translocation in rice is of utmost importance in the development of rice varieties that are tolerant to low Fe availability and with high seed levels of Fe. In recent years, the mechanisms underlying Fe transport and homeostasis have been revealed, providing opportunities to increase the Fe content of rice grain. As excess Fe is toxic to cells, plants have developed sophisticated mechanisms to control Fe flow, making it difficult to alter Fe transport. Thus, choosing appropriate chelators and Fe transporters driven by appropriate promoters seems to be the key in developing rice that is tolerant to low Fe availability and which accumulates high grain levels of Fe. Many recent studies have been aimed at increasing the Fe content of rice. Here, we summarize these efforts and review recent progress in understanding the mechanisms of Fe transport.

Keywords Biofortification · Deoxymugineic acid · Iron · Nicotianamine · Rice · YSLs

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Introduction

Iron (Fe) is an essential micronutrient for all higher organisms. Fe deficiency is one of the most prevalent micronutrient deficiencies in humans, causing 0.8 million deaths annually (WHO 2002) and affecting approximately 2 billion people (Stoltzfus and Dreyfuss 1998). A common approach to mitigating Fe deficiency is to promote healthy food, supplementation, and food fortification (Haas et al. 2005), but poor families cannot afford these strategies, especially those living in developing countries. Moreover, Fe biofortification is difficult as many beneficial Fe compounds (e.g., $FeSO_4$) are unpalatable, and less soluble Fe compounds are poorly absorbed (Hurrell 2002). Thus, increasing the Fe content of grain has great potential in combating Fe deficiency and will have a dramatic impact on human health (Clemens et al. 2002; Guerinot 2001). Increasing the Fe content of rice is a difficult task for several reasons. Although soil is abundant in Fe, plants cannot utilize it as it is chiefly present as largely insoluble Fe(III) compounds. The fact that calcareous soils with a high pH account for 30% of the world's cultivated soils (Chen and Barak 1982) makes the situation worse. On the other hand, Fe is a transition metal that readily accepts and donates electrons. This property of Fe makes it essential for plants while, at the same time, making it toxic through the production of reactive oxygen species (Halliwell and Gutteridge 1986). Thus, plants have developed sophisticated mechanisms to absorb Fe from soil and to transport it from root to shoot and grain. To acquire Fe from soil, graminaceous plants secrete small molecules called mugineic acid family phytosiderophores (MAs) that solubilize Fe (Takagi 1976), and Fe (III)–MA complexes are readily taken up by specific transporter at the root surface.

In recent years, much progress has been made in elucidating the MA biosynthetic pathway, identifying proteins that transport Fe in various forms, and uncovering the transcription factors controlling the Fe homeostasis. In this article, we summarize recent advances in our understanding of the molecular mechanisms underlying Fe uptake and transport in plants, efforts to increase plant tolerance to Fe deficiency, and attempts to increase the rice grain Fe content.

Genes involved in Fe homeostasis

The Fe homeostasis system in graminaceous plants utilizes a complex network of enzymes as well as Fe chelators like citrate, nicotianamine (NA), and deoxymugineic acid (DMA); transporters; and transcription factors. This network has been elucidated in great detail, although many parts of the puzzle have yet to be characterized. The genes controlling Fe homeostasis can be grouped into three broad categories: (1) enzymes, especially those involved in MA biosynthesis; (2) transporters of Fe in different forms; and (3) transcription factors controlling the expression of genes involved in Fe homeostasis.

NA and DMA biosynthesis

As mentioned above, graminaceous plants solubilize soil Fe by secreting Fe(III) chelators called MAs from their roots (Takagi 1976; Takagi et al. 1984). The resulting Fe(III)–MA complexes are then absorbed into the roots by Fe(III)–MA transporter.

The MA biosynthetic pathway has been characterized in detail. MAs are synthesized from L-Met (Mori and Nishizawa 1987). NA synthase (NAS) catalyzes the trimerization of *S*-adenosyl Met (SAM) to NA (Higuchi et al. 1994, 1999), which is then converted to a 3′-keto intermediate through the transfer of an amino group using NA aminotransferase (NAAT) (Kanazawa et al. 1995). The subsequent reduction of the 3′-carbon in the keto intermediate produces DMA, which is the first MA synthesized in this pathway. The biosynthetic pathway of all MAs is the same from L-Met to DMA; however, the subsequent steps differ depending on the plant species and cultivar (Ma et al. 1999). The production and secretion of MAs significantly increase in response to Fe deficiency, and tolerance to Fe deficiency in graminaceous plants is strongly correlated with the MAs secreted. For example, rice secretes only DMA in relatively low amounts and is thus sensitive to low Fe availability. In contrast, barley secretes large amounts of many MAs, including MA, 3-hydroxymugineic acid, and 3-epi-hydroxymugineic acid, and is therefore more tolerant to low Fe availability (Römheld and Marschner 1990; Singh et al. 1993).

