Biofortification of Rice Grains for Increased Iron Content

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Abstract Dietary iron (Fe) deficiency affects 14% of the world population with significant health impacts. Biofortification is the process of increasing the density of vitamins and minerals in a crop, through conventional breeding, biotechnology approaches, or agronomic practices. This process has recently been shown to successfully alleviate micronutrient deficiency for populations with limited access to diverse diets in several countries (<https://www.harvestplus.org/>). The Fe breeding target in the HarvestPlus program was set based on average rice consumption to fulfil 30% of the Estimated Average Requirement of Fe in women and children. In this review, we present the reported transgenic approaches to increase grain Fe. Insertion of a single or multiple genes encoding iron storage protein, metal transporter, or enzyme involved in the biosynthesis of metal chelator in the rice genome was shown to be a viable approach to significantly increase grain-Fe density. The most successful approach to reach the Fe breeding target was by overexpression of multiple genes. Despite this success, a significant effort of 8–10 years needs to be dedicated from the proof of concept to varietal release. This includes large-scale plant transformation, event selection, collection of data for premarket safety assurance, securing biosafety permits for consumption and propagation, and collection of data for variety registration.

Keywords Rice · Grain · Iron · Biofortification · Genetic engineering

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1 Introduction

Nutritional deficiency is an important global health concern that affects approximately 1.86 billion people worldwide, and 61% of it is caused by dietary iron (Fe) deficiency (James et al. 2018). Iron deficiency anemia results in decreased work productivity, increased maternal mortality, increased child mortality, slowed child development, and increased susceptibility to infectious diseases (Stoltzfus 2001). Iron deficiency anemia is estimated to be responsible for 800,000 deaths/year (WHO 2002).

Although providing access to more diverse diets is the ideal solution to alleviate micronutrient deficiency, this may not be achieved in the near future in developing and less developed countries. Iron supplementation and industrial fortification have been shown to alleviate micronutrient deficiencies but require continuous significant budget allocation at the government or household level. Despite the potential to diminish iron deficiency in the population, this may not help people living in remote rural areas because of the lack of infrastructure, purchasing power, or access to markets and healthcare systems (Mayer et al. 2008). Biofortification, a process of increasing the concentration of micronutrient in the edible part of a crop through conventional plant breeding, transgenic methods, and agronomic practices, offers a feasible and cost-effective approach, complementing other efforts to reach rural populations (Bouis and Saltzman 2017).

2 Target Concentration for Fe in Polished Rice

The Estimated Average Requirement (EAR) of iron for non-pregnant, non-lactating women is 1460 mg/day, and for children 4–6 years old is 500 mg/day (WHO/FAO 2004). Current studies show that 90% of the iron remains in the grain after processing, while the nutritionist assumption is that 10% of the iron is bioavailable. With 400 g/day per capita rice consumption for an adult woman and 120 g/day for children 4–6 years old, the [HarvestPlus](#) program set 13 µg/g as the final target concentration for Fe in polished rice to achieve 30% of the EAR (Bouis et al. 2011).

3 Iron Biofortification via Conventional Plant Breeding

Initial screening of the germplasm collection at the International Rice Research Institute (IRRI) showed that the range in Fe concentration in brown rice among 1138 genotypes tested was 6.3–24.4 µg/g (Gregorio et al. 1999). However, the variation in Fe concentration in milled rice becomes narrow due to the high proportion of Fe lost during milling. A study on the iron concentration of brown and milled rice of six varieties collected from ten commercial rice mills in one province in Vietnam

showed that the percentage of Fe loss due to milling ranged from 65% to 82% (Hoa and Lan 2004). Furthermore, the maximal iron concentration in milled rice was 8 µg/g over 11,337 genotypes from the International Center for Tropical Agriculture (CIAT) core collection (Martínez et al. 2010). At IRRI, the highest Fe concentration of 7.4 µg/g in polished rice was achieved by classical breeding (Virk et al. 2006, 2007).

