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Understanding the Mechanism of Iron Metabolism and Bioavailability in Cereals towards Biofortification

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Abstract

Iron (Fe) deficiency remains a critical global health issue, particularly affecting vulnerable populations such as children and pregnant women. Biofortification, the process of enhancing the micronutrients content in staple food crops, holds promise in addressing this challenge. This review aims to elucidate the mechanisms underlying Fe metabolism in cereals, thus focusing on strategies for Fe biofortification. Understanding the molecular mechanisms governing Fe uptake and transport in plants is essential for targeted breeding efforts to enhance the Fe content. Plants employ distinct strategies for Fe uptake from the soil, such as reduction-based and chelation-based approaches, influenced by environmental factors like soil pH. Long-distance Fe transport within plants involves intricate pathways mediated by transporter proteins and regulatory genes. Environmental factors, including soil properties and agricultural practices, influence Fe bioavailability in crops apart from Fe accumulation. Thus, strategies to enhance Fe absorption such as reducing phytic acid content are crucial for improving the nutritional quality of biofortified crops. Various *in vitro*, animal and human studies have assessed the bioavailability of Fe in biofortified crops, highlighting the potential for addressing Fe deficiency through dietary interventions. Combining genetic approaches with an understanding of physiological mechanisms can hasten grain Fe enrichment efforts, resulting in better outcomes through biofortification programmes.

Key words: Cereals, Biofortification, Fe metabolism, Bioavailability.

Introduction

The Green revolution has almost achieved food security across the world addressing the hunger through increasing the production of major staple foods including rice, wheat and other cereals. The prevalent reliance on carbohydrate-rich diets, coupled with restricted dietary diversity due to limited purchasing power in low or middle-income countries, is exacerbating hidden hunger, also known as micronutrient malnutrition (Black *et al.,* 2013). The significance of the nutritional quality of the diets has been underscored by United Nation's (UN) Sustainable Development Goal-2 targeting to eliminate hunger, accomplish food security and enhanced nutrition and promote sustainable agriculture (Lowe, 2021). According to the World Health Organization (WHO) estimates, around 40% of children below five years, 37% of pregnant women and 30% women between 15 to 49 years suffer Fe deficiency (https://www.who.int/). The requirements for Fe almost doubles between 1 and 6 years of age and also during adolescence/puberty, thus children, adolescents, women of gestation reproductive age and pregnant women are the most vulnerable to Fe deficiency (WHO, 2005; Abbaspour *et al.,* 2014). Almost 70% of the Fe in human body is

found in red blood cells (RBC) as haemoglobin and in muscles as in myoglobin facilitating the circulation and metabolism of oxygen among various tissues (McDowell, 1992; Hurrell, 1997). One-fourth of Fe is stored as ferritin to maintain Fe homeostasis and to support important cellular processes (Knovich *et al.,* 2009). For plants also, Fe is a critical essential element and deficiency of Fe is directly related to the reduction in crop productivity and quality (Grotz and Guerinot, 2006).

Increasing Fe content in food grains

Agronomic biofortification is the application of external Fe salts to the plant parts to increase Fe content in grains. However, it is simple and effective, additional cost required for the purchase of Fe salts and labour for the application hinders the wide adoption of agronomic biofortification. Genetic biofortification is a proven approach to enhance Fe content in cereals especially in pearl millet and wheat (Neeraja *et al*., 2022). Biofortification can be achieved through either traditional breeding or genetic engineering. The conventional breeding methodology is based on the existence or availability of genetic variability, crossing to combine the high Fe and yields, selection of desirable recombinants from the segregating material and their stabilization to be released as varieties (Vasconcelos *et al.,* 2017). For the use of marker assisted selection (MAS), several attempts were or being made to identify genomic regions and candidate genes associated with high Fe content in target edible tissues using approaches like QTL mapping, GWAS and genomic selection across crops (Srivastava *et al.,* 2020; Gupta *et al.,* 2021; Swamy *et al.,* 2021). Recent identification of ZmNAC78, a transcription factor associated with high Fe levels in maize appears to be promising for MAS (Yan *et al*., 2023). Genetic engineering approach has demonstrated its potential for enhancing Fe content in cereals, however its adoption is restrained by constraints of regulatory authorities worldwide (Garg *et al*., 2018). Using gene editing strategy, one or a few nucleotides can be changed, existing alleles can be replaced and new genes can be inserted precisely and can be inherited stably (Huang *et al.,* 2016). Genome editing for targeted gene editing through Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) involves Cas9/13, RNA-guided DNA endonucleases guided by a single guided RNA (sgRNA) resulting in a complex at the target site (Roy and Soni, 2021; Ahmad *et al.,* 2020). Gene editing of *OsNRAMP2* increased the grain Fe content in rice (Chang *et al*., 2022). Knocking out anti-nutrient genes responsible for accumulation of heavy metals and phytic acid can reduce the accumulation anti-nutrients. Disruption of inositol penta phosphate 2-kinase 1 (*IPK1*) gene increased grain Fe content in wheat (Aggarwal *et al.,* 2018). Supposed to be transgene free technology, the gene editing appears to be promising with supportive regulatory framework across the world. For targeted breeding efforts to increase Fe content in cereals, deciphering the molecular mechanism of Fe translocation and remobilization into grains is very critical. In the present review, we summarized the uptake and transport mechanisms of Fe and the associated genes in model plants and cereals.