The genes involved in MA biosynthesis have been characterized in rice; three genes, *OsNAS1–3*, are responsible for the trimerization of SAM (Inoue et al. 2003). OsNAAT1 converts NA to a 3′-keto intermediate in rice (Inoue et al. 2008), which is further converted to DMA by OsDMAS1 (Fig. 1; Bashir and Nishizawa 2006; Bashir et al. 2006). In barley, MAs are secreted in a diurnal fashion (Takagi et al. 1984). The diurnal secretion of MAs has not been fully characterized in rice, although the expression of MA family genes is believed to change in a diurnal fashion (Nozoye et al. 2004; Inoue et al. 2009).

In addition to these enzymes, the activity of rice glutathione reductase is also reported to be regulated by Fe deficiency (Bashir et al. 2007). Furthermore, the expression of genes with unknown functions is also regulated by Fe deficiency, and a knockout mutant for a mitochondrial Fe-regulated gene shows sensitivity to Fe deficiency compared to wild-type (WT) plants (Ishimaru et al. 2009).

Transporters involved in Fe homeostasis

Fe transport is a complex process involving a number of genes belonging to different families. Among the transporters involved in Fe homeostasis, the YSL family (Koike

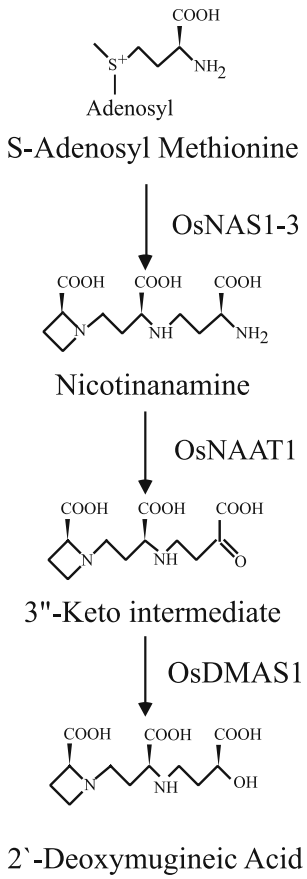

Fig. 1 Biosynthetic pathway of MAs in rice. Three molecules of *S*-adenosyl methionine are combined by OsNAS1–3 to form NA. The amino group of NA is transferred by OsNAAT1, and the resultant 3′-keto intermediate is reduced to DMA by OsDMAS1. The subsequent steps differ with the plant species and cultivar.

et al. 2004), rice Fe-regulated transporters (OsIRT1–2; Bughio et al. 2002; Ishimaru et al. 2006), and citrate transporters (Inoue et al. 2004; Yokosho et al. 2009) have been characterized in detail.

Maize *yellow stripe 1* (*YS1*), which encodes an Fe(III)–MA transporter (Curie et al. 2001), belongs to an oligopeptide family of transporters that function as proton-coupled symporters for various DMA-bound metals (Yen et al. 2001; Schaaf et al. 2004). The rice genome contains 18 putative YSL family genes (Koike et al. 2004), among which *OsYSL2*, *OsYSL15*, and *OsYSL18* have been characterized in detail (Aoyama et al. 2009; Inoue et al. 2009; Ishimaru et al. 2010; Koike et al. 2004). The protein encoded by *OsYSL15* transports Fe(III)–DMA from the rhizosphere to roots (Inoue et al. 2009), whereas *OsYSL18* encodes a functional Fe(III)–DMA transporter involved in DMA-mediated Fe distribution in reproductive organs (Aoyama et al. 2009). In comparison, the translation product of *OsYSL2* has been identified as an Fe–NA or Mn–NA transporter (Koike et al. 2004).