To reach the Fe target of 13 µg/g in polished rice, transgenic approaches are potential options because of the low concentration of Fe found in the rice gene pool. Efforts have been made to study the physiology as well as the genetic basis and biochemical mechanisms involved in Fe uptake and translocation in crops and model plants. These studies have facilitated the detection of the limiting factors that could be manipulated to increase Fe concentration in rice grain.

4 Iron Uptake and Translocation

Iron is an important micronutrient required in various processes such as photosynthesis and respiration. Based on the strategy they use to uptake iron from the rhizosphere, higher plants can be categorized into three different groups (Connorton et al. 2017): (1) Strategy I plants (all dicotyledonous plants and non-graminaceous monocots) that rely on the reduction of ferric Fe(III) to ferrous Fe(II), (2) Strategy II plants (graminaceous monocots) that rely on the chelation strategy involving phytosiderophore secretion, and (3) a combination of both. *Arabidopsis* has been used as a model plant to study Strategy I plants. Some major genes responsible for iron uptake using this strategy have been identified: iron-regulated transporter 1 (*IRT1*) (Eide et al. 1996), ferric-chelate oxidase 2 (*FRO2*) (Robinson et al. 1999), and HC-ATPase (HA) genes (Kobayashi and Nishizawa 2012).

Rice has been used as a model plant to study Strategy II plants. Plants in this group that include the most important cereals in the world secrete phytosiderophore (PS) in the rhizosphere. PS is a high-Fe-affinity organic molecule from the mugineic acid family (Bashir et al. 2017; Borrill et al. 2014; Kobayashi et al. 2005; Suzuki et al. 2006). Figure 1 presents the basic scheme for the genes involved in iron homeostasis in rice.

Mugineic acid is synthesized through a conserved pathway that starts from S-adenosyl-L-methionine. It is then followed by sequential reactions catalyzed by nicotianamine synthase (NAS), nicotianamine aminotransferase (NAAT), and deoxymugineic acid synthase (DMAS) enzymes, producing 20-deoxymugineic acid (DMA), a precursor of different types of mugineic acids (Bashir et al. 2006; Higuchi et al. 1999; Kobayashi and Nishizawa 2012; Takahashi et al. 1999). In rice roots, secretion of DMA is influenced by the expression of the TOM1 gene encoding efflux transporter of DMA (Nozoye et al. 2011). YELLOW STRIPE 1 (YS1) and YELLOW STRIPE 1-like (YSL1) transporters are known for their role in facilitating the uptake of Fe–MA complexes into root cells (Curie et al. 2001; Inoue et al. 2009). After reduction by ascorbate, the Fe(III)–DMA complex is likely converted

to Fe(II)–NA, and then excreted to the xylem. The Fe may create complexes primarily with citrate and some with DMA for further transport (Yoneyama et al. 2015).

Aside from using Strategy II, rice may be able to uptake Fe(II) directly, as indicated by the presence of a ferrous transporter (OsIRT1) in the genome. Other metal transporters involved in Strategy I and Strategy II were also identified such as natural resistance-associated macrophage protein (NRAMP) and ZIP (zinc-regulated transporter, IRT-like protein) family (Cailliatte et al. 2010; Guerinot 2000; Lanquar et al. 2005).

Fe translocation in higher plants is a complex process involving xylem loading/unloading, phloem loading/unloading, and reabsorption (Kim and Guerinot 2007). Different chelators such as citrate, nicotianamine (NA), and MAs play an essential role in symplast metal homeostasis (Garcia-Oliveira et al. 2018). FERRIC REDUCTASE DEFECTIVE-LIKE 1 (OsFRDL1) in rice (Fig. 1) is known to encode a citrate transporter involved in the transport of Fe-citrate complex (Inoue et al. 2004; Yokosho et al. 2009).

The rice YSL family encoding influx transporter consists of 18 members (Curie et al. 2009). The OsYSL2 transporter is a carrier of Fe(II)–NA and is involved in iron transport to sink tissues (Koike et al. 2004). OsYSL15 transports Fe(III)–DMA and is involved in Fe uptake in the root and long-distance Fe transport. The Fe transporter OsYSL18 may play a specific role in fertilization, as indicated by specific expression in the pollen and pollen tubes. OsYSL18 may also be involved in Fe transport in the phloem (Kobayashi and Nishizawa 2012). As indicated by their vascular tissue expression in rice, OsIRT1 and OsTOM1 may be involved in Fe translocation within the plant as well (Ishimaru et al. 2006; Nozoye et al. 2011).

