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Review Article

Iron Uptake Strategies and Different Genes in Rice Biofortification

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Abstract

Biofortification is a new health strategy aimed at preventing iron and micronutrient shortages in staple food crops by increasing their concentration and nutritional quality. Understanding the uptake, transit, and outflow of essential elements in rice is crucial for increasing iron and other nutrients in the endosperm. The study utilized various genes to enhance iron storage, transport, translocation, uptake, and translocation in white grains, including soyferH1, OsNAS1, OsYSL2, IDS3, and OsVIT1. AtNRAMP1, AtNRAMP3, and AtNRAMP4 are involved in the transport of iron and cadmium in Arabidopsis, with AtNRAMP1 being primarily expressed in roots. The expression of two transgenes, nicotianamine synthase and ferritin, in the rice endosperm of Japonica rice and other cultivars has led to a significant increase in iron content.

Keywords: Iron, Rice, Biofortification, Genes.

Abbreviations: Deoxymugeneic Acid **(DMA)**; Nicotianamine **(NA)**; Nicotianamine Synthase **(NAS)**; Vacuolar Iron Transporter 1 **(VIT1)**; Deoxy-Mugineic Acid **(DMA)**; Kilodalton **(kDa)**; Iron-Deficiency Anemia **(IDA)**; Potential of Hydrogen **(pH)**; Iron Regulated Transporter 1 **(IRT1)**; Mugineic Acid **(MA)**; Yellow Stripe 1 **(YS1)**; Fatty Acid Desaturase **(FAD-3)**; Iron Deficiency Specific 2 **(IDS2)**; Myo-Inositol-3-Phosphate Synthase **(MIPS)**; Phytic Acid **(PA)**; Inorganic Phosphate **(Pi)**; Inositol Triphosphate Kinases **(ITPK)**; Genetically Modified **(GM)**; Inositol Pentakisphosphate Kinase **(IPK)**

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INTRODUCTION

Rice grains, rich in genetic variability and popular among resource-poor populations, are ideal for biofortification to enrich with essential micronutrients. (Graham et al. 1999). Iron is absorbed directly in rice or as a compound chelated by mugineic acid phytosiderophores such as 2′ -deoxymugeneic acid (DMA) (Fu et al. 2010). NA synthase (NAS) catalyses the biosynthesis of nicotianamine (NA), a precursor to DMA (Inoue et al. 2003). Different NAS genes have been utilized to generate GM rice (Johnson et al. 2011; Wirth et al. 2009; Masuda et al. 2012). In rice, three NAS genes (OsNAS1, OsNAS2, and OsNAS3) have been discovered (Inoue et al. 2003). OsNAS2 as one of the orthologues since it has been found to be more effective for rice grain Fe augmentation when overexpressed (Johnson et al. 2011). The aph4 gene-encoding enzyme hygromycin B phosphor-transferase as a selective marker (Kaster et al. 2010). Nine countries have approved the sale of foods containing this protein, including the United States, Canada, Japan, Australia, South Korea, New Zealand, Taiwan, Mexico, and Indonesia (ISAAA GM Approval Database, 2015).

Ferritin is an iron storage protein that can hold up to 4,500 Fe atoms per molecule (Briat et al. 1997) and is an important iron source in vegetarian diets (Lönnerdal et al. 2009). Ferritin, a safe protein found in legumes, has been suggested as a potential target for increasing iron content in rice grains and transforming cereals (Borg et al. 2012; Vasconcelos et al. 2003). FERRITIN is a hollow protein structure made up of 24 protein subunits that can store up to 4500 ferric molecules (Harrison and Arosio 1996). FERRITIN enhanced iron content in polished rice grains by 2- to 3.7-fold when expressed under the direction of an endosperms pecific promoter in rice (Goto et al. 1999; Lucca et al. 2001; Oliva et al. 2014; Qu et al. 2005; Vasconcelos et al. 2003). The expression of both FERRITIN and NAS boosts the iron content of rice grains in a synergistic manner. The combination expression of bean FERRITIN (PvFERRITIN) and Arabidopsis NAS1 (AtNAS1) boosted iron and zinc levels in rice endosperm by 6-fold and 1.3-fold, respectively (Wirth et al. 2009). More recently, it was discovered that rice lines with rice NAS2 and soybean FERRITIN expression had polished grain iron and zinc contents that were 7.5 and 3.3 times higher, respectively. (Trijatmiko et al. 2016). Overexpression of NAS also frequently leads to favorable increases in the zinc concentration, which are most likely caused by an increase in PS synthesis because zinc also forms complexes with PS (Schaaf et al. 2004).

Several genes involved in metal homeostasis in rice regulate mineral absorption, distribution, and translocation (Gross et al. 2003, Palmgren et al. 2008). Wirth et al. 2009) Rice endosperms increased by six times with targeted expression of nicotianamine synthase and ferritin genes, possibly the highest iron content in genetically engineered rice (Paul et al. 2012; Goto et al. 1999; Lucca et al. 2001; Qu et al. 2005). Attempts to improve Fe availability by modulating the expression of phytase, a phytic acid degrading enzyme, have also been described (Wirth et al. 2009).

The regulation of transporters that regulate metal distribution within plant cells could enhance metal homeostasis and increase metal accumulation in seeds (Bashir et al. 2016). The expression of vacuolar iron transporter 1 (VIT1) significantly influences Fe localisation in Arabidopsis seeds, making vacuolar transporters of particular relevance (Kim et al. 2006). VIT1 and VIT2 are functional vacuolar transporters in rice that help to maintain Fe, Mn, and Zn homeostasis. Increased metal buildup in rice seeds is caused by disrupting the expression of OsVIT1 and/or OsVIT2 (Zhang et al. 2012). Changing the expression of a mitochondrial Fe transporter, on the other hand, has a deleterious effect on Fe localization in rice seeds (Bashir et al. 2013). Combining several ways to increase metal concentrations in edible plant sections may yield greater results than a single approach (Masuda et al. 2012). The expression of vacuolar iron transporter 1 (VIT1) significantly influences Fe localisation in Arabidopsis seeds, making vacuolar transporters of particular relevance (Johnson et al. 2011; Lee et al. 2012; Masuda et al. 2009). Iron transporter genes YELLOW STRIPE LIKE 2 (OsYSL2) and IRON REGULATED TRANSPORTER 1 (OsIRT1) were also inserted into rice, either alone or in combination with genes linked with MA synthesis, and various degrees of iron buildup in rice grains were identified (Ishimaru et al. 2010; Lee and An, 2009; Tan et al. 2015).