Translocation of Fe from root to loading in grains

Available literature and bioinformatics resources focusing on candidate genes and gene families associated with the translocation of Fe from the roots to the grain-loading process in crops *viz.,* rice, wheat and maize are summarized below.

Fe uptake from soil to roots

Plants employ two distinct strategies for Fe uptake from the soil:

1. Strategy I, known as the reduction-based strategy, is activated by non-grass plants when they experience Fe deficiency.
2. Strategy II, referred to as the chelation-based strategy, is triggered in grasses (**Figure 1**).

Strategy I: Reduction-based strategy

The Strategy I (reduction-based strategy), is predominantly employed by non-graminaceous plants. This reduction-based strategy hinges on the activity of the Fe-regulated transporter 1 (*IRT1*). Here is how it works:

- In Strategy I, the available Fe^{3+} in the rhizosphere is first converted into Fe^{2+} through a reduction process by the plant before it can be taken up.
- When plants are under Fe-deficient conditions, they release protons (H^{+}) into the rhizosphere, which leads to a decrease in pH in the immediate root vicinity. This acidification process is facilitated by ATPases, which utilize ATP to pump protons into the rhizosphere (Kim and Guerinot, 2007).
- As the pH decreases in the rhizosphere, the solubility of ferric oxides (Fe^{3+}) increases.
- Furthermore, an enzyme called ferric reductase oxidase 2 (*FRO2*) aids in the reduction of ferric oxides (Fe^{3+}) to ferrous oxide (Fe^{2+}), using NADPH-dependent Fe^{3+} chelate reductase. This conversion makes Fe more soluble.
- Subsequently, the soluble ferrous oxide (Fe^{2+}) is transported from the rhizosphere to the roots, primarily through a transporter controlled by *IRT1* **(Figure 1)** (Ishimaru *et al.,* 2006).

Strategy II: chelation-based strategy

Strategy II (chelation-based strategy) is primarily employed by graminaceous plants *viz.,* maize, wheat and rice from the grass family for Fe uptake. Here is how it works:

- Strategy II transport activities are controlled by transporters known as mugineic acid (*TOM1*) and yellow stripe 1 (*YS1*).
- In Strategy II, ferric ions (Fe^{3+}) present in the rhizosphere are transported into the root cytosol with the help of soluble phyto-siderophores. These siderophores are natural Fe chelators with a high affinity for Fe^{3+} transport (Morrissey and Guerinot, 2009).
- Among these chelators, the mugineic acid (MA) belonging to family of phytosiderophores (PS) is particularly effective in binding to Fe^{3+}. Different plant species secrete various members of the MA family, depending on their specific needs. For instance, rice primarily releases 2′ deoxymugineic acid (DMA), while barley releases two different types of MAs, *viz.,* 3’ epihydroxymugineic acid (epi HMA) and 3’ epihydroxy 2′ deoxymugineic acid (epi HDMA), near the rhizosphere through the TOM1 transporter (Ishimaru *et al.,* 2006).
- These MAs efficiently form Fe^{3+}-MA complexes.
- The YS1 transporter then transported the formed complexes into the root (Schaff *et al.,* 2004 and Ishimaru *et al.,* 2006). This chelation-based strategy enhances the uptake of Fe in graminaceous plants.

