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Iron Biofortification of Rice: Progress and Prospects

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<http://dx.doi.org/10.5772/intechopen.73572>

Abstract

Biofortification is the process of improving the bioavailability of essential nutrients in food crops either through conventional breeding or modern biotechnology techniques. Rice is one of the most demanding staple foods worldwide. Most global population live on a diet based on rice as the main carbohydrate source that serve as suitable target for biofortification. In general, polished grain or white rice contains nutritionally insufficient concentration of iron (Fe) to meet the daily requirements in diets. Therefore, iron biofortification in rice offers an inexpensive and sustainable solution to mitigate iron deficiency. However, understanding on the mechanism and genes involved in iron uptake in rice is a prerequisite for successful iron biofortification. In this chapter, the overview of iron uptake strategies in plants and as well as different iron-biofortified approaches used in rice will be outlined. Then, the challenges and future prospects of rice iron biofortification to improve global human health will also be discussed.

Keywords: agronomic practices, conventional plant breeding, genetic engineering, iron biofortification, *Oryza sativa*, transgenic rice

1. Introduction

Rice is one of the most consumed staple foods worldwide. In developing countries, people often rely on rice as their sole source of nutrition [1]. However, polished grain, known as white rice, contains limited amount of essential nutrients to sustain a good health and development [2]. Hence, those who are incapable to afford other micronutrients-rich nonstaple food for their balance diet are often at the highest risk for micronutrients deficiencies [3].

Iron deficiency is a common health disorder affecting nearly 2 billion people worldwide with other mineral and vitamin deficiency [4, 5]. Common effects of iron deficiency include anemia and impaired growth development in pregnant women and preschool children [6]. It can be easily addressed through dietary diversification, micronutrient supplements, medicines, and surgery depending of the severity of the condition [5, 7]. However, such treatments may not be available to everyone due to limitations such as geographical and financial capabilities [4]. In addition, iron is the most difficult mineral to be used in food fortification because the most soluble and absorbable compounds (e.g., FeSO_4) alters the taste or color of fortified food making it unappetizing while the least soluble compounds (e.g., $\text{Fe}_4(\text{P}_2\text{O}_7)_3$) are poorly absorbed by human body [8, 9]. Hence, food fortification is not a sustainable solution to mitigate iron deficiency.

Government bodies and nonprofit organization could play an important role to combat micronutrient deficiency by providing adequate food, supplements, and medicine supplies to rural areas. Nevertheless, it may not be an effective long-term solution because it is highly dependent on continuous investment, appropriate infrastructures, and transportation [10]. Hence, an alternative solution through biofortification is seen to be more efficient and cost-friendly in mitigating to micronutrient deficiency.

Iron biofortification, the process of improving the bioavailability of iron in food crops can be achieved via agronomic practices, conventional breeding, and genetic engineering. Biofortification through agronomic practices can be performed through fertilizer or foliar feeding [11]. Agronomic practices need to take bioavailability of iron at different stages into account as not all of the nutrients are transferred [12]. Several crucial factors may contribute to the nutrient loss at different stages such as bioavailability of nutrient uptake from the soil, nutrient distribution in different parts of the plants, milling or dehusking during food processing, and the ability of human to absorb and utilize the nutrients [13]. These factors need to be considered carefully to ensure successful iron biofortification through agronomic practices.

Meanwhile, conventional plant breeding involves identifying and selection of parent line, which contains desirable traits found in both parent plants. Parent lines are then crossed over for a few generations until daughter plants with both desirable nutrient and agronomic traits are observed and selected [14]. For instance, iron bean is one of the successful products through conventional plant breeding with high iron content, high bioavailability, and high yield [15, 16]. In addition, the advancement of modern biotechnology techniques, such as marker-assisted selection, improves the efficiency and precision in identification of potential lines in daughter plants [17].