Plant cells have low cytosolic-free Fe concentrations due to iron stored in harmless forms in chloroplasts, mitochondria, and vacuoles, used for Fe heme or Fe-S cluster synthesis. (Finney and O' Halloran, 2003). When the external Fe supply is insufficient, vacuoles play a crucial role in storing excess Fe and releasing it into the cytosol (Kim and Guerinot, 2007; Lanquar et al. 2010; Pich et al. 2001). The vacuolar membrane-located VACUOLAR IRON TRANSPORTER 1 (AtVIT1) is important for iron uptake into vacuoles in Arabidopsis (Kim et al. 2006). Specific members of the NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN (NRAMP) family, on the other hand, mediate iron efflux from vacuoles to the cytosol. Depending on the plant species, NRAMP transporters are found in intracellular vesicles, the vacuole, or the plasma membrane (Bereczky et al. 2003; Lanquar et al. 2005; Takahashi et al. 2011; Thomine et al. 2003). AtNRAMP1, AtNRAMP3, and AtNRAMP4 mediate iron and cadmium transport in Arabidopsis, while AtNRAMP1 is preferentially expressed in roots (Curie et al. 2000; Thomine et al. 2000). AtNRAMP3 and AtNRAMP4 are vacuolar membrane proteins whose genes are activated by iron deprivation (Lanquar et al. 2005, 2010; Thomine et al. 2003). OsNRAMP1, one of seven NRAMP homolog's

discovered in rice, is involved in iron uptake and is confined to the plasma membrane (Curie et al. 2000; Takahashi et al. 2011). In the opinion of Gross et al. 2003, substantial research has been done on the similarities between the NRAMP genes in rice and Arabidopsis. The endosperm-specific expression of PvFER alone or in combination with constitutive AtNAS1 expression, along with the expressed AtNRAMP3 under the control of either the rice 18-kDa Oleosin (Ole18) or maize UBIQUITIN (Ubi) promoters. To improve endosperm iron content, vacuolar iron reserves have been used. The effective integration of intracellular and intercellular iron mobilization for rice biofortification techniques.

Biofortification Of Rice

Biofortification enhances rice's micronutrient content, aiding in alleviating deficiencies in rice-consuming individuals with limited access to diverse diets and healthcare (Datta, et al. 2000). Different ways have been strategized worldwide under rice biofortification research programmes for sustaining, increasing, and introducing new micronutrients in rice grain.

Iron-Rich Rice

One of the most important minerals for human health is iron. Rice's iron content rises dramatically. Paddy (rough rice) has 38 ppm of iron, which is decreased to 8.8 ppm in brown rice after processing and to 4.1 ppm in milled rice (Dexter et al. 1998). In another study, iron concentration in brown rice was lowered from 19 ppm to roughly 4 ppm in polished grains (a 4.75-fold drop) (Masuda et al. 2009). In underdeveloped nations, having enough iron in rice would help to sustain the health of children and pregnant women. Iron deficiency produces Iron-deficiency anemia (IDA), which has major health repercussions for people, especially children and women. IDA affects 32.9% of the world's population, with the risk of infection being higher in sub-Saharan Africa and South Asia (Kassebaum et al. 2014). It impairs children's cognitive development, weakens their immune systems, and raises their risk of morbidity. IDA can also have a negative impact on productivity, cause premature deliveries, and raise the chance of death in women. In such nations, the development of high iron milled rice as part of a biofortification initiative could be useful in combating IDA.

Plant Iron Uptake Strategies

Plants primarily obtain iron from the rhizosphere, with soil iron availability influenced by pH and redox potential (Morrissey et al. 2009). With a higher pH, iron becomes less soluble and is found in the form of insoluble ferric oxides. Iron, on the other hand, becomes more soluble at low pH and can be easily absorbed by plant roots (Kim et al. 2007). Different uptake methods significantly influence plant micronutrient uptake and distribution, allowing plants to absorb the appropriate amount without causing toxicity (Welch et al. 2002). The reduction-based technique and the chelation-based strategy are used for iron uptake from the soil, just as they are for iron uptake in plants (Ishimaru et al. 2006; Wirth et al. 2009). Graminaceous plants utilize a chelation-based method, while non-graminaceous plants employ a reduction-based technique. Rice employs a combination of reduction- and chelation-based techniques, as shown in (**Figure 1**).

Strategy I is a reductionist strategy

Non-graminaceous plants use a reduction-based method. This method entails converting available Fe^{3+} to Fe^{2+} before it is taken into the plant system through reduction activity. In reduction-based strategy, non graminaceous plant will release protons toward the rhizosphere to decrease the pH in the surrounding soil under Fe-deficient condition. ATPase is thought to be responsible for releasing protons into the rhizosphere and lowering the pH of the surrounding rhizosphere (Kim et al. 2007). The solubility of Fe^{3+} in the rhizosphere will rise as pH drops. In addition, with the help of ferric reductase oxidase 2, NADPH-dependent Fe^{3+}-chelate reductase converts Fe^{3+} into a more soluble form of Fe^{2+}. The iron regulated transporter 1 (*IRT1*)-controlled ferric ion transporter will then transfer Fe^{2+} into the roots (Ishimaru et al. 2006) (**Figure 1**).