Certain crops, including rice, are capable of employing a combination of both reduction based (Strategy I) and chelation based (Strategy II) strategies for Fe uptake from the rhizosphere into the roots. Here is how it works

- In this combined strategy, plants directly absorb the soluble ferrous oxide (Fe^{2+}) from the rhizosphere, which can be richer in Fe^{2+} compared to Fe^{3+}. This is facilitated through transporters like *IRT1* and/or *IRT2* (Kim and Guerinot, 2007; Sperotto *et al.,* 2012).
- Simultaneously, via Strategy II, Fe^{3+}-MA complexes are formed in the rhizosphere. These Fe^{3+}-MA complexes are then transported into the root’s cytosol using transporters like Yellow stripe -like 15 (*YSL15*) (Ishimaru *et al.,* 2006).
- Instead of relying solely on direct Fe^{2+} uptake from the rhizosphere, rice successfully utilizes both reduction and chelation-based strategies, ensuring sufficient Fe is absorbed from the rhizosphere into the root cytosol through the Fe^{3+}-MA complexes (see **Figure 1**) (Ishimaru *et al.,* 2006).

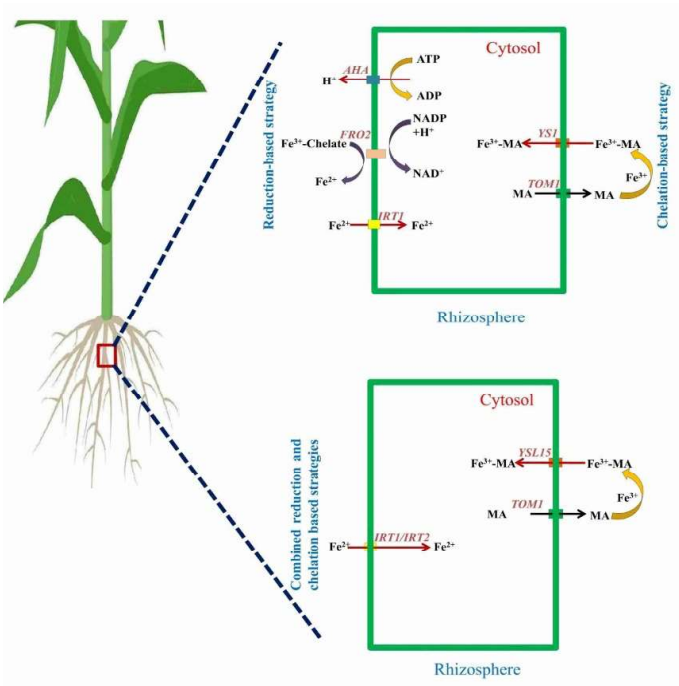

Figure 1: Fe uptake strategy I and II in plants

The expression of different sets of ferric reduction oxidase (*FRO)* genes in various locations suggests their role in Fe uptake in different plant tissues. *FRO2* corresponding gene to the yeast Fe (III) reductase 1 (*FRE1*) was identified in Arabidopsis based on its sequence similarity (Robinson *et al.,* 1999). *FRO2* is a primary *FER* expressed in the epidermal cells of Fe inadequate roots. In low Fe growth condition, the over expression of *FRO2* makes plants more resistant (Connolly *et al.,* 2003). Root specific FRO genes (*FRO2, FRO3* and *FRO5*) are expressed in roots, specifically FRO3 gene expressed in the vascular cylinder of roots. Shoot specific FRO genes (*FRO6, FRO7* and *FRO8*) are expressed in shoot (Feng *et al.,* 2006). The orthologs of *IRT1* gene have been identified which combines both Strategy I and II for Fe uptake in rice. Unlike Arabidopsis, the *LeIRT1* and *LeIRT2* genes are expressed in the roots of tomato in both the Fe deficient and sufficient roots, especially *LeIRT1*, shown induction under Fe deficiency (Eckhardt *et al.,* 2001). Ethylene is also associated in the stress adaptation like Fe deficiency in rice, although not in barley (Wu *et al.,* 2011). Additionally, Methylthioribose kinase (*MTK*) and S-adenosyl methionine synthetase (*SAM*) genes are expressed under Fe deficiency conditions in chelation-based strategy plants *viz.,* rice and maize (Liu *et al.,* 2015).