To date, with the recent advancement of genetic engineering technologies served as a platform, which inspires many researchers in exploring alternative solution through genetic modification. Genetic engineering involves in removing, altering, or inserting specific sequence into the plant genome, which provides a better flexibility by silencing or overexpressing desirable gene sequences for desirable traits [18, 19]. Genetic engineering is an excellent method to obtain desirable micronutrient levels in a more effective manner by targeting specific gene of interest. However, successful biofortification via genetic engineering requires extensive knowledge and understanding of iron uptake, trafficking, and homeostasis mechanisms in plants to prevent undesirable side effects.

2. Iron uptake strategies in plants

Plants acquire iron mainly from the rhizosphere. There are abundant of iron in the soil, but only minute quantities of iron are absorbed by the plants. The availability of iron is dependent on the soil pH and soil redox potential [7]. Iron becomes less soluble in higher pH and it can be found in the form of insoluble ferric oxides. In contrast, iron becomes more soluble in low pH and they can be readily absorbed by the plant roots [20].

Micronutrient uptake and distribution in plants are heavily controlled and regulated by different uptake strategies. This allows the required amount of micronutrients to be absorbed into the plant but not high enough to exhibit toxicity effect [21]. Similarly for iron uptake in plants, there are two strategies used for iron uptake from the soil, namely reduction-based strategy and chelation-based strategy [22, 23]. Graminaceous plants are able to utilize chelation-based strategy while nongraminaceous plants utilize reduction-based strategy. However, rice is able to utilize combination of both reduction-based and chelation-based strategies as shown in **Figure 1** [22, 23].

2.1. Strategy I: reduction-based strategy

Reduction-based strategy is utilized by nongraminaceous plants. This strategy involves reducing available Fe^{3+} through the reduction activity into Fe^{2+} before being absorbed into the plant system. In reduction-based strategy, nongraminaceous plant will release protons toward the rhizosphere to decrease the pH in the surrounding soil under Fe-deficient condition. Kim [20] suggested that ATPase are responsible for releasing protons into the rhizosphere and reducing the pH of surrounding rhizosphere. The decrease in pH will increase the solubility of Fe^{3+} in the rhizosphere. In addition, NADPH-dependent Fe^{3+} -chelate reductase reduces Fe^{3+} into a more soluble form of Fe^{2+} with the help of ferric reductase oxidase 2 (FRO2). Then, Fe^{2+} will be transported into the roots via ferric ion transporter controlled by iron regulated transporter 1 (IRT1) [22].

2.2. Strategy II: chelation-based strategy

Grasses families such as maize, wheat, and rice are known as graminaceous plants. In response to iron deficiency, these plants are able to increase iron uptake through chelation-based strategy. Chelation-based strategy transports Fe^{3+} from rhizosphere into the roots with the help of soluble siderophores. Mugineic acid (MA) family phytosiderophores are natural iron chelators and they have a higher affinity toward Fe^{3+} [7]. Depending on different species, different sets of MAs will be released by the plant to surrounding rhizosphere via transporter of MAs (TOM1). For instance, rice will secrete only 2'-deoxymugineic acid (DMA), while barley secretes different types of MA such as MA, 3-epihydroxymugineic acid (epi-HMA), and 3-epihydroxy-2'-deoxymugineic acid (epi-HDMA) [22]. During iron deficiency, graminaceous plants will secrete MAs into the rhizosphere to solubilize sparingly soluble iron in rhizosphere. MAs will bind Fe^{3+} efficiently forming Fe^{3+} -MA complexes. The complexes will be transported into the root via yellow stripe 1 (YS1) transporter [22, 24].