Strategy II: An approach based on chelation

Graminaceous plants, such as maize, wheat, and rice, are members of the grass family. These plants use a chelation-based method to improve iron uptake in response to iron deprivation. With the use of soluble siderophores, a chelation-based approach transfers Fe^{3+} from the rhizosphere into the roots. Natural iron chelators, the Mugineic acid (MA) family phytosiderophores have a greater affinity for Fe^{3+} (Morrissey et al. 2009). Different sets of MAs will be delivered by the plant to the surrounding rhizosphere via MA transporter depending on the species (TOM1). Rice, for example, produces 2′-deoxymugineic acid (DMA), but barley produces MA, 3-epihydroxymugineic acid (epi-HMA), and 3-epihydroxy-2′-deoxymugineic acid (epi-HDMA) (Ishimaru et al. 2006). Graminaceous plants produce MAs into the rhizosphere to solubilize sparingly soluble iron in the rhizosphere during iron deprivation. MAs will efficiently bind Fe^{3+}, creating Fe^{3+}-MA complexes. Yellow stripe 1 (*YS1*) transporter will transport the complexes into the root (Ishimaru et al. 2006; Schaaf et al. 2004) **(Figure 1)**.

Rice Iron Uptake Mechanism

For iron uptake, some graminaceous plants, particularly rice, can use a combination of reduction-based and

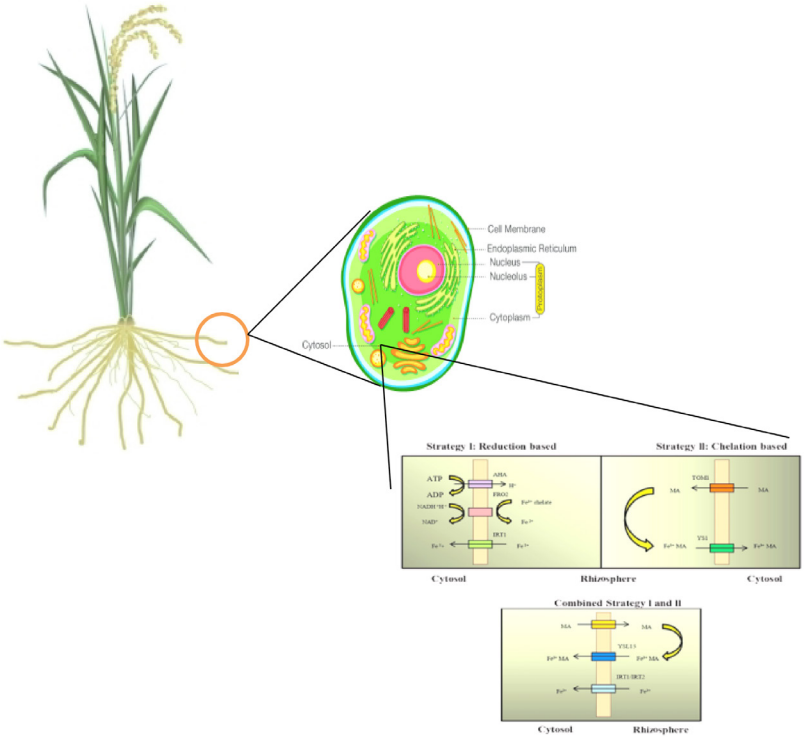

Figure 1: Graminaceous And Non-Graminaceous Plants Have Different Strategies For Iron Uptake.

chelation-based techniques. The rice obtains Fe^{3+} from the environment *via* the strategy I-like system and Fe^{2+} directly from the environment via *IRT1* or *IRT2*. However, as compared to non-graminaceous plants, there is no rise in Fe^{3+}-chelate reductase levels in the roots (Kim et al. 2007). The adaptability of rice when grown in a submerged and anaerobic environment high in Fe^{2+} relative to Fe^{3+} is one possible explanation (Sperotto et al. 2012). MAs will be secreted into the rhizosphere to interact with Fe^{3+}, and the complexes will be carried into the root by YS-like 15 proteins, similar to method II (*YSL15*). Rice, when compared to direct Fe^{2+} uptake, is able to absorb iron from the environment more efficiently through Fe^{3+}-MA complexes (Ishimaru et al. 2006).

Genetic Engineering in Rice

Ferritin is a protein that can store up to 4500 iron atoms in a complex and non-toxic state and is found in almost every organism (Boonyaves, 2017). As a result of the insertion of the ferritin gene, the amount of iron in rice varieties increases. The initial step in iron biofortification is to boost ferritin expression by introducing soybean ferritin (*SoyaferH1* and *SoyaferH2*) genes into rice. Overexpression of genes involved in *mugineic acid* (MA) biosynthesis, such as NA (nicotinamine) production, is a second strategy for improving iron transport in plants. The NAS gene can catalyse the synthesis of nicotinamine from S-adenosyl methionine. NA has been discovered to be a natural metal chelator for Fe (II) and Fe (III) in higher plants, and it is implicated in plant homeostasis and metal translocation. *OSNAS1*, *OSNAS2*, and *OSNAS3* are three nicotianamine synthetase (1, 2 & 3) genes involved in long-distance transportation in plants, and each NAS gene is regulated differently in various parts of the rice plant in response to iron deficiency (De-Xian Kok, 2017). Manipulation of several genes has been successfully accomplished in rice. The phytase gene is being used to boost nutritional content in crops. Antinutrient reduction is a feasible goal, but it should be approached with caution because antinutrients serve critical roles in plant metabolism and human food. Phytase can catalyse the hydrolysis of phytic acid, releasing phosphate and chelated minerals in the process (Weich, 2002). Because of its significant metal cation binding capabilities, phytic acid, for example, can lessen the incidence of colon and breast cancer (De-Xian Kok, 2017). Amino acids can also be implanted in rice by expressing seed-specific genes from beans, such as -phaseolin, pea legumin, soyabean glycinin, and sesame. (Singh Sindhu et al. 1997). Polyunsaturated fatty acids are another vital food that helps to lower harmful cholesterol levels in the body. The expression of the *omega-3* fatty acid desaturase (*FAD-3*) gene increases -linolenic acid (an unsaturated fatty acid) in rice (Anai et al. 2003). Golden rice has been established as a good source of provitamin A (-carotene) and is another notable accomplishment of genetic engineering (Ye & Beyer, 2000). Carotene desaturase and the PSY gene, which

code for a protein, are both expressed, and are thought to regulate disease load (Ye & Beyer, 2000). Normal rice cannot accumulate carotene in its endosperm, but golden rice can. The - carotene biosynthetic pathway was responsible for this transgenic process (Burkhardt et al. 1997) **(Figure 2)**.

