Plant Cell Tiss Organ Cult (2013) 113:363–373
DOI 10.1007/s11240-012-0286-7

REVIEW

Loading and bioavailability of iron in cereal grains

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Received: 17 August 2012 / Accepted: 29 December 2012 / Published online: 10 January 2013

Abstract Cereal plants take up iron from the soil via a phytosiderophore-mediated chelation system. Following root absorption, iron is transported through the xylem and phloem of the plant with the help of a variety of efflux and influx transporters belonging to the Zrt Irt-like protein (ZIP) and yellow stripe-like (YSL) protein families. Iron-regulated transporter1, a member of the ZIP family, mobilises ferrous [Fe(II)] ions, while several YSL family members such as YSL2, YSL15 and YSL18 can transport both ferric [Fe(II)] and ferrous [F'III)] ions into developing grains via chelation with mugineic acid or its derivatives. The iron is accumulated largely in the outer aleurone layer and embryo of the grains, which are removed during milling, leaving behind consumable endosperm that contains a very low amount of iron. This review highlights the uptake, transport and loading mechanisms for iron in cereal grains and provides an overview of strategies adopted for developing highly iron-enriched grains.

Keywords Cereals · Phytosiderophores · Transporters · Ferritin · Biotechnology · Iron

Introduction

Iron is an essential nutritional mineral and its deficiency can lead to the development of Iron Deficiency Anaemia (IDA) along with impaired brain development due to the malfunctioning of the central nervous system (Lozoff and Georgieff 2006). It also affects cognitive functions such as learning and memory as well as the body's immune system (Gordon 2003). Worldwide, 2 billion people are anaemic, the major cause being iron deficiency (WHO 2008). WHO estimates that IDA affects 88 % of pregnant and 74 % of non-pregnant women and 60 % of children, especially in Africa and Southeast Asia (WHO 2002). In India, approximately 14 million men, 28 million pregnant women and 85 million children suffer from anaemia every year (King 2002; Stein et al. 2008). The major contributing factor to iron deficiency is the low amount of bioavailable iron found in dietary foods (Glahn et al. 2002).

Iron bioavailability can be increased by the use of many available supplementary additives, but most of these are as expensive as are commercially available iron supplement capsules (Hurell 2002). Some additives also exhibit undesirable colour reactions in the food and promote fat oxidation in stored cereals, which are disadvantageous (Bovell-benjamin et al. 1999; Hurell 2002). Although the FAO/WHO Expert Committee on Food Additives approved these components for iron fortification strategies, still they have not implemented at the country level (FAO/WHO 1999). Hence, it is better to develop iron-biofortified foods naturally by the application of technology rather than add iron supplementary chemicals to food or use iron supplement capsules (Datta and Khush 2002).

In India and other Asian countries, most people consume cereal products as their staple foods, and coincidently, the people of these countries have the highest IDA percentages in the world (Gillespie and Haddad 2001). Therefore, increasing the levels of bioavailable iron in their staple food crops is highly desirable. However, edible cereal grains such as rice contain very low amounts of iron due to

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the removal of embryo and aleurone layers during milling (Ozturk et al. 2006). Genetic engineering has been established as a promising strategy to improve the iron content in edible tissues and found the new platform of biotechnological research in mineral nutrition.

This review mainly focuses on the different biotechnological approaches that have been employed to overcome the probable barriers to iron loading in cereal grains as well as possible approaches to be applied to future iron-biofortification programmes.

The important criteria for iron loading are the soil iron status and the sensitivity of higher plants towards it. Most of the higher plants grow in aerobic conditions, and approximately 30 % of cultivated land is alkaline in nature. In this calcareous or alkaline condition, Fe is present in the insoluble ferric form, which cannot be absorbed by the plants (Kim and Guerinot 2007). Apart from the soil iron status, iron insolubility is another reason for plant iron deficiency (Guerinot 2001). In order to overcome iron deficiency, higher plants have evolved several adaptive mechanisms to increase iron solubility and uptake from soil through their roots. Plants induce several biochemical responses that increase the solubility of iron in the soil and make it easily accessible through the roots. Higher plants possess 2 different types of iron uptake mechanisms (Marschner et al. 1986). The reduction-based Strategy I is employed by non-graminaceous monocots. In this strategy, a plasma membrane-coupled reductase protein, namely, Ferric Reduction Oxidase 2 (FRO2) converts insoluble soil ferric ions [Fe(III)] into more-soluble ferrous ions [Fe(II)] through the oxidation of a reducing agent such as NADPH. Along with the reductase, the plasma membrane H^+ATPases actively take part in this uptake mechanism by lowering the pH of the soil and creating an electrochemical gradient between soil particles and the plasma membranes of the root hairs. Thus, the energy-driven Proton Motive Force (PMF) facilitates the uptake of different solutes through their respective carriers and channels in the root epidermis. Hence, under iron-limiting conditions, the reduction of Fe(III) to Fe(II) is an obligatory step for iron uptake after which the plant automatically switches off its reduction mechanism (Kim and Guerinot 2007).