5 Iron Biofortification via Genetic Engineering

Several efforts have been conducted to increase Fe concentration in rice grains. These studies can be categorized into different approaches: (1) overexpression of geneencoding iron storage protein, (2) overexpression of geneencoding enzyme involved in the biosynthesis of metal chelator, (3) overexpression of geneencoding metal transporter, and (4) a combination of two or three approaches (Tables 1 and 2).

Fe concentration of 38.1 µg/g in brown rice was achieved by endosperm-specific expression of a soybean Fe storage protein, SoyFerH1 (Goto et al. 1999). Similar approaches using the *SoyferH1* gene driven by different promoters (OsGluB1, OsGluB4, OsGlb1, ZmUbi-1) in different backgrounds (Swarna, IR68144, BR29, IR64, M12) were reported (Slamet-Loedin et al. 2015). Stable Fe concentrations of 9.2 or 7.6 µg/g over several generations were obtained (Khalekuzzaman et al. 2006; Oliva et al. 2014). Overexpression of the *OsFer2* gene was also studied and Fe concentration of 15.9 µg/g, vis-à-vis 7 µg/g in control variety Pusa-Sugandh II, was observed (Paul et al. 2012).

Another approach in improving grain-Fe concentration in rice is by increasing the expression of genes encoding enzymes involved in the biosynthesis of metal

Table 1 Summary of transgenic approaches to develop iron-rich milled rice

	Mechanism involved	Iron ([c] in µg/g)		Growth condition	Cultivar	References
		TG	WT			
Rice gene overexpression						
<i>OsNAS1</i>	Iron uptake and translocation	Up to ~40	~20	Greenhouse	Japonica cv. EYI 105	Díaz-Benito et al. (2018)
<i>OsNAS2</i>	Iron uptake and translocation	~10	~4	Greenhouse	Japonica cv. Dongjin	Lee et al. (2012)
<i>OsNAS3</i>	Iron uptake and translocation	~12	~4	Greenhouse	Japonica cv. EYI 105	Lee et al. (2009b)
<i>OsNAS1, OsNAS2, OsNAS3</i>	Iron uptake and translocation	Up to ~19	~4.5	Greenhouse	Japonica cv. Nipponbare	Johnson et al. (2011)
<i>OsYSL2</i>	Iron uptake and translocation	~7.5	~1.8	Greenhouse	Japonica cv. Tsukinohikari	Ishimaru et al. (2010)
<i>Osfer2</i>	Storage	Up to ~15.9	~7	Greenhouse	Indica cv. Pusa-Sugandh II	Paul et al. (2012)
Rice gene silencing/knockdown mutant						
<i>OsVIT2</i>	Inter-cellular/intra-cellular transport and storage	~8	~5		Japonica cv. Dongjin	Bashir et al. (2013)
<i>OsYSL9</i>	Inter-cellular/intra-cellular transport and storage	Up to ~2.5	1		Japonica cv. Tsukinohikari	Senoura et al. (2017)
<i>OsDMAS1</i>	Inter-cellular/intra-cellular transport and storage	~5	~5		Japonica cv. Dongjin	Bashir et al. (2017)
Rice gene knockout mutant						
<i>OsVMT</i>	Inter-cellular/intra-cellular transport and storage	9	4	Greenhouse	Japonica cv. Nipponbare	Che et al. (2019)
Overexpression of genes from different species						
<i>HvNAS1</i>	Iron uptake and translocation	~8.5	~4	Greenhouse	Japonica cv. Tsukinohikari	Masuda et al. (2009)