Genetic Engineering for Iron Biofortification

Genetic engineering tools have revolutionized molecular research, enabling the creation of transgenic plants and analyzing gene function through gene expression modulation. They include importing gene of interest from other closely-related, RNA interference (RNAi) gene silencing, and overexpression of gene of interest (Abdallah et al. 2015; Abdallah et al. 2015). In comparison to agronomic and conventional plant breeding, genetic engineering methods can provide a more efficient and reliable way for studying the link between genotype and phenotype (Gaj et al. 2013; Yin et al. 2017). As a result, genetic engineering is being favoured as a biofortification method for increasing the iron content of rice grains. Different transgenic procedures (**Table 1**), as well as combinations of transgenic approaches (**Table 2**), have been tried and effectively employed to increase the iron content of rice grain.

Iron storage in rice is improved by ferritin genes

Ferritin is a protein found in all living organisms that can store up to 4500 complex, harmless iron atoms (Theil et al. 2003; Boonyaves et al. 2017). The iron complex in soybean ferritin is readily available for human body absorption via the intestine's iron uptake mechanism (Theil et al. 2011; Lönnerdal et al. 2009). Thus, increasing ferritin expression by introducing soybean ferritin (SoyferH1 and SoyferH2) genes into rice is the initial step in iron biofortification. SoyferH1 and SoyferH2 are the two forms of ferritin proteins found in soybeans, and both ferritin genes are regulated by endosperm-specific promoters (Masuda et al. 2012). When compared to transgenic rice with ferritin genes expression driven by a single endosperm specific promoter (Oryza sativa Globulin (OsGlb) and Oryza sativa Glutelin (OsGluB1) promoter, expression of multiple endosperm specific promoters (Oryza sativa Globulin (OsGlb) and Oryza sativa Glutelin (OsGluB1) promoter (Qu et al. 2005). Overexpression of soybean ferritin in rice, on the other hand, has been demonstrated by a twofold increase in iron concentration in endosperm compared to wild-type rice (Goto et al. 1999; Vasconcelos et al. 2003; Lucca et al. 2002; Oliva et al. 2014; Paul et al. 2012). However, introducing SoyferH2 into rice plants is favoured because SoyferH1 is more vulnerable to protease digestion, causing structural changes, whereas SoyferH2 is more resistant to protease digestion (Masuda et al. 2013; Trijatmiko et al. 2016). Rice plants containing a single soybean ferritin gene did not enhance iron concentration in rice grains, which was surprising (Qu et al. 2005; Masuda et al. 2013). This shows that ferritin gene expression is influenced by soil composition, and that overexpression of ferritin genes as a single transgenic strategy may be ineffective in treating iron shortage (Qu et al. 2005; Lephuthing et al. 2017) **(Table 1)**.

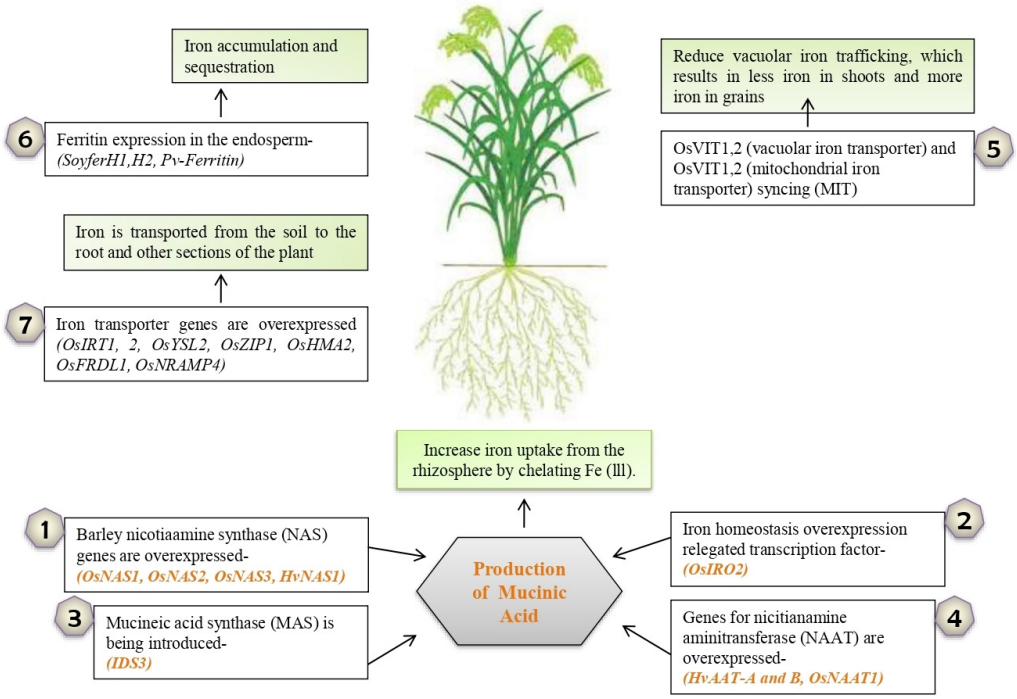

Figure 2. Rice Major Iron Biofortification Strategies.

Table 1. Iron Biofortification Strategy In Rice Focuses On Genes Involved In Iron Storage, Transport, Influx, Uptake, Translocation And Phytic Acid Accumulation.