Iron nutrition in cereal plants

Iron uptake

Graminaceous plants possess a different type of iron acquisition mechanism referred to as the Strategy II mechanism. Cereals including rice, wheat, and maize acquire iron through secretion from their roots of low molecular weight compounds called phytosiderophores that contain Mugineic Acid (MA) and its derivatives. MA binds to iron mainly in the ferric form and solubilises it in the rhizosphere for reabsorption through the roots (Kawai et al. 2001). MAs are amino acid derivatives, their precursor being L-methionine. Intermediates in the MA biosynthesis pathway, for instance the Deoxymugineic Acid (DMA), 3-Hydroxymugineic Acid (HMA) and 3-epihydroxymugineic acid, can also chelate soil iron particles. During iron deficiency, there is a positive correlation between the amount of secreted MAs and the iron sensitivity of the secreting plant (Negishi et al. 2002). Nicotianamine, an intermediate in the MA biosynthetic pathway, plays a crucial role in long-distance transport of iron in plants (Takahashi et al. 2003). Hence, MAs efficiently mobilise Fe and serve an important role in Fe acquisition for grasses growing on calcareous soils.

Iron transport through roots

After absorption through the roots, reduced ferrous ions are transported across the root epidermal membrane by specific carriers or channel proteins such as the ZRT IRT-like Protein (ZIP) family transporters (Kim and Guerinot 2007). Alternatively, different types of epidermal and endodermal efflux transporters mainly transport Fe-phytosiderophore (Fe-PS) complexes into the vascular bundles of the plants (Conte and walker 2011). Fe and other metals are transported both symplastically and apoplastically within the root system. In rice and barley, Efflux Transporters (ETs) such as Transporter of Mugineic Acid 1 (*Os*TOM1), *Os*TOM2 and *Hv*TOM1 bind to DMA and export it from the root to the soil. DMA chelates the soil Fe(III) and is further absorbed as the DMA-Fe(III) complex through the Yellow Stripe-Like (YSL) type of Influx Transporters (ITs) present in the root (Nozoye et al. 2011; Fig. 1). Along with the TOM proteins, another group of rice efflux transporters, viz., Phenolics Efflux Zero1 (PEZ1), helps to solubilise the apoplasmic precipitated iron by secretion of phenolics and caffeic acid (Ishimaru et al. 2011).

Dual strategy of iron transport in rice

Most importantly, rice plants possess a dual uptake system for iron acquisition. Under waterlogged conditions, Fe is present in the Fe(II) form and is easily accessible through the Iron-regulated Transporter (IRT); this occurs in paddy fields where the Fe(II) form is more prevalent due to the low availability of oxygen in the soil (Kim and Guerinot 2007). To acclimatise to the conditions of lower Fe(III) concentrations in the soil, rice plants secrete low quantities of DMA and thus exhibit a combination of the Strategy I and Strategy II iron uptake mechanisms in which both TOM and IRT transporter proteins are used.

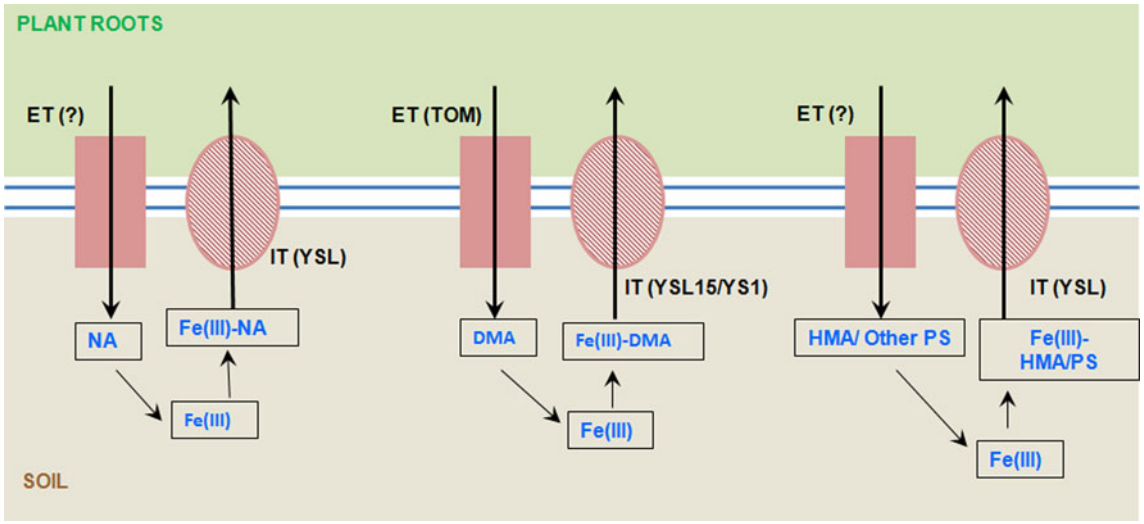

Fig. 1 Iron uptake from soil by cereal plants and translocation through roots (*ET* Efflux transporter, *IT* Influx transporter, *NA* Nicotianamine Acid, *TOM* Transporter of Mugineic acid, *HMA* 3-Hydroxymugineic acid, *DMA* Deoxymugineic Acid, *PS* Phytosiderophore, *YSL* Yellow Stripe Like, *YS* Yellow Stripe)

Long distance transport through xylem and phloem

Metals are mainly transported apoplastically to the older leaves of plants through the xylem. Different ETs are present in the root endodermis and pericycle membrane to transport the metals. In rice and barley plants, TOM1 helps to transport Fe(III) into the xylem as well as into the phloem in the DMA-chelated form (Nozoye et al. 2011). In rice, Fe(III) is also transported as a citrate-chelated form through the Ferric Reductase Defective-Like (FRDL) channel transporter, whereas Fe(II) is mainly bound to Nicotianamic Acid (NA) and translocated as NA–Fe(II) in the xylem (Yokosho et al. 2009; Kakei et al. 2009). In the nodal region of graminaceous plants, nutrients are transferred to the phloem from the xylem and then translocated to the flag leaf and spike regions (Yamaji and Ma 2009).