	Mechanism involved	Iron ([c] in µg/g)		Growth condition	Cultivar	References
		TG	WT			
<i>HvYSL1</i>	Iron uptake and translocation	Up to ~9	~4		Japonica cv. EYI 105	Banakar et al. (2017a)
<i>AtIRT1</i>	Iron uptake and translocation	Up to ~4.86	~2.28	Greenhouse	Japonica cv. Taipei 309	Boonyaves et al. (2016)
<i>SoyferH1</i>	Storage	Up to ~37	~10	Screenhouse	Indica cv. IR68144	Vasconcelos et al. (2003)
<i>SoyferH1</i>	Storage	Up to ~9.2	~3.8	Greenhouse	Indica cv. BR29	Khalekuzzaman et al. (2006)
<i>SoyferH1</i>	Storage	Up to ~16	~6.75	Greenhouse	Indica cv. Swarna	Paul et al. (2014)
<i>SoyferH1</i>	Storage	Up to ~7.6	~3.3	Greenhouse	Indica cv. IR64	Oliva et al. (2014)
Multigene overexpression of genes from different species						
<i>SoyferH2</i> + <i>HvNAS1</i> + <i>OsYSL2</i>	Combined mechanisms	Up to ~4	~1	Paddy field	Japonica cv. Tsukinohikari	Masuda et al. (2012)
<i>HvNAS1</i> , <i>HvNAS1</i> + <i>HvNAAT</i> , <i>IDS3</i>	Combined mechanisms	Up to ~7.3	~5.8	Paddy field	Japonica cv. Tsukinohikari	Suzuki et al. (2008)
<i>SoyferH2</i> + <i>HvNAS1</i> + <i>OsYSL2</i>	Combined mechanisms	~6.3 (~5.02)	~3.2 (~1.46)	Greenhouse	Tropical Japonica cv. Paw San Yin	Aung et al. (2013)
<i>AtNAS1</i> + <i>Pvfer</i> + <i>Aphytase</i>	Combined mechanisms	Up to ~7	~1	Hydroponic	Japonica cv. Taipei 309	Wirth et al. (2009)
<i>AtIRT1</i> , <i>Pvfer</i> , <i>AtNAS1</i>	Combined mechanisms	Up to ~10.46	~2.7	Greenhouse	Japonica cv. Nipponbare	Boonyaves et al. (2017)
<i>SoyferH1</i> + <i>OsNAS2</i>	Combined mechanisms	~15	~2.5	Paddy field	Indica cv. IR64	Trijatmiko et al. (2016)
<i>AtNAS1</i> + <i>AtFRD3</i> + <i>Pvfer</i> , <i>AtFRD3</i> + <i>Pvfer</i>	Combined mechanisms	Up to ~11.08	~2.05	Greenhouse	Japonica cv. Nipponbare	Wu et al. (2018)

(continued)

Table 1 (continued)

	Mechanism involved	Iron ([c] in µg/g)		Growth condition	Cultivar	References
		TG	WT			
<i>AtNAS1</i> + <i>Pvifer</i> + <i>AtNRAMP3</i> , <i>Pvifer</i> + <i>AtNRAMP3</i>	Combined mechanisms	Up to ~13.65	~2.72	Greenhouse	Indica cv. IR64	Wu et al. (2019)
<i>AtNAS1</i> + <i>Pvifer</i> + <i>ZmPSY</i> + <i>PtCRT1</i>	Combined mechanisms	Up to ~6.02	~1.82	Greenhouse	Japonica cv. Nipponbare	Singh et al. (2017)
<i>OsNAS1</i> , <i>HvHAATb</i> , <i>OsNAS1</i> + <i>HvHAATb</i>	Combined mechanisms	Up to ~18	~4	Hydroponic	Japonica cv. EYI 105	Banakar et al. (2017b)
<i>OsNAS1</i> , <i>HvNAATb</i> , <i>OsNAS1</i> + <i>HvNAATb</i>	Combined mechanisms	Up to ~55	~20	Greenhouse	Japonica cv. EYI 105	Díaz-Benito et al. (2018)
<i>SoyferH2</i> + <i>HvNAS1</i> + <i>HvNAAT</i> ; <i>A-B</i> + <i>IDS3</i>	Combined mechanisms	Fourfold		Greenhouse	Japonica cv. Tsukinohikari	Masuda et al. (2013)