Approach	Genes-Promoter Used	Rice Species/Cultivars	Fold of Fe Increase	References
Improving iron storage via ferritin genes	OsGluB1 pro- SoyferH1	Japonica cv. Kitaake	1.5 fold (brown grain)	(Qu et al. 2005)
	OsGluB1 pro- SoyferH1	Japonica cv. Kitaake	2 fold (polished grain)	(Goto et al. 1999)
	OsGluA2 pro- Osfer2	Indica cv. Pusa-Sugandh II	2.1 fold (polished grain)	(Paul et al. 2012)
	OsGluB1 pro- SoyferH1	Japonica cv. Taipei 309	2.2 fold (brown grain)	(Lucca et al. 2002)
	OsGluB1 pro- SoyferH1	Indica cv. IR64	3.4 fold (polished grain)	(Oliva et al. 2014)
	OsGluB1 pro- SoyferH1	Indica cv. IR68144	3.7 fold (polished grain)	Vasconcelos et al. 2003)
	OsGluB1 pro soyferH1	Indica cv. BR29	9.2 µg/g of Fe (white)	(Khalekuzzaman et al. 2005)
Enhancing iron transport via NAS gene	35S pro- HvNAS1	Japonica cv. Tsukinohikari	2.3 fold (polished grain)	(Masuda et al. 2009)
	Maize Ubiquitin pro-OsNAS2	Japonica cv. Kitaake	2.9 fold (polished grain)	(Lee et al. 2012)
	Maize Ubiquitin pro-OsNAS3	Japonica cv. Dongjin	2.9 fold (polished grain)	(Lee et al. 2009)
	35S pro- OsNAS1, 2, 3	Japonica cv. Nipponbare	4 fold (polished grain)	(Johnson et al. 2011)
	OsActin 1pro-HvNAS1	Japonica cv. Tsukinohikari	4.5- fold Fe (white)	(Masuda et al. 2009)
Enhancing iron influx via OsYSL2 gene	OsSUT1 pro- OsYSL2	Japonica cv. Tsukinohikari	4.4 fold (polished grain)	(Ishimaru et al. 2010)
Enhancing iron uptake and Translocation via IDS3 gene	35S pro-barley 20-kb IDS3 genome fragment	Japonica cv. Tsukinohikari	1.3 fold (brown grain)	(Suzuki et al. 2008)
	35S pro-barley 20-kb IDS3 genome fragment	Japonica cv. Tsukinohikari	1.4 fold (polished grain)	(Masuda et al. 2008)
Enhancing iron translocation via silencing OsVITs genes	OsVIT1 or OsVIT2 T-DNA insertion line	Japonica cv. Zhonghua11 and Japonica cv. Dongjin	1.4 fold (brown grain)	(Zhang et al. 2012)
	OsVIT2 T-DNA insertion line	Japonica cv. Dongjin	1.8 fold (polished grain)	(Bashir et al. 2013)
Reduction in phytic acid accumulation	Ole 18pro-IPK 1	Indica cv. Pusa sugandhi II	1.8- fold Fe (white)	(Ali et al. 2013)

Iron transport in rice is improved by NAS genes

The second strategy involves overexpressing genes involved in MA production, like nicotianamine synthase, to enhance iron transport in the plant. The synthesis of nicotianamine (NA) from S-adenosyl methionine can be catalysed by NAS (Wirth et al. 2009). NA is a natural metal chelator for Fe (II) and Fe (III) that is found in all higher plants and is involved in metal translocation and homeostasis (Morrissey et al. 2009; Hell 2003; Takahashi et al. 2003; Koike et al. 2004). *OsNAS1*, O*sNAS2*, and *OsNAS3* are the three NAS genes found in rice. These genes are involved in long-distance transportation in plants, and each NAS gene is regulated differently in response to iron deficiency in different areas of the plant (Singh et al. 2017; Inoue et al. 2003). Overexpression of the NAS gene increases MA release into the rhizosphere, resulting in increased iron uptake into the plant via chelation (Wirth et al. 2009; Bashir et al. 2010; Takahashi et al. 2003). Overexpression of rice *OsNAS1*, *OsNAS2*, and *OsNAS3* (Johnson et al. 2011), *OsNAS2* (Lee et al. 2012), *OsNAS3* (Lee et al. 2009), and barley *HvNAS1* (Masuda et al. 2009) genes has been shown to enhance iron content in polished grain by more than threefold **(Table 1)**.

The OsYSL2 gene improves iron influx into seeds

A total of 18 different YSL (yellow stripe-like) genes were identified in rice (Koike et al. 2004). The rice YSL2 (OsYSL2) is the main focus in this approach as this gene plays an important role as a metal-chelator transporter involved in translocation and accumulation of iron in endosperm (Koike et al. 2004; Ishimaru et al. 2010). OsYSL2 was found to be highly expressed in leaves of iron-deficient rice plants in contrast to other parts of the plant where no expression was detected. Therefore, hypothesized that this transporter is involved in long-distance transport of iron-NA complexes via phloem in response to iron deficiency in rice plant (Koike et al. 2004). Consistently, it was discovered that the iron influx into the rice endosperm could be controlled through iron nicotianamine transporter OsYSL2 (Schroeder et al. 2013). In comparison to wild-type rice, (Ishimaru et al. 2010) successfully demonstrated that disrupting the OsYSL2 gene in rice reduced the iron concentration in both brown rice and polished grain by 18 and 39 percent, respectively, with higher iron buildup in roots. Furthermore, with increased expression of OsYSL2 using the rice sucrose transporter (OsSUT1) promoter, (Ishimaru et al. 2010) were able to increase the iron content in rice grain up to 4-fold in polished grain. However, OsYSL2 overexpression has the opposite

Table 2. Multiple Transgenes Were Combined For Iron Biofortification In Rice.