The most important transport is phloem-mediated, through which Fe and Zn are transported symplastically to the youngest leaves, floral parts and developing seeds (Wang et al. 2011). In the phloem, several ZIP and YSL family transporters play an important role in Fe and Zn transport. In *Arabidopsis*, barley and rice plants, different divalent cations are transported selectively through particular ZIP family members (Waters and Sankaran 2011). In rice, among the ZIP family members, *Os*ZIP1, 3, 5 and 7 are only associated with Zn^{2+} transport, while *Os*Zip4 and 8 transport both Zn^{2+} and Fe^{2+} and are expressed in the vascular tissues, leaf mesophyll cells and apical meristems of plants (Lee et al. 2010). One of the important members of the ZIP family, Iron-Regulated Transporter 1 (IRT1), facilitates the transport of NA-Fe(II) into the phloem, whereas Fe(III) is transported via chelation with DMA or NA through the TOM1 efflux channel and several YSL family members, respectively (Nozoye et al. 2011; Curie et al. 2009). Different *Os*YSL members are expressed in different tissues of the rice plant, as are other transporter group members (Bashir et al. 2010; Table 1).

Loading and accumulation of iron in seeds

In cereal plants, senescent leaves and roots actually transport Fe through their phloem into developing seeds,

Table 1 Iron transporters and their expression in cells/tissues of rice

Transporters	Expression in cells/tissues
*Os*IRT1	Epidermis, pericycle, companion cells in root, Xylem parenchyma, phloem parenchyma, companion cells in shoot, mesophyll and bundle sheath cells of lamina
*Os*FRDL1	Pericycle and companion cells in root, Xylem parenchyma, phloem parenchyma and companion cells in shoot mesophyll and bundle sheath cells of lamina
*Os*YSL1-4,9-11 and *Os*YSL18	Xylem parenchyma, phloem parenchyma, companion cells in shoot, mesophyll and bundle sheath cells of lamina, floral tissues
*Os*YSL2	Root pericycle and companion cells in root along with the above-mentioned tissues
*Os*YSL 5-7,14 and *Os*YSL17	Epidermis, cortex and stele in root
*Os*YSL12	Cortex and stele of roots
*Os*YSL15	Epidermis, pericycle, companion cells in root
*Os*YSL16	Epidermis, cortex, endodermis and stele of root

thus making the grains or seeds the principal area of exploration for iron and other mineral nutrition. In the castor bean, a novel Fe(III)-binding 17 kDa protein, viz., Iron Transporter Protein (ITP), has been found in the phloem (Kruger et al. 2002), which indicates that, while NA mobilises Fe(III) in and out of the phloem, the actual transport occurs via ITP within the phloem (Fig. 2). Wheat remobilises 77 % of iron in the shoots to the seeds, whereas rice transports only 4 % (Morrissey and Guerinot 2009). Generally, in grain, nutrients and iron are transported into the maternal seed coat region through the phloem and then translocated into the apoplastic region between the maternal seed coat and the aleurone or endosperm through different types of efflux and influx transporters such as ZIP, Nramp and the YSLs (Tauris et al. 2009; Fig. 2).

What are the barriers to iron loading in the grain endosperm?

In developing countries, cereals are the staple food that provides more than 50 % of calories, but unfortunately, the consumable endosperm of the grain helps very little in iron nutrition due to its low iron content. Several iron-loading barriers are as follows: Firstly, a variety of transporters and chelating molecules such as YSL, IRT, NA and DMA are localised in the outer tissue region of the resting seed (Fig. 2) and transport iron into the inner endosperm only during germination, a process that clearly indicates low Fe-bioavailability in the endosperm (Walker and Waters 2011). Secondly, the presence of very low amounts of ferritin protein moieties in the endosperm tissue of the mature grain (Stein et al. 2009) is a major drawback to iron