Several endogenous or housekeeping genes have been associated with grain Fe content in rice. For example, the ubiquitin activating enzyme (*UBA*), a small globular protein involved in the ubiquitination process, has shown a significant positive correlation with Fe concentration in rice grains. This suggests a potential role for *UBA* in Fe homeostasis, in addition to the reported ubiquitin-conjugating and ligase genes (Bej *et al.,* 2020). In both rice and Arabidopsis, genes related to acquisition, uptake and transport of Fe through both Strategies I and II have been identified and annotated. In Fe deficiency condition, six candidate genes have been associated with Fe in maize and these genes are associated in various aspects of Fe homeostasis, including Fe(III)-phytosiderophore transporter, Fe transport to vacuoles and transcriptional factors that regulate Fe-related gene expression (Curie *et al.,* 2001; Kobayashi *et al.,* 2014).

Long-distance Fe transport

Following the Fe transport from the rhizosphere into the root symplast, Fe is needed for chelating compounds. Then the Fe-chelator complexes are transported into the stele, following a diffusion gradient across intercellular connections. At this stage, Fe efflux is required to release Fe into the xylem vessels within the apoplastic space. However, the exact pathway of Fe efflux is not yet fully understood. In plants, especially Arabidopsis, three transporter proteins, known as Fe-regulated transporters (*IREGs*) or ferroportins (*FPTs*) are localized to the root epidermal cells. It is predicted that these transporters are involved in Fe-dependent nickel detoxification (Schaaf *et al.,* 2006). *AtIREG1/ FPT1* is bound to the plasma membrane of stele cells, indicating a potential role in releasing Fe into the xylem tracheas (Kim and Guerinot, 2007). *FRD3* is a

citrate efflux long-distance Fe transporter associated to *MATE* family. *MATE* gene is expressed in the root pericycle and vascular cylinder, indicating its role in citrate efflux into the root pericycle and xylem vessels. However, in the xylem, the concentration of Fe is reduced and it accumulates in the shoot apoplast due to *FRD3's* involvement in bypassing long-distance Fe transport. This apoplastic movement of Fe, transfers it from cell to cell through intercellular spaces or walls, allowing Fe to move from the roots to the shoots and from the xylem to the phloem. This may compensate for Fe transport mediated by the xylem (Green and Rogers, 2004). Several genes play a role in the mechanism of Fe uptake from the xylem vessels into the plasma membrane of leaf cells. FRO and ZIP genes are expressed in shoots and the basal part of flowers, signifying their role in Fe uptake in aerial tissues (Vert *et al.,* 2002). The mechanism of Fe transport through the phloem is also noteworthy, as it provides a feasible means of Fe transport, particularly when the Fe levels are insufficient in developing tissues/organs *viz.,* apices, seeds and root tips if relying solely on xylem vessels. In phloem sap, the alkaline pH (>7) is favourable for maintaining Fe and Fe chelates in a soluble form. Phloem transport is also involved in the remobilization of Fe from older to younger leaves, where the alkaline pH in the phloem sap facilitates the binding of Fe to chelators to keep it soluble (Kim and Guerinot, 2007).

Chloronera (*chln*) is a mutant tomato that exhibits the role of nicotianamine in long-distance Fe transport. The *chln* gene encodes NAS in the mutant tomato and illustrates the role of nicotianamine in the transport of Fe over long distances. The *chln* gene was recognized due to the interveinal leaf chlorosis it caused in young leaves, although it led to increased Fe accumulation in roots. This phenomenon suggests that nicotianamine can act as a shuttle, chelating Fe^{2+} from Fe(III)-DMA during phloem loading and unloading, facilitating Fe^{2+}/Fe^{3+} transformation and specific Fe(II)-NA transport within the phloem. *OsYSL1* transporters, similar to maize YS1, play a key role in the transport of Fe (III)-PS and Fe (II)-NA complexes. In rice total 18 putative YSL genes are identified in its genome and *OsYSL2* is essential gene for transporting Fe(II)-NA and Mn(II)-NA (Koike *et al.,* 2004). The temporal and spatial expression of YSL family genes indicating their role in Fe uptake mechanisms. The expression of *AtYSL1* mRNA is increased in the vasculature of roots and shoots, specifically in the xylem tubes and is detected in young siliques and the chalazal zone of the embryo, indicating the role of YSLs in Fe loading of seeds. *AtYSL1* and *AtYSL3* shows a similar expression pattern in the vasculature of shoots and reproductive organs (Takahashi *et al.,* 2003). Over all in Strategy II plants, the YSL genes are important in the long-distance Fe transport mechanism. In maize, the *YS1* gene is expressed in both roots and shoots (Curie *et al.,* 2001). Several *OsYSL* genes *viz., OsYSL2* and *OsYSL13* being preferentially expressed in shoots particularly *OsYSL6, OsYSL14* and *OsYSL16* are over expressed in both roots and shoots (Koike *et al.,* 2004). *OsYSL2* is a crucial gene overexpressed in the vascular bundles of the panicle neck and the sieve element cells of the phloem in flowers and developing seeds.