Rice also possesses the ability to transport Fe^{2+}. In rice, two genes, *OsIRT1* and *OsIRT2*, have been cloned and characterized. The proteins encoded by each gene localize to the plasma membrane and have been shown to complement the growth defect of a yeast Fe uptake mutant, confirming that they are functional Fe transporters (Bughio et al. 2002; Ishimaru et al. 2006). OsFRDL1, a member of the multidrug and toxic compound extrusion transporter family, is a citrate transporter localized to pericycle cells that is important for efficient Fe translocation to shoots (Inoue et al. 2004; Yokosho et al. 2009).

Transcription factors controlling the Fe homeostasis in rice

Transcription factors controlling the expression of genes involved in Fe uptake and translocation have also been identified. Two novel Fe deficiency-responsive *cis*-acting elements in barley, Fe deficiency-responsive elements 1 and 2 (IDE1 and IDE2; Kobayashi et al. 2003), were the first elements found to control the expression of genes involved in micronutrient homeostasis. When expressed in rice, IDE1 and IDE2 induce Fe deficiency-responsive gene expression in roots and leaves (Kobayashi et al. 2004). Sequences similar to IDE1 or IDE2 are present in various Fe deficiency-inducible promoters in barley, rice, tobacco, and *Arabidopsis* (Ducos et al. 2005; Kobayashi et al. 2003, 2005), suggesting that gene regulatory mechanisms involving IDEs are also functional in nongraminaceous plant species. The transcription factors for IDE1 and IDE2 have been characterized; IDE-binding factor 1 (*IDEF1*) and *IDEF2* specifically bind to *IDE1* and *IDE2*, respectively (Kobayashi et al. 2007; Ogo et al. 2008). *IDEF1* and *IDEF2* belong to uncharacterized branches of the plant-specific transcription factor families ABI3/VP1 and NAC, respectively. *IDEF1* recognizes the sequence CATGC within *IDE1*, whereas *IDEF2* predominantly recognizes CA[A/C]G[T/C][T/C/A][T/C/A] within *IDE2* as its core binding site. *IDEF1* and *IDEF2* transcripts are constitutively expressed in rice roots and leaves (Kobayashi et al. 2010).

Moreover, the bHLH transcription factor gene *OsIRO2*, which binds specifically to CACGTGG and is regulated by Fe deficiency, has also been characterized (Ogo et al. 2006). *OsIRO2* expression controls the expression of various Fe deficiency-induced genes in rice roots, including genes involved in MA biosynthesis (*OsNAS1*, *OsNAS2*, *OsNAAT1*, and *OsDMAS1*) and Fe(III)–MA transport. *OsIRO2* also affects the expression of Fe deficiency-inducible transcription factor genes possessing OsIRO2-binding core sequences in their promoter regions (Ogo et al. 2007). *OsIRO2* itself harbors multiple *IDEF1*-binding core sequences in its promoter region and is regulated by *IDEF1* (Kobayashi et al. 2007). *IDEF1* expression is correlated with that of *OsIRO2*, *OsYSL15*, *OsIRT1*, *OsYSL2*, *OsNAS1*, *OsNAS2*, *OsNAS3*, and *OsDMAS1* just after the onset of Fe deficiency, suggesting that *IDEF1* is essential for the early onset of the Fe deficiency response (Kobayashi et al. 2009).

Fe homeostasis during germination

Seed germination is a complex process that is regulated by various internal and external factors, including an intricate network of hormone signaling pathways, light, and water. Transporters that are involved in both vacuolar Fe influx and efflux have been identified, and they have been shown to be essential for germination and seedling development in *Arabidopsis* (Lanquar et al. 2005; Kim et al. 2006). Rice seeds contain not only Fe but also NA and DMA, and the amount of DMA is significantly higher than that of NA (Usuda et al. 2009; Masuda et al. 2009). During germination, Fe is mobilized along with NA or DMA, as revealed by an analysis of rice seeds grown without Fe (Takahashi et al. 2009).

Recent technological advances have made it possible to visualize Fe localization in seeds. The localization of Fe in germinating rice grains was observed using X-ray fluorescence imaging (Takahashi et al. 2009). During germination, Fe is detectable in the dorsal vascular bundle, aleurone layer, and endosperm (Fig. 2a), while in the embryo, it localizes to the scutellum and vascular bundle of the scutellum 12 h after sowing. Twenty-four hours after sowing, Fe remains detectable in the dorsal vascular bundle, is dispersed in the scutellum, and accumulates in the coleoptiles. Fe can also be seen in the epithelium and endosperm near the scutellum. Thirty-six hours after sowing, Fe can be observed in root tips. At this stage in