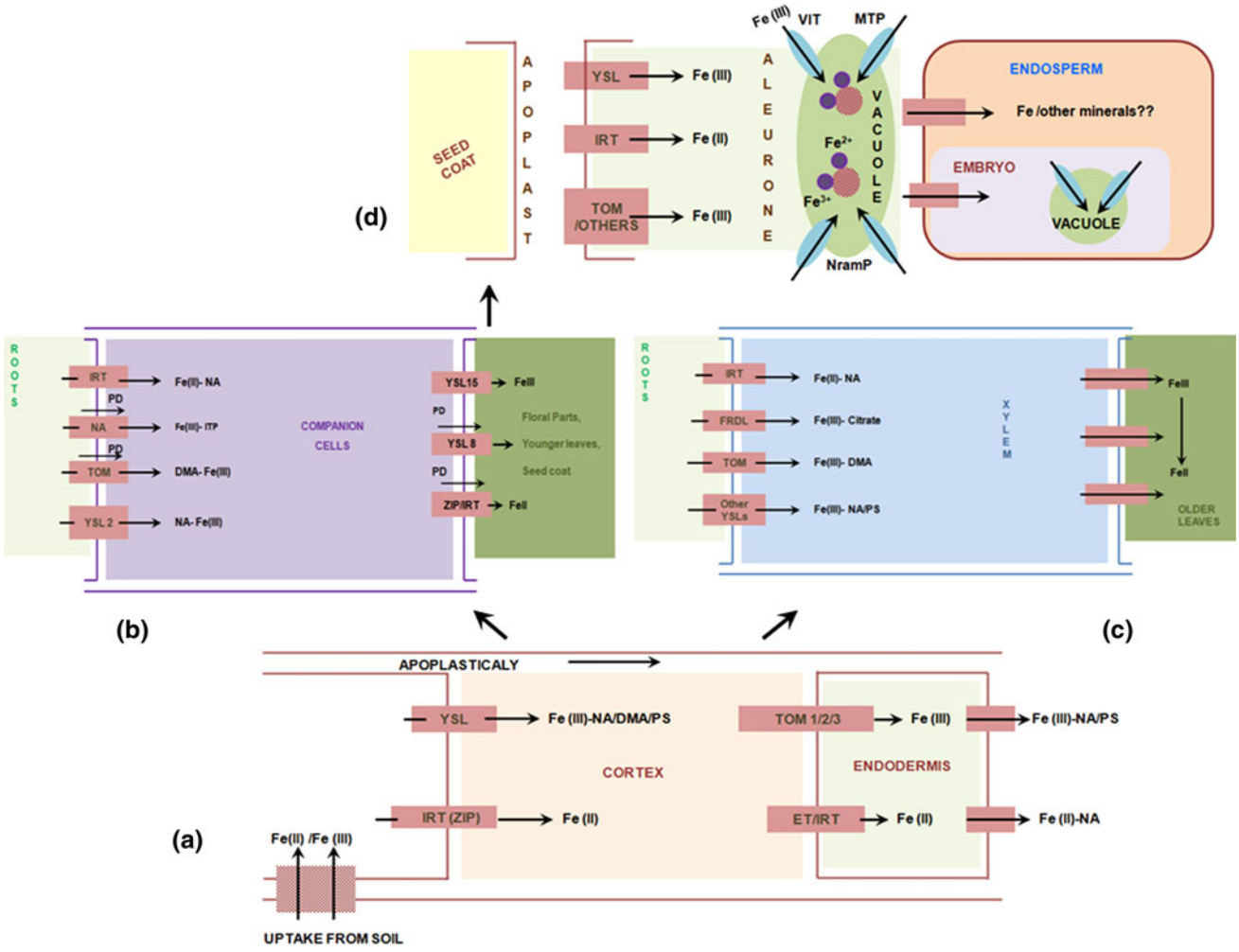

Fig. 2 Iron transport and loading from **a** roots to **d** seeds via **c** xylem and **b** phloem (modified from Morrissey and Guerinot 2009 and Waters and Sankaran 2011, *IRT* Iron Regulated Transporter, *ZIP* ZRT IRT-like protein, *PS* Phytosiderophore, *TOM* Transporter of Mugineic acid, *NA* Nicotianamine Acid, *DMA* Deoxymugineic Acid, *YSL* Yellow Stripe Like, *PD* Plasmodesmata, *ITP* Iron Transporter Protein, *MTP* Metal Transporter Protein, *VIT* Vacuolar Iron Transporter, *FRDL* Ferric Reductase Defective-Like)

bioavailability. In most cereal grains, the presence of phytic acid is another large concern for iron nutrition, as iron and zinc are localised in the phytate-chelated globoid form, mainly within the vacuole of the seed coat, peripheral aleurone and embryo (Ozturk et al. 2006; Cakmak et al. 2010), and thus cannot be mobilised into the endosperm. Ferric ions move into the vacuole of the aleurone layer through several types of Vacuolar Iron Transporter (VIT) and Nramp types of transporter families (Fig. 2). Nano-scale secondary ion mass spectrometry has confirmed the presence of phytate-chelated forms of metals such as Mn, Zn and Fe in the vacuole, further indicating the low quantities of minerals in the seeds (Ravet et al. 2009; Smart et al. 2010).

To overcome these barriers, several promising biotechnological approaches have been studied to provide better iron nutrition to malnourished people (Fig. 3).

Biotechnological interventions

Tissue-specific overexpression of the *ferric reductase* gene in rice plants

In rice plants, the activity of FRO2 is very low, even under Fe-deficient conditions. The expression of *Osfro1* is observed under Cu-, Zn- and Mn-deficient conditions, whereas *Osfro2* is expressed only under Fe-deficient conditions and restricted to the shoots (Ishimaru et al. 2007). Therefore, to increase root ferric reductase activity, *Atfro2* was introduced into rice plants, but the transgenic plant did not show any transgene expression due to the weak activity of the native promoter (Vasconcelos et al. 2004). The failure was circumvented by the introduction of the yeast ferric reductase gene *fre1*, under the control of the root-specific *irt1* promoter, which enhanced iron uptake in Fe-deficient rice plants due to the resulting higher ferric reductase activity. However, the amount of iron in the resulting transgenic rice grains was similar to that in non-transgenic brown rice (Ishimaru et al. 2007). Therefore, it is evident that *fre1* plays a role in increasing the adaptability of plants to iron-deficient conditions, but it is not effective for iron enrichment in the grain.