S.No.	Genes-Promoter Used	Rice Species/Cultivars	Fold of Fe Increase	References
1.	OsGlb1 pro-Pvferritin 35S pro-AtNAS1 OsGlb pro-Afphytase	Japonica cv. Taipei 309	6.3 fold (polished grain)	(Wirth et al. 2009)
2.	OsGluB1 pro-SoyferH2 OsGlb1 pro-SoyferH2 HvNAS1, HvNAAT-A,-B and IDS3 genome fragments	Japonica cv. Tsukinohikari	4 fold (polished grain)	(Masuda et al. 2013)
3.	MsENOD12B pro-AtIRT1 OsGlb1 pro-Pvferritin 35S pro-AtNAS1 OsGlb pro-Afphytase	Japonica cv. Taipei 309	4.3 fold (polished grain)	(Boonyaves et al. 2016)
4.	Native AtIRT1 pro-AtIRT1 OsGlb1 pro-Pvferritin 35S pro-AtNAS1 OsGluB1 pro-SoyferH2	Japonica cv. Nipponbare	4.7 fold (polished grain)	(Boonyaves et al. 2017)
5.	*OsGlb1* pro-*SoyferH2* *OsAct* pro-*HvNAS1* *OsSUT1* pro-*OsYSL2* *OsGlb1* pro-*OsYSL2*	Japonica cv. Tsukinohikari	6 fold (brown grain)	(Masuda et al. 2012)
6.	*OsGluB1* pro-*SoyferH2* *OsGlb1* pro-*SoyferH2* *OsAct* pro-*HvNAS1* *OsSUT1* pro-*OsYSL2* *OsGlb1* pro-*OsYSL2*	Tropical *Japonica* *cv*. Paw San Yin	3.4 fold (polished grain)	(Aung et al. 2013)
7.	*GluA2* pro-*SoyferH1* *CaMV35S* pro-*OsNAS2*	Idiaca *cv*. IR64	6 fold (polished grain)	(Trijatmiko et al. 2016)

impact of OsYSL2 gene silencing in transgenic rice, leading to a higher iron content in the roots than in the rice grains and shoots. Without a doubt, expressing OsYSL2 under the control of the OsSUT1 promoter is a potential strategy for iron biofortification of rice grains. **(Table 1)**.

Iron uptake and translocation are improved by the IDS3 gene

MAs, as noted in Section 2.2, are natural iron chelators that form complexes with iron to aid in the transfer of iron from the rhizosphere into the plant. Different sets of MAs genes were discovered in barley, indicating that it has the ability to synthesis various forms of MAs via the MAs biosynthetic pathway (Bashir et al. 2006; Masuda et al. 2013). Furthermore, the presence of iron deficiency specific clones no. 2 (IDS2) and no. 3 (IDS3) in barley helps to battle iron deficiency (Masuda et al. 2013). In response to iron deprivation, the IDS genes promote the production of several types of MAs via DMA, and these genes are strongly expressed in roots (Kobayashi et al. 2001). Rice, on the other hand, lacks the ability to synthesis other forms of MAs besides DMA since it lacks both the IDS2 and IDS3 genes. When opposed to rice, barley has a different collection of MAs that allows it to be more tolerant of iron deficiency. The IDS3 gene from barley allows transgenic rice to synthesis and secretes various types of MAs into the rhizosphere (Kobayashi et al. 2001). In addition, formation of Fe (III)-MA complex has a better stability as compared to Fe (III)-DMA complex when grown in a slightly acidic soil (Von Wirén et al. 2000). This could improve iron translocation in rice, reducing iron insufficiency and boosting rice plant tolerance to iron deficiency. Furthermore, the synthesis of Fe (III)-MA complex is more stable than Fe (III)-DMA complex when grown in a slightly acidic soil (Von Wirén et al. 2000). Furthermore, (Masuda et al. 2008; Suzuki et al. 2008) found that when cultivated in either Fe-sufficient or Fe-deficient soil, IDS3 rice lines may raise Fe concentrations by 1.4 and 1.3-fold for both polished and brown grains, respectively, compared to wild-type rice. As a result, the presence of the IDS3 gene can increase iron accumulation in rice grain even when grown in iron-deficient soil, as well as increase tolerance to iron deficiency. **(Table 1)**.

Iron translocation is aided by the OsVIT gene

The functional characterization of rice vacuolar iron transporter genes was published by Zhang et al. in 2012 (OsVIT1 and OsVIT2). These genes were discovered to be expressed at low levels throughout the plant, but substantial levels of expression of OsVIT genes were found in the flag leaves. These genes are involved in the tonoplast-mediated transfer of Zn^{2+} and Fe^{2+} into vacuoles (Kim et al. 2006). Furthermore, knocking down the OsVIT genes

greatly increases Fe and Zn accumulation in rice grains while decreasing Fe and Zn accumulation in flag leaves (Bashir et al. 2013). OsVIT1 and OsVIT2 gene knockouts resulted in a 1.4-fold increase in iron concentration in rice grain (Zhang et al., 2012; Bashir et al., 2013). This method, however, can only be used if the transgenic rice is grown in unpolluted soil. This is because studies have shown that when rice is planted in polluted soil, it accumulates Cd^{2+} concentrations (Zhang et al. 2012) (**Table 1, 2**).

Combination of several transgenic

Rice has undergone numerous gene manipulations. By introducing Arabidopsis thaliana NAS1 (AtNAS1), Phaseolus vulgaris ferritin (Pvferritin), and Aspergillus fumigatus phytase (Afphytase) genes into rice, (Wirth et al. 2009) demonstrated the synergism of three separate genes expression with a 6-fold increase in iron content in rice. The primary goal of adding phytase genes into rice is to lower the iron antinutrient phytate. Some foods contain antinutrients such as Phytic acid (PA), which binds tightly to metal cations like iron and zinc, rendering them insoluble (White et al. 2005). Phytase can catalyse the hydrolysis of Phytic acid (PA), which releases phosphate and chelated minerals (Welch et al. 2002). The enzyme responsible for the degradation of such components is missing from the human digestive system (Wirth et al. 2009). Antinutrient reduction is a viable strategy for increasing nutrient content in crops, but it should be used with caution because many antinutrients serve critical functions in plant metabolism and human diet (Welch et al. 2002; Velu et al. 2014). Antinutrients play a role in plant resistance to pests, diseases, and abiotic stress while also acting as anticarcinogens in human diets (Saied et al. 1998; Zhou et al. 2009; Chrispeels et al. 1991; Zajdel et al. 2013). PA, for example, can lessen the incidence of both colon and breast cancer due to its high metal cation binding capacities (Thompson et al. 1991; Zhou et al. 2009). Furthermore, PA has antioxidant properties by inhibiting the generation of iron-mediated hydroxyl radicals ($^{\cdot}OH$) in food and the gastrointestinal tract, which would cause lipid peroxidation and tissue damage (Zhou et al. 2009; Chrispeels et al. 1991). Antinutrient lectin, on the other hand, was shown to be responsible for plant defence by demonstrating cytotoxic actions when consumed by pests and animals (Zajdel et al. 2013). Introducing a mixture of various genes involved for MA synthesis into rice (Fer-NAS-NAAT-IDS3 lines) resulted in a 4-fold increase in iron accumulation in endosperm, according to (Masuda et al. 2013) (**Table 2**).