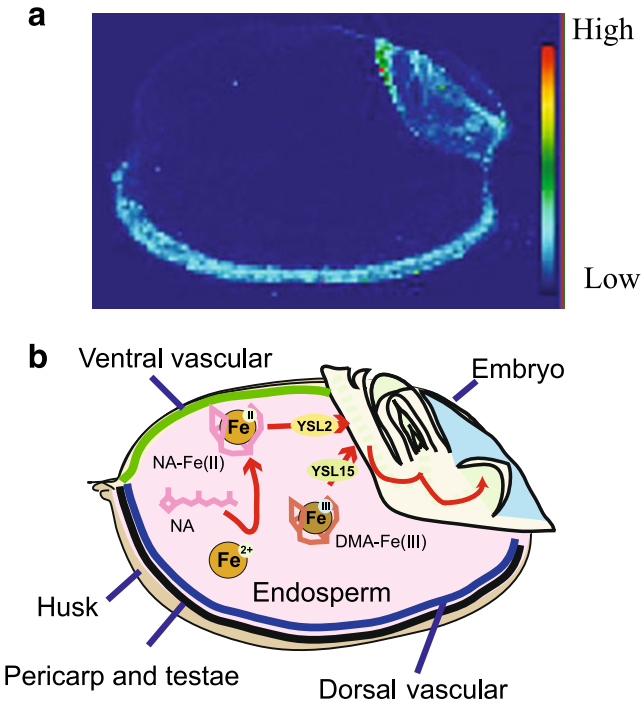

Fig. 2 Fe distribution during germination. Fe localization in seeds (**a**) and a schematic diagram (**b**) showing the production of NA and DMA and Fe distribution during germination. Modified from Nozoye et al. (2007).

the rice embryo, Fe localizes not only to the epithelium, scutellum, and coleoptile, but also to the leaf primordium and radicle (Takahashi et al. 2009).

More than 50% of Fe deficiency-inducible genes are highly expressed during germination, and 76% of Fe deficiency-inducible genes change their expression during seed germination. The expression of various Fe deficiency-related genes has been analyzed during germination through promoter-GUS analysis. *IDEF1* and *IDEF2* are expressed constitutively during germination in the embryo and endosperm (Kobayashi et al. 2010). Moreover, the expression of various genes involved in Fe homeostasis, including *OsNAS2–3* and *OsNAAT1*, increases sharply after germination, indicating that NA and DMA production increases (Nozoye et al. 2007). The expression of the Fe transporters *OsYSL2* (Fe–NA transporter) and *OsIRT1* has been observed during germination. NA seems to be produced in the endosperm, while DMA is produced in the embryo, especially in the coleorhizae and bud scales. NA and DMA production is also overlapped in the embryo (Nozoye et al. 2007). Hence, NA and DMA are synthesized during germination and are involved in Fe translocation during germination. Furthermore, the expression of *OsIRT1* indicates that rice can utilize both Fe^{2+} and Fe^{3+} during germination (Nozoye et al. 2007; Takahashi et al. 2009). The role of Fe transporters during germination has also been studied using knockdown plants. In *OsYSL2* RNAi plants, OsYSL2 does not translocate enough Fe to the roots and shoots of seedlings during germination, resulting in growth defects (Ishimaru et al. 2010). These data are supported by the finding that *OsYSL2* is expressed mainly in the epithelium, vascular bundle of the scutellum, and leaf primordium (Nozoye et al. 2007), suggesting that OsYSL2 is also important for Fe translocation from seeds. The growth of *OsYSL15* knockdown seedlings is arrested at early growth stages, including germination, and can be rescued by the supply of high levels of Fe, indicating that *OsYSL15* plays a crucial role in Fe uptake and homeostasis during early growth (Inoue et al. 2009). Changes in Fe localization, in the expression of Fe transporters, and growth defects in knockdown mutants of *OsYSL2* and *OsYSL15* suggest that these genes play a critical role in Fe translocation during germination (Fig. 2b).

Fe uptake from soil

As discussed above, soil contains abundant Fe; however, most of it is unavailable due to poor solubility. Therefore, plants have developed sophisticated and tightly regulated mechanisms for acquiring Fe from soil, and these can be grouped into two strategies (Marschner et al. 1986). Nongraminaceous plants lower the soil pH by enhancing phenolic and proton excretion into the rhizosphere, reducing the Fe to a more soluble ferrous form at the root surface by inducing the expression of ferric-chelate reductase and by transport of the resulting ferrous ions across the root plasma membrane through IRT1 (Vert et al. 2002).