Gene manipulation in a phytosiderophore-mediated chelation strategy

In cereal plants, MAs are the most important members of the transporter families, among which nicotianamine plays the central role because it chelates both ferrous and ferric ions (von Wiren et al. 1999). Most of the genes involved in the MA biosynthesis pathway have been characterised (Nozoye et al. 2007). In the biosynthetic pathway for mugineic acids, the *nas* gene produces the nicotianamine acid synthase (NAS) enzyme that catalyses the transformation of L-methionine to nicotianamine, which further produces an intermediate 'oxo' acid through the nicotianamine amino acid transferase (NAAT) enzyme encoded by the *naat* gene. Deoxymugineic acid (DMA), the first product of the mugineic acid family, is then synthesised by the *dmas* gene product. DMA subsequently produces epi-hydroxymugineic acid and MA with the help of the *ids3* and *ids2* gene products. In rice plants, variant *nas*, *naat* and *dmas* genes have been isolated and functionally characterised (Nozoye et al. 2007).

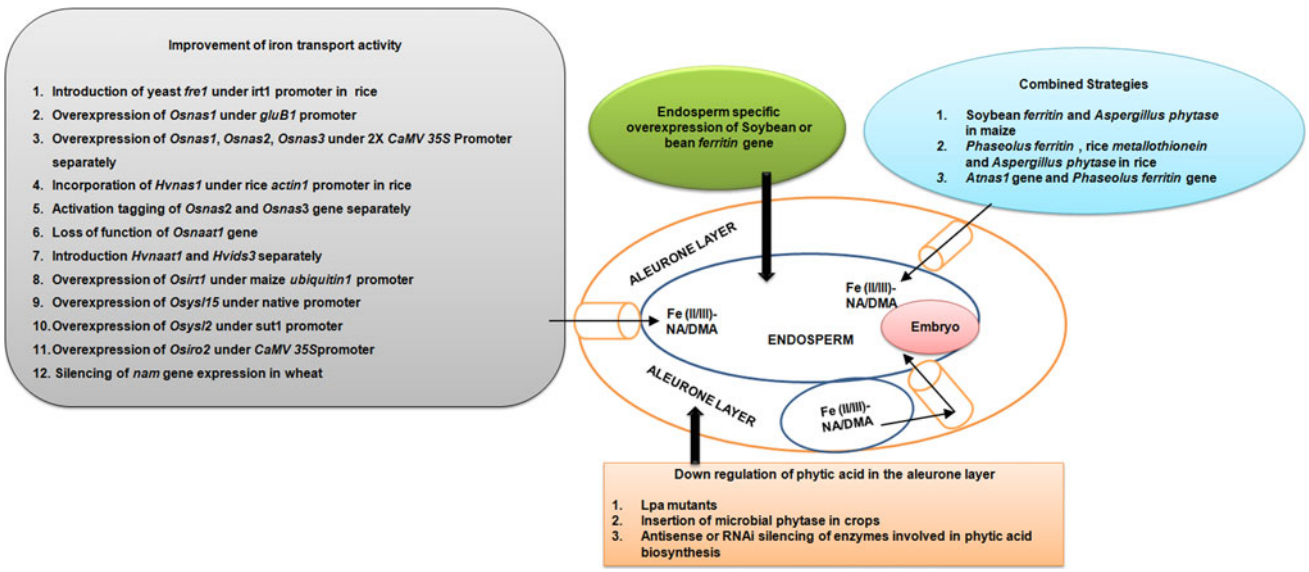

Fig. 3 Different biotechnological approaches adopted for development of iron enriched cereal grains (*NA* Nicotianamine Acid, *DMA* Deoxymugineic Acid)

Rice seed-specific overexpression of the *Osnas1* gene under the control of the *GlutelinB1* promoter increased grain iron bioavailability along with zinc enrichment (Zheng et al. 2010). Furthermore, large amounts of nicotianamine production in rice plants have been reported through the overexpression of three *Osnas* genes, namely, *Osnas1*, *Osnas2* and *Osnas3*, under the control of a double *CaMV*35S promoter that ultimately increased grain iron content up to 2.4-, 3.5- and 2.7-fold, respectively (Johnson et al. 2011). Prior reports had suggested that incorporation of the *Hvnas1* gene under the control of the rice *actin1* promoter was a successful attempt at iron enrichment of seeds (Masuda et al. 2009). Another strategy, the activation tagging of the *Osnas3* gene, yielded a twofold enrichment of the iron content in the seeds (Lee et al. 2009a). A similar approach was applied to the *Osnas2* gene, which ultimately showed a 2.7-fold zinc enhancement in rice seeds (Lee et al. 2011). Introduction of other MA biosynthesis genes such as *Hvnaat1* and *Hvids3* led to better tolerance of the transgenic rice plants to calcareous soils as well as higher amounts of iron and zinc transported into the seeds compared with non-transgenic plants (Takahashi et al. 2001; Suzuki et al. 2008). A loss-of-function mutation in the *Osnaat1* gene also resulted in 1.8- and 3.8-fold higher iron accumulation in brown and white rice, respectively, and may be used as a potent tool in future iron biofortification programmes (Cheng et al. 2007).