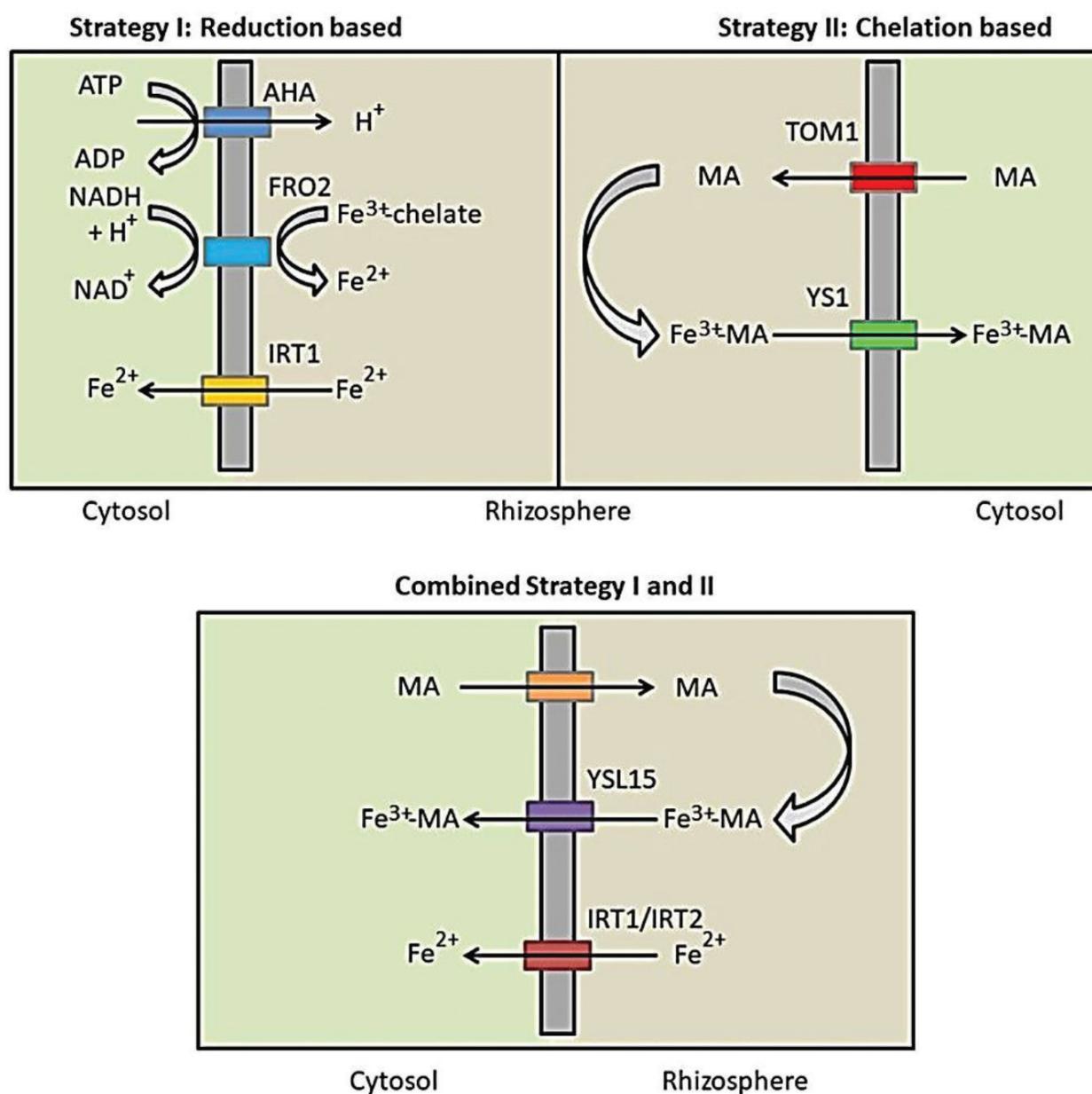


Figure 1. Iron uptake strategy by graminaceous plants and nongraminaceous plants.

2.3. Iron uptake mechanism in rice

Some graminaceous plants, in particular rice, can undergo combined strategies of reduction-based strategy and chelation-based strategy for iron uptake. Rice acquires Fe³⁺ via strategy I-like system and Fe²⁺ directly from the surroundings via IRT1 or IRT2. However, there is no increase in Fe³⁺-chelate reductase levels detected in the roots as compared to nongraminaceous plants [20]. Possible explanation is that adaptation of rice when grown in submerged and anaerobic environment rich in Fe²⁺ compared to Fe³⁺ [10]. Similarly to strategy II, MAs will be secreted into the rhizosphere to bind with Fe³⁺ and the complexes will be transported into the root via YS-like 15 (YSL15). Between both strategies, rice is able to uptake iron from the surrounding more efficiently through Fe³⁺-MA complexes as compared to direct Fe²⁺ uptake [22].