The genes of the MA pathway are significantly regulated by Fe deficiency. *OsNAS1–2* are tightly regulated in response to Fe deficiency in roots and shoots, while the expression of *OsNAS3*, which is mainly expressed in Fe-sufficient shoots, is induced in roots but suppressed in shoots in response to Fe deficiency (Inoue et al. 2003). The expression of *OsNAAT1* is strong in Fe-deficient roots, particularly in companion and pericycle cells (Inoue et al. 2008). The expression of *OsDMAS1* is also responsive to Fe deficiency (Bashir et al. 2006). The expression patterns and localization of *OsNAS1–2*, *OsNAAT1*, and *OsDMAS1* (root companion cells, pericycle, cortex, and epidermis/exodermis [Fig. 3a]) are quite similar, indicating that these genes work together to produce DMA for Fe acquisition and translocation.

Among Fe transporters, *OsYSL15* is predominantly expressed in the epidermis/exodermis and phloem cells under conditions of Fe deficiency and only in phloem under Fe sufficiency (Inoue et al. 2009). The growth of *OsYSL15*

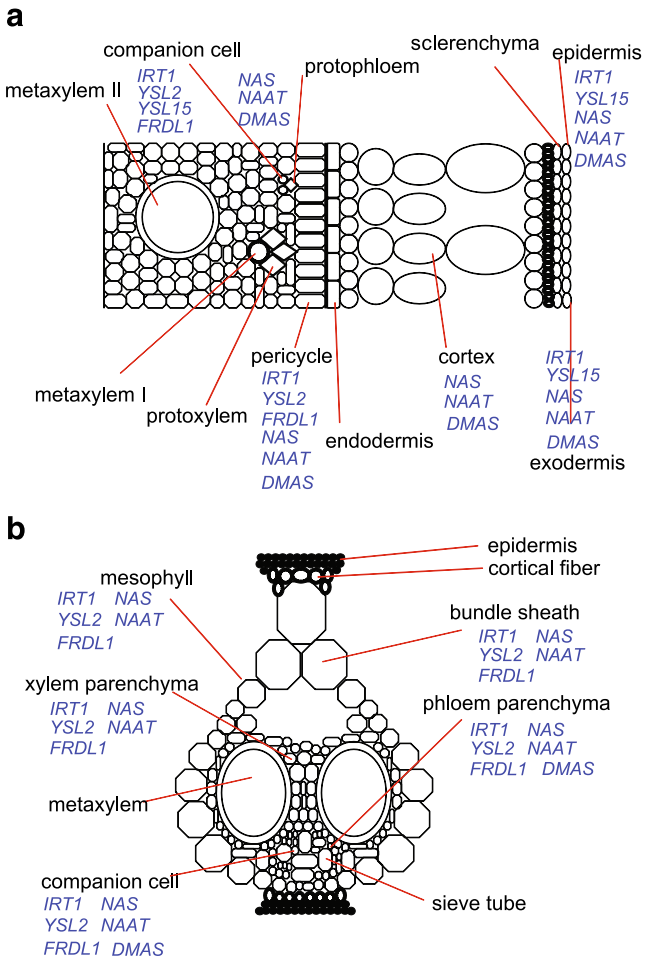

Fig. 3 Tissue-specific expression of Fe homeostasis-related genes in rice roots (**a**) and shoots (**b**).

RNAi seedlings is arrested during early growth and can be rescued by the supply of high levels of Fe (Inoue et al. 2009). *Osysl15* T-DNA mutants are chlorotic under Fe-deficient conditions and have reduced Fe concentrations in their shoots, roots, and seeds (Lee et al. 2009a), indicating that *OsYSL15* plays a crucial role in Fe uptake and homeostasis (Inoue et al. 2009; Lee et al. 2009a).

Rice is unique in the sense that it utilizes both strategies. Besides secreting DMA, it also absorbs Fe^{2+}, which is more abundant than Fe^{3+} under the submerged conditions to which rice is well adapted (Ishimaru et al. 2006). Although two homologs of ferric-chelate reductase are present in rice, the expression of these genes is not observed under Fe-deficient or Fe-sufficient conditions, and the level of Fe^{3+} chelate reductase activity is very low compared to that in other plants (Ishimaru et al. 2006). As rice plants are adapted to grow under submerged conditions where Fe^{2+} is abundant, they may have lost the ability to reduce Fe^{3+} through the development of a functional Fe^{2+}-regulated transporter, but not ferric-chelate reductase (Chang 2003; Ishimaru et al. 2006). *OsIRT1* and *OsIRT2* expression is observed only in Fe-deficient roots (Ishimaru et al. 2006). The existence of strategies I and II in rice allows it to utilize both ferrous and ferric iron, depending on their availability and environmental conditions.