Overexpression of different groups of transporters

Overexpression of transporters has had a positive impact on grain iron concentration, as reflected in several biotechnological applications. IRT is a very common member of the ZIP family of transporters, which are mainly responsible for transporting ferrous ions (Vert et al. 2002). In iron-deficient conditions, the expression of *Atirt1* is increased in the external root cell layers, and the protein's function has been identified as participating in mono-ubiquitin-dependent endocytosis (Barberon et al. 2011). The expression of the *Atirt2* gene is similar to that of *irt1* but is localised to the subapical cell layers of the root (Vert et al. 2001). Recently, the *At*IRT3 transporter, which mainly transports zinc and iron rather than other heavy metals, has been identified (Lin et al. 2009). Hence, use of *Atirt3* has an advantage over use of *irt1* because no other heavy metal loading occurs except for Fe and Zn. A rice cDNA library screened with rice Expression Sequence Tag (EST, D49213), having high homology to the *Atirt1* gene. Further functional analysis of the homologous region called *Osirt1* demonstrated higher expression under iron-starvation conditions in the roots and shoots. The *Osirt2* gene has also been identified, but its expression was very weak under Fe-deficient conditions (Ishimaru et al. 2007). Overexpression of *Osirt1* under the control of the maize *ubiquitin-1* promoter led to high iron accumulation in the grains (Lee and An 2009), but morphological parameters such as height, tiller number and grain yield of the transgenic plants were altered. The negative impact on the plant's agronomic performance may have been due to the constitutive expression of the IRT1 transporter along with toxicity due to excess Zn and Cd. The overproduction of another transporter protein in *Brassica juncea*, such as the ABC Transporter of Mitochondrion 3 (AtATM3), which helps to transport Fe–S clusters into the cytoplasm from the mitochondria, significantly maintained iron homeostasis in the plants. However, accumulation of heavy metals such as Pb, Cd is a major drawback for its use in an iron biofortification programme, similar to Yeast Cadmium Factor 1 (YCF1) protein-mediated heavy metal acquisition (Bhuiyan et al. 2011a, b).

In cereal plants, the YSL family of transporters is crucial for iron transport. The family has been divided into four groups, but the functional properties of all members are yet to be characterised except for group I, *Os*YSL18 (group IV) and *Hv*YSL5 (group II; Zheng et al. 2011). In rice, 18 YSL family transporters have been reported to date, among which the three YSL transporters *Os*YSL2, *Os*YSL15 and *Os*YSL18 are very promising for iron biofortification purposes (Curie et al. 2009) due to their iron loading capacities in different plant parts (Table 1). *Os*YSL2 transports both Fe(II) and Fe(III)-NA into the leaf primordial and scutellum tissues of the grain (Nozoye et al. 2007; Aoyama et al. 2009), whereas *Os*YSL15 and *Os*YSL18 transport only Fe(III)-DMA into developing seeds and floral parts, respectively (Inoue et al. 2009; Ishimaru et al. 2010). Introduction of these transporters into rice plants redirects higher iron concentrations to the rice grains. In spite of a large amount of iron accumulation, agronomic performance was compromised in the transgenic *Oysl15* overexpressor plants (Lee et al. 2009b), as in the *Osirt1* overexpressor plants. Tissue-specific activity of the transporter has been suggested as a suitable strategy for iron loading into grains without disturbing the morphology of the plants, as exemplified by the overexpression of *Oysl2* under the control of the phloem-specific *sut1* promoter. Polished grain from these transgenic rice plants revealed a 4.4-fold enhancement of seed iron content compared with non-transgenic grain (Ishimaru et al. 2010). An RNAi-YSL2 line of transgenic rice plants also confirmed the involvement of the YSL2 transporter in the iron translocation mechanism in the seeds (Ishimaru et al. 2010) and supports the proposition that RNAi is a potent tool for the development of Genetically Modified (GM) crops (Ali et al. 2010).

Another important YSL family member, *Oysl*18, may be a promising biotechnological tool for future use, but no *Oysl*18 overexpressor or knockdown (antisense/RNAi)

line has yet been reported. Recently developed transgenic rice plants expressing *Hvysl*1 showed increased sensitivity towards alkaline soil and can accumulate more iron in leaves compared to seeds, which indicates that *Hvysl*1 mobilises iron into the plant's vegetative parts (Gomez-Galera et al. 2012).

Different bHLH (111 types in rice) transcriptional factors greatly influence metal transport in plants through a complex signalling mechanism (Hindt and Guerinot 2012). In rice, one of the Fe-deficiency-inducible bHLH transcription factors, called *Os*IRO2, has been identified, and its promoter region was found to contain a sequence similar to Iron Deficiency responsive Elements (IDE). Under Fe-deficiency conditions, IDE binding proteins activate the expression of the *Os*IRO2 protein, which helps to synthesise Fe-regulated gene products through a signal transduction pathway and ultimately facilitates iron translocation to the shoots (Ogo et al. 2007). Overexpression of *Osiro2* under the *CaMV*35S promoter resulted in enhancement of iron uptake from the soil and translocation to the grain (Ogo et al. 2007).