Similarly, a transgenic line expressing AtIRT1, Pvferritin, AtNAS1, and Afphytase was found to increase iron buildup in polished grain by fourfold (Boonyaves et al. 2017; Boonyaves et al. 2016). The OsYSL15 or OsIRT1 genes are mostly expressed in roots, and their expression is increased in response to iron deprivation (Boonyaves et al. 2016). The Fe^{2+} transporter, which is engaged in both strategies I and II, is encoded by the OsIRT1 gene. Although overexpression of OsIRT1 alone can raise iron content in rice grain by 1.3-fold, when combined with other genes, OsIRT1 has the ability to increase iron content even more (Lee et al. 2009). Combination techniques, on the other hand, have been shown to boost the iron content in rice grain by 3.4 and 6 times when introduced into Myanmar and Japanese rice cultivars, respectively (Masuda et al. 2012; Aung et al. 2013). Because the vector inserted comprises two gene cassettes for each gene expression driven by distinct promoters for each gene cassette, both SoyFerH2 and OsYSL2 were highly expressed in transgenic rice (OsSUT1 promoter-OsYSL2, OsGlb promoter- OsYSL2, OsGluB1 promoter-SoyferH2, OsGlb promoter-SoyferH2). Intriguingly, (Trijatmiko et al. 2016) were able to develop transgenic rice that expressed the OsNAS2 and SoyferH1 genes, resulting in a 15g Fe/g (6-fold) increase in polished grain. The transgene construct was discovered to be inserted with inverted repetitions at a single region in the transgenic rice line. This means that several transgene insertions were successful in increasing the iron content of rice (Masuda et al. 2012; Trijatmiko et al. 2016; Aung et al. 2013). However, due to the possibility of epigenetic silencing in transgenic plants, a transgene cassette with duplicated or inverted transgene repeats may not be stable and inherited after several generations (Boonyaves et al. 2017; Kumpatla et al. 1998; Tang et al. 2006; Rajeev et al. 2015). As a result, more research is needed to determine the stability of transgenes or other approaches to maintaining many transgenes through multiple generations.

Rice with low phytates produced using RNAi technology

With the exception of maize, roughly 80% of total phytic acid is accumulated in the aleurone layer of the grains in most cereals. Phytate, a mixture of salts, increases as phytic acid accumulates. Because phytate contains six negatively charged ions, it is an effective chelator of divalent cations such as Fe^{2+}, Zn^{2+}, Ca^{2+}, and Mg^{2+}, lowering their bioavailability. (See Figure 3) Many attempts have been undertaken to lower phytic acid levels in rice by creating mutant types with a low phytic acid phenotype (lpa) (Larson et al. 2000; Kim et al. 2008) (**Figure 3) .**

Although these mutant lines are effective, they negatively affect crop output and overall performance. Transgenic crops were produced as an alternate technique by silencing critical enzymes in the phytic acid biosynthesis pathway using RNA interference (RNAi) (Karmakar et al. 2019; Ali et al. 2010; Kuwano et al. 2009). The enzyme myo-inositol-3-phosphate synthase (MIPS) catalyses the initial step of phytic acid production in rice seeds. Although seed-specific

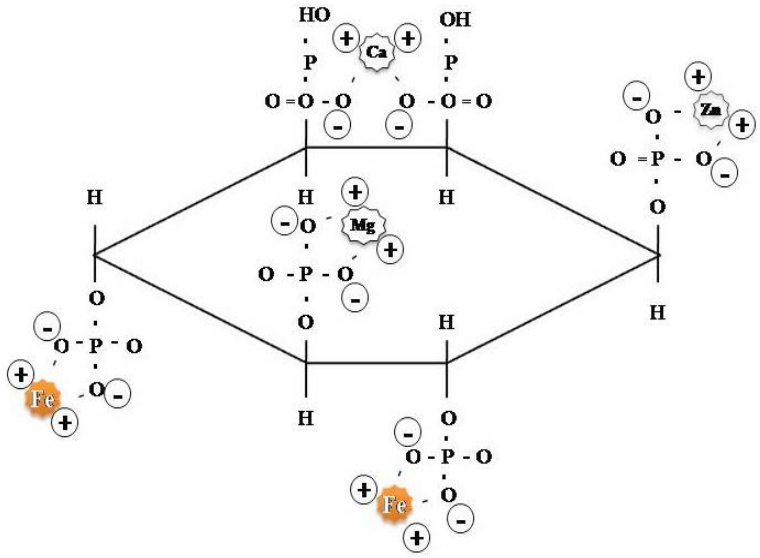

Figure 3. Phytic Acid As A Chelator For Iron (Fe^{2+}) And Other Divalent Cations.