Fe homeostasis during vegetative and reproductive growth

Fe transport from root to shoot and grain is essential for normal plant growth. In rice, Fe can be transported in various forms through the xylem and phloem, including Fe-citrate, DMA–Fe(III), and NA–Fe(II). The knockout of the rice citrate transporter *osfrdl1* results in chlorotic plants with low levels of Fe in the leaves and Fe precipitation in the root stele. Such mutants also show a reduced concentration of citrate and ferric iron in the xylem sap compared to WT rice. *OsFRDL1* expression was first identified in cells involved in long-distance transport as well as in reproductive organs, and, unlike other Fe transporters, *OsFRDL1* expression is not regulated by Fe availability (Inoue et al. 2004). These results suggest a role for *OsFRDL1* in Fe homeostasis through the xylem.

Although DMA is utilized for Fe acquisition from soil, its role in internal Fe homeostasis cannot be overlooked. In rice and barley, DMA has been detected in shoots under Fe-sufficient conditions, and the amount of DMA increases under Fe deficiency. The amount of DMA is higher in rice leaves compared to barley leaves under Fe-sufficient and Fe-deficient conditions (Higuchi et al. 2001a), although barley secretes larger amounts of MAs. *OsDMAS1* promoter-*GUS* activity was not observed in Fe-sufficient rice shoots, although it is expressed in portions of roots involved in long-distance transport, indicating that the DMA detected in Fe-sufficient rice leaves is translocated from roots in a complex with Fe (Bashir et al. 2006; Mori et al. 1991). On the other hand, DMA is at least partially synthesized in Fe-deficient shoots. The DMA synthesized in shoots is thought to be involved in Fe homeostasis in shoots and flowers and does not participate in the acquisition of Fe from soil (Bashir et al. 2006). Similar to roots, the expression of *OsNASs*, *OsNAAT1*, and *OsDMAS1* is significantly overlapped in shoots, indicating that these genes work in coordination to produce DMA (Fig. 3b).

Among the 18 putative YSL family genes in rice, *OsYSL5–7*, *-14*, and *-17* are expressed in the epidermis, cortex, and stele of Fe-sufficient and Fe-deficient roots. On the other hand, the expression of *OsYSL1–4*, *9–11*, and *-18* was not observed in roots, irrespective of their Fe status. *OsYSL12* is expressed in the cortex and stele under Fe-sufficient and Fe-deficient conditions, whereas *OsYSL16* is expressed in the epi-/endodermis and cortex under Fe-sufficient condition and in the epi-/endodermis, cortex,

and stele under Fe-deficient condition (Inoue et al. 2009). Although the expression of these genes is not significantly regulated by Fe, at least in root tissues, the involvement of these genes in Fe homeostasis cannot be ruled out. YSL family genes also exist in nongraminaceous plants, where they transport metal–NA complexes (DiDonato et al. 2004). Phylogenically, the YSL family in plants can be grouped into four subgroups, and among these, *OsYSL1*, *3–4*, *7–8*, and *17–18* form a rice-specific group (Aoyama et al. 2009). In this group, only *YSL18* has been characterized as an Fe–DMA transporter, and the rice-specific nature of this subgroup indicates that other genes may also transport DMA–metal complexes.

Among these genes, *OsYSL2*, *OsYSL15*, and *OsYSL18* have been characterized in detail (Aoyama et al. 2009; Inoue et al. 2009; Ishimaru et al. 2010; Koike et al. 2004). As mentioned above, *OsYSL15* transports Fe(III)–DMA from the rhizosphere to the roots and is involved in internal Fe homeostasis. *OsYSL15* promoter-driven GUS expression was not only observed in leaf tissue but also at the flowering stage (Inoue et al. 2009). These results indicate that *OsYSL15* is involved in Fe transport to rice grains. Furthermore, *OsYSL18* encodes a functional Fe(III)–DMA transporter involved in DMA-mediated Fe distribution in reproductive organs, lamina joints, and phloem cells at the base of the leaf sheath (Aoyama et al. 2009).