In wheat plants, an NAC transcription factor, namely, No Apical Meristem (NAM) protein, helps to mobilise iron and zinc into the developing seeds, as confirmed by an NAM-RNAi knockdown line (Walker and Waters 2011). The *Osnac5* gene has been functionally characterised as an ABA-dependent abiotic stress-related regulator and its maximum expression was demonstrated in the flag leaves of high iron and zinc rice cultivars (Sperotto et al. 2009; Takasaki et al. 2010; Song et al. 2011), which indicates its novelty for future transgenic development. A new class of NA transporter, Zinc-Induced Facilitator1 (ZIF1), identified in *Arabidopsis*, helps to translocate zinc to the vacuoles from the cytoplasm as well as increase the iron trafficking phenomenon through the cytoplasm (Haydon et al. 2012). Cereal plants such as rice and maize also contain several types of Zinc-Induced Facilitator Like (ZIFL) transporters, which may play a crucial role in iron biofortification due to their iron transport activities (Ricachenevsky et al. 2011).

Tissue-specific overexpression of *ferritin*

Ferritin (ca. 450 kDa), the most common source of non-haem iron, can accumulate approximately 4500 ferric ions in its central cavity. The outer protein coat consists of 24 oligopolypeptides (ca. 20 kDa) that are folded together with each other through hydrogen and ionic bonds to form a large cage-like protein shell (Masuda et al. 2010). Ferritin has a central mineral cavity and 12 mineral attachment sites on its inner surface along with 8 entry and exit channels on the outer surface (Theil 2003). Ferritin is mainly localised in the plastidial stroma but is also found in plant mitochondria (Zancani et al. 2004). The de novo synthesis of ferritin is regulated in a very controlled way because Fe-overloading results in the production of Reactive Oxygen Species (ROS), particularly OH^{*}, through the Fenton reaction, which can damage the cellular metabolism (Theil 2003). Recently, it has been noted that Fe binds to NO to form an iron-nitrosyl compound, thus alleviating the cellular iron through upregulation of a variety of transporters and the *ferritin* gene. In contrast, it has been observed that levels of frataxin, a mitochondrial protein, are inversely related to *ferritin* gene expression, which was evident from a frataxin mutant of *Arabidopsis* that showed higher iron compartmentalisation in the root (Ramirez et al. 2011). Apart from iron concentration, H_2O_2 and Abscisic acid (ABA) also upregulate the expression of the different classes of *ferritin* genes, as reported for *Arabidopsis* (Briat et al. 2010). Hence, through a signal cascade mechanism, ferritin plays an important role in iron homeostasis to prevent the cell from iron toxicity or deficiency and acts as a sink for ferric ion, which ultimately helps to alleviate iron uptake problems due to Fe insolubility in the soil.

Due to the high iron storage capacity of the ferritin protein, iron-biofortified rice grain has been generated through the overexpression of soybean and bean *ferritin* genes under the control of the *GlutelinB1* promoter (Goto et al. 1999; Vasconcelos et al. 2003). The milled seeds of the transgenic rice plants exhibited approximately two to threefold enhancement of their iron content along with ~1.6-fold enhancement of zinc. A constitutive promoter such as *ubiquitin-1* contributed to twofold higher iron accumulation in transgenic rice leaves compared to non-transgenic seeds, regardless of total iron content (Drakakai et al. 2000). Hence, use of seed-specific promoters is an important criterion for development of high-iron grain. *Globulin1*, a seed-specific promoter, also showed tenfold higher *ferritin* gene expression in the central part of the endosperm (Qu and Takaiwa 2004). Subsequently, double transformant rice seeds carrying two constructs, namely, *glutelinb1-soyferH*1 and *globulin1-soyferH1* and a single transformant of *globulin1-soyferH1* were established as effective for an iron biofortification programme due to their 5.8 and 11.4-fold enhancement of ferritin protein accumulation in the seeds, respectively (Qu et al. 2005). However, 30 % increment of the iron in brown rice was not consistent with the level of ferritin proteins expressed in the seeds (Qu et al. 2005). The endogenous *ferritin* genes of different cereals may emerge as an alternative tool for iron biofortification. Recently, overexpression of the rice endogenous *ferritin* (*Osfer2*) gene under the control of the *OsglutelinA2* promoter revealed a 2.1-fold enhancement of iron content along with a 1.36-fold zinc enhancement in the transgenic aromatic milled rice grain (Paul et al. 2012). In addition to the iron sequestration property, overexpression

of the alfalfa ferritin protein also caused higher abiotic stress tolerance in grapevine, which indicates its dual role in transgenic plant development (Zok et al. 2010).

Downregulation of phytic acid biosynthesis

In cereal crops, phytic acid (myo-inositol-hexakis phosphate), the major antinutrient, chelates essential nutritional minerals such as Fe, Zn and Ca and makes them indigestible by the human intestine due to its lack of a phytase enzyme (Brinch-Pedersen et al. 2007; Persson et al. 2009; Coulibaly et al. 2011). Synchrotron soft X-ray microscopy and high-resolution secondary ion mass spectrometry clearly revealed the presence of the Fe-phytate chelated form in the aleurone layer globoids of wheat and barley grains (Regvar et al. 2011; Lombi et al. 2011; Moore et al. 2012).