promoters such as *Glutelin B-1* (*GluB-1*) and *Oleosin 18* (*Ole18*) are favoured for maximum phytate formation in seed, as reported by (Feng et al. 2004), this enzyme was targeted for silencing in rice using the *CaMV35S* promoter (Kuwano et al. 2009; Kuwano et al. 2004; Ali et al. 2013). Because MIPS are a precursor for the de novo synthesis of myo-inositol, rice seeds showed a shift in myo-inositol levels after MIPS silencing. As a result, enzymes involved later in phytic acid biosynthesis in rice should be targeted to reduce phytate concentration in seeds without disrupting related important pathways (Ali et al. 2013). Ali et al. (2013) developed a Pusa Sugandhi II (PSII) indica rice cultivar by manipulating the expression of the final step key enzyme inositol-1,3,4,5,6-pentakisphosphate 2-kinase. The transgenic seeds had a 3.85-fold reduction in IPK1 transcripts, which correlated to a significant reduction in phytate levels and an increase in the amount of inorganic phosphate (Pi), and accumulated 1.8-fold more iron in the endosperm without hindering transgenic rice plant growth and development. Seed-specific RNAi-mediated gene silencing of an inositol triphosphate kinases (ITPK) homolog (OsITP/6K-1) resulted in a 46.2 percent reduction in phytic acid in the Khitish indica rice variety, with a 1.3-fold increase in iron accumulation, 1.6-fold zinc, and 3.2-fold Pi bioavailability (Karmakar et al. 2019). Ferritin overexpression in rice plants for the production of 'high iron' rice has been researched via phenotypic and agronomic, the long-term effect of RNAi-mediated suppression of the phytic acid system. Iron buildup in seed and seed morphology were evaluated after Genetically modified (GM) rice plants were maintained for numerous generations to establish homozygous plant lines. There was no change in seed structure in high iron rice. Ferritin overexpression in rice plants for the production of 'high iron' rice has been researched using phenotypic and agronomic performance, the long-term effect of RNAi-mediated suppression of the phytic acid system. Multiple generations of GM rice plants were grown to establish homozygous plant lines and iron buildup in seed and seed.

Phytic acid-bound iron liberation

Expression of fungal (i.e., Aspergillus fumigatus) phytases enzyme in rice is a viable biofortification technique as an alternative to silencing of phytic acid metabolism genes. Phytase catalyses the hydrolysis of phytic acid (phytate), releasing chelated minerals such as Fe^{2+}, Zn^{2+}, Ca^{2+}, and Mg^{2+}, as well as phosphate, resulting in increased mineral bioavailability (Welch et al. 2002). Thermo-tolerant properties of a fumigatus phytase (Afphytase) make it more appropriate for food processing and biofortification of staple crop-related applications (Pasamontes et al. 1997). (Wirth et al. 2009; Boonyaves et al. 2016; Boonyaves et al. 2017) inserted the Afphytase gene into rice, and the resulting GM rice demonstrated enhanced iron buildup in polished grain. Overexpression of the E. coli app A (phytase) gene in the Khitish (indica) rice cultivar resulted in a two fold increase in iron and threefold rise in zinc accumulation in rice seed, as well as a fourfold increase in inorganic phosphorus (Pi) (Bhattacharya et al. 2019). Afphytase was introduced into the rice endosperm along with a rice cysteine-rich metallothionein-like protein (to improve iron absorption) (Lucca et al. 2001). Cysteine aids non-haem iron absorption (Taylor et al. 1986), and each metallothionein (MT) molecule is said to contain a lot of it (20 of 60 amino acids in mammalian MTs (Kagi et al. 1988) and 12 of 74 in plant MTs (Hsieh et al. 1995). The cysteine concentration of seed protein can be raised in grains by endosperm-specific overexpression of MT in rice, resulting in improved

iron bioavailability. The phytase content in the created GM Taipei-309 rice was 130-fold higher (in the grains), and it showed 100% phytic acid breakdown in a test (simulated digestion).

CONCLUSION

Modern breeding offers a complementary technique to investigate and generate Fe-rich rice resources by using Fe absorption, transit, and storage genetic engineering. The current transgenic techniques and mechanisms for Fe bioreinforcement in crops are generalized based on developments in Fe absorption, transport, storage, regulation, and homeostasis. Overexpression of the transporter gene OsIRT1 increased Fe concentrations in rice leaves and grains by 1.7 and 1.1 times, respectively. Rice grain Fe reinforcement may be required to improve its absorption and transport ability, as well as to augment the tissue-specific storage capacity of Fe. Chloroplast-derived ferritin can be expressed in rice endosperm using transgenic technology, which is an effective technique to increase the Fe storage capacity of rice endosperm. Certain techniques, such as upregulating the induced Fe chelator production pathway and regulating and optimising inter-/intracellular transport and storage, have been investigated to increase Fe uptake and transport capacity in overall plants. The upregulation of Fe chelator synthesis NA and PS in rice plants is promoted by overexpression of NAS constitutive and PS synthesis genes, which enhances the grain Fe concentration in polished rice by two to four times. The iron deficiency specific clone 3 (IDS3) genes from cereals can be expressed in rice, causing activation of Fe transport system genes and a 1.4-fold increase in grain Fe level in polished rice. Rice plants with OsYSL15 and OsYSL2 overexpression driven by the promoter of the action or sucrose transporter gene had higher YSL expression in diverse tissues, resulting in a 1.2- to 4.0-fold increase in grain Fe content. RNA interference (RNAi) reduces OsYSL9 expression in rice plants, which also slows Fe transit from the endosperm to the embryo, raising the embryonic Fe concentration. Regulating Fe intracellular transport in rice by lowering OsVIT1 or OsVIT2 expression promotes inter-tissue Fe movement from flag leaves to grains, resulting in an increase in endosperm Fe content. Fe homeostasis regulators in rice plants also have an indirect effect on the Fe transport and accumulation network. The transcription factor Fe-related transcription factor 2 (OsIRO2) regulates the production of Fe chelate, MA, and the Fe-MA transport pathway, for example. A gene-combination technique related to effective Fe transport and storage has yielded a more significant endosperm Fe increase in rice than a single-gene strategy. The CRISPR approach of knocking down the OsVIT2 gene can enhance the quantity of Fe in grains. Studies to lower inositol 1,3,4,5,6-pentakisphosphate 2-kinase (IPK1) gene expression by RNAi have been done since the introduction of RNAi-mediated gene silencing technology.

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