Table 2 Summary of transgenic approaches to develop iron-rich brown rice

	Mechanism involved	Iron ([c] in µg/g)		Growth condition	Cultivar	References
		TG	WT			
Rice gene overexpression						
<i>OsIRT1</i>	Iron uptake and translocation	~12	~10	Paddy field	Japonica cv. Dongjin	Lee and An (2009)
<i>TOM1</i>	Iron uptake and translocation	~18	~15	Hydroponic	Japonica cv. Tsukinohikari	Nozoye et al. (2011)
<i>OsNAS1</i>	Iron uptake and translocation	Up to ~19	~12	Paddy field	Japonica cv. Xiushui 110	Zheng et al. (2010)
<i>OsYSL3</i>	Iron uptake and translocation	~15	~11	Greenhouse	Japonica cv. Zhonghua 11	Zhang et al. (2018)
<i>OsYSL15</i>	Iron uptake and translocation	~14	~12	Paddy field	Japonica cv. Dongjin	Lee et al. (2009a)
<i>OsbHLH</i>	Iron uptake and translocation	~35	~18	Hydroponic	Japonica cv. Nipponbare	Kobayashi et al. (2019)
<i>OsIRO2</i>	Iron deficiency response	Up to ~15.5	~6	Greenhouse	Japonica cv. Tsukinohikari	Ogo et al. (2011)
<i>TOM2</i>	Iron deficiency response			Hydroponic	Japonica cv. Nipponbare	Nozoye et al. (2015)
Rice gene silencing/knockdown mutant						
<i>OsVIT1</i>	Inter-cellular/intra-cellular transport and storage	~26	~20	Paddy field	Japonica cv. Zhonghua 11	Zhang et al. (2012)
<i>OsVIT2</i>	Inter-cellular/intra-cellular transport and storage	~28	~20	Paddy field	Japonica cv. Dongjin	Zhang et al. (2012)
Overexpression of genes of different species						
<i>SoyferH1</i>	Storage	Up to ~38	~14.3	Greenhouse	Japonica cv. Kitaake	Goto et al. (1999)
<i>SoyferH1</i>	Storage	~18	~18	Greenhouse	Indica cv. M12	Drakakaki et al. (2000)
<i>SoyferH1</i>	Storage	Up to ~25	~17	Greenhouse	Japonica cv. Kitaake	Qu et al. (2005)
Multigene overexpression of genes of different species						
<i>Pvifer + rgMT + phyA</i>	Combined mechanisms	~22	~10	Greenhouse	Japonica cv. Taipei 309	Lucca et al. (2002)

chelator. *OsNAS1* overexpression resulted in Fe concentration of 19 $\mu\text{g/g}$ in brown rice; however, the concentration decreased to only 5 $\mu\text{g/g}$ after polishing (Zheng et al. 2010). Co-overexpression of *OsNAS1* and *HvNAAT* genes in japonica rice resulted in Fe concentration of 18 $\mu\text{g/g}$ in the polished grain (Banakar et al. 2017b). Fe concentration of 55 $\mu\text{g/g}$ in the succeeding generation was observed; however, this unusually high Fe concentration suggests either low milling degree or Fe contamination (Díaz-Benito et al. 2018). Overexpression and activation tagging of *OsNAS2*, on the other hand, resulted in 19 $\mu\text{g/g}$ and 10 $\mu\text{g/g}$ Fe concentration in polished rice, respectively (Johnson et al. 2011; Lee et al. 2012). Meanwhile, activation tagging of *OsNAS3* achieved 12 $\mu\text{g/g}$ Fe in polished grain vis-à-vis 4 $\mu\text{g/g}$ Fe in the wild type (Lee et al. 2009b).

Several studies reported increased Fe uptake and translocation by overexpression of genes encoding metal transporter, including *OsYSL2* (Ishimaru et al. 2010), *OsYSL15* (Lee et al. 2009a), and *OsYSL9* (Senoura et al. 2017). *OsYSL2* and *OsYSL15* are responsible for the uptake of Fe(II)–NA and Fe(III)–DMA, respectively, whereas *OsYSL9* is involved in the transport of both complexes. Although only a minimal Fe increase was detected in T1 brown rice of *OsYSL9* and *OsYSL15* OE lines, overexpression of *OsYSL2* resulted in a fourfold increase in Fe concentration in T1 polished rice.