To reduce the phytic acid content in grain, several low phytate crops have already been developed (Raboy 2007), but unfortunately, their growth and seed germination rates are very poor. The biosynthesis of phytic acid is a complex metabolic pathway and is regulated by metabolic enzymes such as Myo Inositol-Phosphate-Synthase (MIPS) and multiple groups of Inositol Phosphate Kinases (IPKs), which are the main metabolic enzymes involved in the phytic acid biosynthetic pathway (Suzuki et al. 2007). Hence, the reduction using antisense technology of RINO1 protein, which is involved in a specific step of the phytic acid biosynthetic pathway, is a promising biotechnological approach to increase iron bioavailability in seeds (Fig. 3; Kuwano et al. 2009). In *Arabidopsis*, downregulation of *ipk1* and *ipk2* resulted in 93 % less phytate in seeds (Stevenson-Paulik et al. 2005), while in maize and soybean, low phytate seeds have been produced through tissue-specific silencing of an ABC transporter (Shi et al. 2007).

Combined approaches

The expression of the different iron and metal transporter, carrier and storage proteins are highly dependent on the external as well as the internal iron status and other metal concentrations in the plant. Hence, biotechnological approaches incorporating two machineries such as combining transporters and iron storage molecules are most promising for an iron biofortification programme (Fig. 3). Transgenic maize seeds contained 20–70 %-enhanced iron levels when the plants were transformed with *Aspergillus* phytase and soybean *ferritin* genes together (Drakakai et al. 2005). Another successful combination with a *Phaseolus ferritin*, rice metallothionenin (*rgmt*) and the *Aspergillus* phytase (*phyA*) gene showed enrichment of the iron content

Table 2 Selected potential future efforts for enhancing iron content in cereal grains

Activity	Strategy
Overexpression of *Osnaat1* and *Osdmas* genes	High amount of iron chelation and uptake from soil
Overexpression of *Osysl18 gene*	Iron transport to floral parts
Overexpression of *Osnac5* gene	Mobilization of iron and zinc to the floral parts and grains
Identification and introduction of different *tom* genes from cereals	Iron transport through roots to shoots
Overexpression of specific ENA transporter	Iron transport to the mature seeds
Isolation, characterization and incorporation of specific *itp* genes	Iron transport through phloem
Overexpression of specific *Osnramp* genes	Iron transport to the mature grains
Overexpression of *Osfrdl1* gene	Iron transport from xylem to seeds via floral parts
Identification and overexpression of specific *zifl* gene	Iron trafficking through phloem
Overexpression of endogenous *ferritin* gene	Iron bioavailability due to higher amount storing molecules
Downregulation of phytic acid biosynthesis pathway through silencing of different inositol kinase expression	Iron bioavailability due to reduced amount of phytic acid in aleurone layer
Characterization of different signaling molecules involved in cellular iron homeostasis like calcein, frataxin etc. in cereals and their application such as downreguation of frataxin	Iron transport to the seeds without hampering the cellular iron homeostasis
Identification of different transcriptional factors involved in ferritin and different transporters upregulation or downregulation and their application	Iron transport and storage without disturbing the cellular iron concentration
Combined strategies:	Iron uptake, transport and loading in the seeds, which attributed to higher iron biavailability
Overexpression of different phytosiderophore biosynthesis involved genes with *ysl*, *ena*, *itp*, *irt* etc.	
Overexpression of different groups of transporter along with ferritin	
Overexpression of different transporters with silencing of phytic acid	
Overexpression of ferritin protein with silencing of phytic acid	
Higher amount of ascorbic acid and citrate production in the plants	Iron transport through xylem and phloem to the seeds

in rice grain (Lucca et al. 2001). Approximately sevenfold higher levels of cysteine residues in soluble seed proteins along with 130-fold more phytase helped to improve the iron bioavailability of rice grain. Recently, transgenic rice plants harbouring both the *Atnas1* gene under the control of the *CaMV*35S promoter and *Phaseolus ferritin* under the control of the endosperm-specific *globulin* promoter provided five to sixfold enhancement of iron accumulation in grain (Writh et al. 2009).

Future gateway to iron bioavailability

To maintain cellular iron homeostasis, plants can control the entire pathway of iron uptake, transport and loading through upregulation and downregulation of a variety of transporters, cell signalling, and iron storage molecules. Nevertheless, cell signalling mechanisms and the role of signalling molecules such as frataxin, calcein and Fe–S cluster protein molecules will be emphasised more in the future to increase cellular iron levels without hampering iron homeostasis. Even now, more iron and zinc transporters and their upregulation by particular transcriptional factors are yet to be discovered. Therefore, more research needs to be focused mainly on the function and localisation of different transporters, efflux and influx proteins, their signal transduction mechanisms and their different biotechnological implementations in grains for enhanced bioavailability (Table 2). Furthermore, it is important to analyse the bioavailability of the enhanced iron levels and to perform detailed studies of the agronomic performances of the transgenic crops.

Acknowledgments We record our special thanks to the Department of Biotechnology (DBT) of Government of India and University Grants Commission (UGC) for financial support for different research projects in the laboratories.

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