Members of the NRAMP family genes are intermediaries in the uptake of divalent cations (Thomine *et al.,* 2003) and in mutant yeast, *AtNRAMP4* can complement the Fe uptake, indicating their role in Fe transport (Thomine *et al.,* 2003). In roots, *NRAMP1* is expressed to take up Fe from the soil and is induced by Fe deficiency. Under Fe-deficient conditions, *NRAMP1* targets the intracellular membrane and remobilizing the Fe into the cytosol (Thomine *et al.,* 2000). In tomato, *NRAMP1* and *NRAMP3* genes are localized to vacuolar, intracellular vesicle and plasma membrane (Bereczky *et al.,* 2003). In Arabidopsis, two *NRAMP* genes *viz., AtNRAMP3* and *AtNRAMP4* are localized to the vacuolar membrane (Lanquar *et al.,* 2005). The *NRAMP1, NRAMP3* and *NRAMP4*

genes are over expressed in response to Fe deficiency. When the *NRAMP3* gene is overexpressed in plants, the over-expression of Fe uptake genes, *viz., FRO2* and *IRT1*, is downregulated, indicating that *NRAMP3* remobilizes the vacuolar Fe into the cytosol (Thomine *et al.,* 2003). Fe is stored as ferritin in the plastid stroma of plant cells. Ferritin is a Fe storage protein capable of storing up to 4,500 Fe atoms. Arabidopsis has four genes (AtFer1-4) encoding ferritin. The transcript of AtFer1, 3 and 4 is expressed upon excess Fe treatment in both roots and leaves (Petit *et al.,* 2001). However, despite the abundance of Fe, the mechanism of Fe uptake into chloroplasts is not well understood. Studies of Fe uptake with isolated chloroplasts have suggested that the mechanism is light dependent and requires Fe (III) chelate reductase activity in barley (Bughio *et al.,* 1997).

Contribution of environmental factors

When the soils are aerobic or of higher pH, Fe is oxidized and fixed as insoluble ferric oxides and at the same time, as Fe is highly reactive, when it is present in excess it becomes toxic. Therefore, plants have developed a control system for Fe (Morrissey and Guerinot, 2009; Grillet *et al.,* 2014). Application of animal manure and plant residues modifies properties of the soils and reported to increase Fe and Zn availability, however foliar applications found to be more than soil nutrient application in increasing grain nutrient contents (Wei *et al.,* 2012; Prasad *et al.,* 2014; Velu *et al.,* 2015).

Bioavailability

Research on bioavailability of nutrients has received greater attention in the past decades. For improvement of Fe absorption, a ratio of phytic acid (PA): iron (Fe), <1:1, is requisite without any enhancers (Hurrell and Egli, 2010). Developing the biofortified crop with high nutritive content is not the only concern but also the bioavailability of the nutrients in human gut (Neeraja *et al.,* 2017). Antinutrient *viz.,* phytic acid inhibit absorption of minerals and hinder the bioavailability of Fe from the ingested food (Kumar *et al.,* 2017). Phytic acid represents 80% of the phosphorous in plants (Bohn *et al.,* 2008). However, variation in the phytic acid content is not attributed to the Fe bioavailability. In maize, by expressing a fungal phytase, 3-fold increase in bioavailable Fe and decrease in phytic acid is reported (Drakakaki *et al.,* 2005). Fe bioavailability is measured by *in vitro* methods *viz.,* Caco-2 cell model. The caco-2 cells are the colonic carcinoma cells that are morphologically and functionally similar to the epithelial cells lining the small intestine. Animal studies using rodent models have been used in bioavailability studies on Zn and carotenoids, but this model seems to be a poor choice to assess Fe bioavailability. High Fe bioavailability in biofortified food crops was observed using isotopic human studies, however they are time consuming and very expensive, which has limited their use (La Frano *et al.,* 2014). Around 23 articles evaluated the bioavailability in biofortified crops, of which eight were animal studies, seven were *in vitro* studies and eight were human studies. A combination of these *in vitro*, animal and human studies will be an effective approach for investigating the efficacy of biofortification programmes (Dias *et al.,* 2018).