NA, besides being an MA precursor, also serves as a chelator of divalent metals (Takahashi et al. 2003). In rice, *OsYSL2* has been identified as an Fe–NA transporter (Koike et al. 2004). *OsYSL2* is expressed at the reproductive stage, and it seems to contribute to Fe accumulation in seeds (Koike et al. 2004). Furthermore, *OsYSL2* RNAi and 35S-*OsYSL2* lines have been characterized (Ishimaru et al. 2010). At the vegetative stage, Fe and Mn concentrations decreased in the shoots of *OsYSL2* RNAi plants, while the Fe concentration increased in the roots. At the reproductive stage, Fe translocation to the shoots and seeds was suppressed in *OsYSL2* RNAi plants. The Fe and Mn concentrations also decreased in *OsYSL2* RNAi seeds, especially in the endosperm. Surprisingly, the Fe concentration in *OsYSL2* overexpressors was lower in seeds and shoots but higher in roots, compared with WT plants, indicating the role of *OsYSL2* in Fe homeostasis.

Transgenic approaches to mitigating Fe deficiency in plants and humans

Rice tolerant to low Fe availability

Attempts to increase Fe uptake and ultimately increase tolerance to low Fe availability have utilized several approaches. Rice plants tolerant to low Fe availability were first produced through strengthening of the strategy II system with the heterologous expression of *HvNAAT* genes. Plants harboring *HvNAATA-B* secrete large amounts of phytosiderophores compared to WT plants and are tolerant to alkaline soils (Takahashi et al. 2001). Furthermore, several genes from barley, including *HvNAS1* and *HvNAAT*, in combination with *HvNAS1* and *IDS3* were introduced into rice to increase Fe deficiency tolerance under alkaline conditions. These plants showed significantly enhanced tolerance to low Fe availability when grown in calcareous soils (Suzuki et al. 2008). Furthermore, the yeast ferric reductase gene *FRE1* was reconstructed for expression in plants (*refre1*; reconstructed *FRE1*; Oki et al. 2004) and then mutagenized and screened, so that it could work under alkaline conditions, and the reconstructed gene was introduced into rice under control of the *IRT1* promoter (Ishimaru et al. 2007). Transgenic rice harboring *refre1/372* transported more Fe compared to vector controls, as revealed by positron emission tracer imaging system analysis. These transformants exhibited enhanced tolerance to low Fe availability in both hydroponic culture and calcareous soils and produced eight times more grain in calcareous soils compared to the vector control (Ishimaru et al. 2007). Rice plants overexpressing *OsIRT1* also showed enhanced tolerance to Fe deficiency in paddy fields. These plants accumulated higher levels of Fe and Zn in shoots, roots, and mature seeds, indicating that *OsIRT1* can be used to enhance micronutrient levels in rice grains (Lee and An 2009). The efforts to increase tolerance to Fe deficiency in alkaline soils have been reviewed recently (Kobayashi et al. 2008).

Biofortification of rice with Fe

Different approaches have been adopted to increase the edible Fe in rice seed. In rice, as the pericarp, testae, aleurone layers, and embryos are removed during processing to improve the quality and shelf-life of the product, leaving only the endosperm as the edible part (Matsuo and Hoshikawa

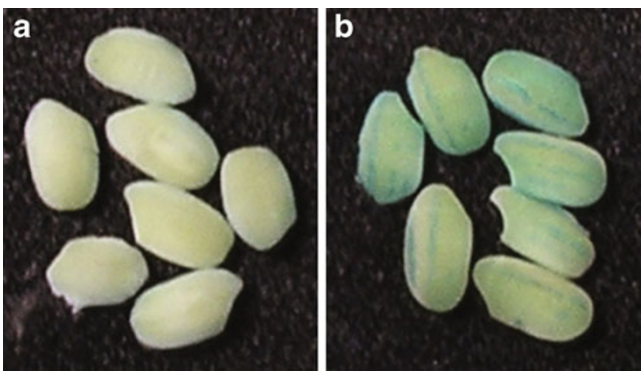

Fig. 4 Fe localization in the seeds of WT plants as determined by Perl's staining. WT (**a**) and transgenic rice (**b**).