Recently, two studies reported significant Fe concentration increases in rice grains by overexpressing multiple genes. Wu et al. (2019) overexpressed the *AtNAS1*, *Pvfer*, and *AtNRAMP3* genes, resulting in 13.65 $\mu\text{g/g}$ Fe in polished grains under greenhouse conditions. Trijatmiko et al. (2016), on the other hand, reported an Fe concentration of 15 $\mu\text{g/g}$ in polished grains under field conditions by overexpressing nicotianamine synthase (*OsNAS2*) and soybean ferritin (*SoyferH-1*) genes. This high-Fe rice event did not show a yield penalty in field trials in the Philippines and Colombia. The grain quality of the transgenic event was similar to that of the IR64 genotype background used for transformation. These two studies show the potential for further advanced development of a biofortified rice product with elevated Fe concentration.

6 Future Directions

There is little prospect of achieving the target Fe concentration to reach 30% of the EAR via conventional plant breeding because of limited genetic variation in the Fe concentration in polished grains within the global rice germplasm collection. On the contrary, recent studies show that the target concentration can be achieved via genetic engineering. Under the current regulations in different countries, it usually takes 8–10 years from proof of concept to market release of genetically modified (GM) crops (Mumm 2013). The best performing events need to be selected from large-scale transformation. These events, aside from showing stable and acceptable trait expression, should have a simple integration of transgenes and have no

disruption of endogenous genes with important phenotypic manifestation. Significant efforts need to be dedicated to collecting data for premarket safety assurance of the potential product, such as detailed molecular characterization of the event, safety of newly expressed proteins, novel protein expression and dietary exposure analysis, comparative nutritional analysis, and some environmental safety data collected from multi-location and multi-season field trials. After a biosafety permit for propagation of the event has been secured, developers need to follow similar procedures as in conventional breeding of a product for variety registration.

High Fe content is a consumer trait. To facilitate adoption by farmers, this micronutrient trait needs to be combined with agronomic traits. The most prospective agronomic trait for farmer adoption is higher yield. For this purpose, the possibility to incorporate the high-Fe trait into hybrid rice needs to be explored. The high-yield trait obtained through heterosis can be combined with nutritional traits. In addition, we observed that overexpression of some genes for Fe enhancement might cause unintended effects such as a yield decrease when the plants were in a homozygous condition. However, this is not detrimental when only one allele is present in hemizygous condition, and in some cases the micronutrient concentration can be retained in the hemizygous condition. In such a situation, hybrid rice can be a solution to achieve higher micronutrient concentration using a wider gene pool.

Recent developments in the regulation of genome-edited crops in different countries have attracted many scientists to work on genome editing. In the United States, certain categories of modified plants would be exempted from the regulations if the product can also be created through conventional breeding (APHIS 2020). In Argentina, a resolution on New Breeding Techniques (NBT) was passed in 2015, which rules that, if a transgene is not used or a transgene is used but is removed in the final product, it will not be classified as a GM product (Friedrichs et al. 2019). Precise genome editing technology that produces a double-stranded break in the genome, followed by the repair of this break that leads to a mutation or deletion, may result in a product that meets the non-GM regulatory classification.

Increased Fe concentration in polished grains was observed on the T-DNA insertion mutant of *OsVIT2* (Bashir et al. 2013). The insertion of the T-DNA in the promoter region in this mutant led to the knockdown of the *OsVIT2* gene (Bashir et al. 2013). Genome editing can be used to mutate the regulatory elements of genes involved in Fe homeostasis. This type of editing could result in altered expression of the genes and consequently enhanced Fe concentration in rice grains.

Although genome editing has great potential to ease the burden of regulatory requirements, genetic engineering will still be the primary tool to achieve the target Fe concentration. Overexpression of other genes involved in Fe homeostasis needs to be explored. Special attention needs to be given to the possible yield decrease in transgenic plants. Fine-tuning the expression of the genes by choosing a moderate constitutive promoter or tissue- and/or stage-specific promoter may need to be tested to avoid any yield penalty.

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