Caco-2 cell bioassay was reported as the best approach to evaluate the nutritive quality of Fe biofortified beans (*Phaseolus vulgaris*) and these varieties have high absorption than normal bean variety. Caco-2 cell model can also disclose the effects of antinutrients like phytic acid. Fe absorption studies in 61 Rwandese women with low Fe status revealed no significant difference in the Fe absorption from Fe rich beans than normal beans, which might be due to high phytic acid and polyphenols in beans (Petry *et al.,* 2012). In Pea, phytic acid decrease by 60% has increased bioavailability of Fe in Caco-2 cell studies and improvement of Fe bioavailability by 50-100% was identified in *lpa* lines than in controls (Warkentin *et al.,* 2012; Liu *et al.,* 2015). Cognitive performance, especially the efficiency of search and the speed

of retrieval on memory tasks, was improved in 18-25 women tested by consuming the Fe biofortified beans (86.1 ppm) compared with the normal beans (Murray-Kolb *et al.,* 2017). High ferritin formation in the Caco-2 cells with digests having $FeSO_4$ and ascorbic acid than the digests with $FeSO_4$ and citric acid was reported by (Glahn *et al.,* 1998). The effects of Fe status by consuming the Fe-biofortified rice was tested in 191 women in Philippines which resulted in increase of Fe stores in the women (Haas *et al.,* 2005). Meta-analysis on Fe bioavailability in different types of millets, (*in vitro* and *in vivo*) showed variation in the Fe levels ranging from 2 to 8 mg/100 g and 13.2% significant increase in haemoglobin levels. Enhancement of Fe bioavailability by 3.4 to 2.2 times is noted in women by following traditional methods like fermentation and germination (Anitha *et al.,* 2021). Randomised efficacy trials in the Fe profiles like serum ferritin, soluble transferrin receptor, total body Fe etc. were conducted in the Philippines, India and Rwanda in different crops like rice, pearl millet and beans. Cognitive performance in attention and memory domains were significantly improved by Fe biofortified crops compared with conventional crops (Finkelstein *et al.,* 2019). When the Indian school children, between 12-16 years of age, fed with Fe biofortified pearl millet continuously for six months, showed increased light physical activity and decreased sedentary time in children (Pompano *et al.,* 2022). Decrease in pathogenic bacteria and increase in beneficial bacteria in the gut is reported by dietary intake of Fe biofortified foods (Gomes *et al.,* 2021). The bioavailability of Fe can be enhanced from 12.1 to 16.4 ppm by following different processing techniques like popping, malting etc. (Neeraja *et al.,* 2017). The Fe bioavailability can also be altered by different methods of cooking and digestion in the intestine. During heating, Fe^{2+} or Fe^{3+} are released from Fe (III) hydroxides in ferritin (Hoppler *et al.,* 2008) and in the food matrix they are chelated by phytic acid (Moore *et al.,* 2018; Perfecto *et al.,* 2018). Since, the primary inhibitors of Fe bioavailability in food crops are phytic acid and polyphenolic compounds, breeding low phytate genotypes is now being targeted in different crops.

Conclusion

With more than half of the world's women and children suffering from Fe deficiency, all the available strategies should be adopted for improving Fe status in food grains. Biofortification is one of the proven approaches for enhancing Fe content in cereals such as pearl millet and in other crops such as beans. Limited genetic variability for grain Fe content is a major constraint in cereals, thus extensive germplasm screening should be done. High throughput sequencing of germplasm accessions, genome wide association mapping and RNA seq analyses could lead to the candidate genes associated with high grain Fe content. Genome editing offers favorable opportunities to increase the grain Fe content by modifying known candidate genes for Fe metabolism. Converging the physiological and molecular mechanisms of Fe transport and translocation along with methods to improve bioavailability could pave way to the success Fe Biofortification targeting nutritional security.

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