1993), efforts have been focused on increasing the Fe content of the rice endosperm. The first attempt to increase the endosperm Fe content in rice employed the overexpression of ferritin (Goto et al. 1999), which has the unique ability to store up to 4,500 Fe atoms (Harrison and Arosio 1996). Plants expressing ferritin accumulated more Fe in the seed endosperm compared to WT plants (Goto et al. 1999; Vasconcelos et al. 2003). Transgenic rice plants expressing soybean ferritin under control of the seed-specific rice *Gluterin-B1* promoter accumulated up to three times more seed Fe compared to WT plants (Goto et al. 1999), although Fe accumulation did not parallel the high expression level of ferritin in rice seeds (Qu et al. 2005). A twofold increase in the Fe content of rice seeds carrying *FERRITIN* from *Phaseolus vulgaris* has been reported (Lucca et al. 2001). However, an attempt to increase the bioavailability of Fe by expressing a thermo-tolerant phytase from *Aspergillus fumigatus* and a cysteine-rich metallothionine-like protein in rice met with limited success (Lucca et al. 2001). These results indicate that in addition to increased Fe storage in seeds, efforts to enhance Fe uptake from soil and its translocation are essential to further increase the Fe content of rice endosperm. Rice transformed with barley *IDS3* (a mugineic acid-synthesizing dioxygenase gene) accumulated more Fe in the grain (Masuda et al. 2008). Transgenic lines harboring *HvNAS1* driven by the *35 S* or *actin1* promoter accumulated more Fe and Zn in polished T2 seeds, showing a positive correlation between Fe and NA/DMA concentrations in seeds (Masuda et al. 2009). These results suggest that NA overproduction enhances the translocation of Fe and Zn to rice grains. Higuchi et al. (2001a, b) produced rice lines expressing *HvNAS1*, resulting in an increased NA content in the roots and leaves under Fe-sufficient conditions. However, the seed Fe and Zn concentrations did not increase in plants grown under Fe-deficient conditions in calcareous paddy fields or under Fe-sufficient conditions in andosol paddy fields (Masuda et al. 2008; Suzuki et al. 2008). This difference in Fe accumulation in rice seeds is thought to be related to expression of the NAS transgene and subsequent NA accumulation. Under Fe-sufficient conditions, the NA concentration in 35S-*HvNAS1* lines was higher than that in lines harboring the *HvNAS1* genomic fragment (Higuchi et al. 2001a). Moreover, in 35S-*HvNAS1* lines, the NAS transgene was presumably expressed throughout the rice plant, which would promote widespread Fe circulation and bioavailability.

Transgenic plants expressing *AtNAS1* and *Pvferritin* simultaneously accumulated six times more Fe in the endosperm than WT plants, showing that this combination exerts a synergistic effect on Fe uptake and storage (Wirth et al. 2009). However, a similar approach using *HvNAS1* in combination with soybean *ferritin* did not increase the seed Fe content (Masuda et al. 2009). Activation tagging for *OsNAS3* also resulted in increased Fe accumulation in roots, shoots, and seeds (Lee et al. 2009b). *OsNAS3*-activated plants grown on a paddy field accumulated three times more Fe than WT plants and 9.6 times more NA in their seeds. This Fe is bioavailable, as shown by a feeding test using mice (Lee et al. 2009b).

In addition to increased Fe storage and uptake, increased Fe translocation may significantly enhance the seed Fe content, given that the strong expression of *OsIRT1* in rice resulted in a marginal increase in seed Fe (Lee and An 2009). Rice plants overexpressing *OsYSL15* accumulated up to 29% more Fe than WT plants (Lee et al. 2009a). Recently, it was demonstrated that the expression of *OsYSL2*, if driven by a suitable promoter, resulted in a significant increase in grain Fe (Ishimaru et al. 2010). The expression of *OsYSL2*, when controlled by the sucrose transporter promoter, increased the Fe concentration in polished rice up to 4.4-fold compared to WT, indicating that the control of gene expression in terms of temporal and spatial expression could lead to the creation of high Fe rice seeds (Fig. 4).

Conclusions

Despite significant effort and progress, the problem of Fe deficiency cannot be solved immediately, and there is still room for the development of better rice varieties through a combinatorial approach. For example, although lines overexpressing *OsNAS1*, *OsNAAT1*, *refre1*, *OsIRO2*, or *IDEF2* show enhanced tolerance to low Fe availability, the combination of these genes may further enhance tolerance to Fe deficiency in alkaline soils. In particular, the expression of *DMAS1* regulated by an appropriate promoter may significantly enhance tolerance to Fe deficiency by increasing MA production. Similarly, different approaches to achieve high Fe rice may be combined to breed varieties that accumulate more Fe in the rice endosperm.

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