3. Iron biofortification via agronomic practices

Agronomic biofortification is a traditional biofortification approach, which involves micronutrient uptake from the surrounding soil and translocation into the edible parts of the plants. Effective agronomic biofortification are determined by various factors due to the potential nutrient loss during the transition at different stages such as from the soil to the plants, plants to food, and finally to humans [13, 25, 26]. Soil conditions such as pH, soil composition, aeration, and moisture are important for iron availability and uptake in plants [13, 27]. As mentioned in Section 2.1, higher plants are able to release protons to the surrounding soil to increase iron solubility and pH of surrounding rhizosphere in order to enhance iron availability and uptake. Similarly through soil management, properties of the soil could be altered to increase iron availability and uptake in plants by utilizing organic wastes such as plant residues and animal manure [27, 28]. Besides, organic wastes is able to enhance the soil properties, nutrient bioavailability, cation exchange capacity, and water holding capacity while providing a constant and slower nutrient release [13, 29]. However, application of organic wastes alone is insufficient to mitigate iron deficiency and it requires combination application with iron fertilizer [13].

Iron availability in the soil can be enhanced through fertilizer application onto the soil or foliar feeding application directly onto the leaves of the crop. Iron fertilizer via foliar feeding enhance iron uptake and efficient translocation into rice as compared to soil fertilizer [30–32]. However, the fertilizers are often removed by the rain and they require reapplication each time after raining, which are costly and dangerous to the environment [13, 33]. Conversely, the application of iron fertilizer through the soil is inefficient due to strong binding between iron and the soil, which reduces iron uptake efficiency in plants [13, 15].

In addition, macronutrient also plays a crucial role in iron biofortification in plants. Previously, several studies on positive interactions between iron and zinc concentration in grains with nitrogen, phosphorus, and potassium (NPK) fertilizer have been reported [10, 27, 32, 34]. The presence of nitrogen alone was reported to increase iron content in brown rice by 15% and addition of potassium is able to further increase the iron content in rice grain [35]. This is because nitrogen and phosphorus are involved in root development, shoot transport and re-localization, which improves the translocation of iron into rice grain [13, 15, 27, 33]. On the other hand, the presence of phosphorus is able to reduce toxicity in plants at the cost of reduce uptake of both iron and zinc uptake in plant due to dilution effect [13]. Hence, combined application of both NPK fertilizer and iron fertilizer could be a potential approach to increase iron bioavailability in rice [13].

4. Iron biofortification via conventional plant breeding

Conventional plant breeding has been practiced for centuries to improve the properties of food crops by identifying and developing parent plants with desired characteristics, crossing the parent plants, and selecting offspring with desired agronomic traits inherited from both parent plants [14]. An example of a product developed via plant breeding is high iron rice variety (IR68144) with high yield, disease tolerant, good tolerant to mineral deficient, and excellent seed vigor. The IR68144 rice variety was developed through crossing between

semi dwarf rice cultivar, IR8 and Taichung (Native)-1. Meanwhile, IR8 is a product developed through crossing between Chinese dwarf rice variety “Dee-Geo-woo-gen” (DGWG) and Indonesia high yield rice variety “Peta” [36]. The Taichung (Native)-1 is a product of crossing between DGWG and a traditional tall variety ‘Tsai-Yuan-Chung’, which produces high yield and dwarf variety. Crossing between IR8 and Taichung (Native)-1 allows the development of new rice cultivar, which is semi-dwarf and contains high yield properties [37]. This rice variety is able to produce 21 $\mu\text{g/g}$ (2-fold) of iron concentration in brown rice [35]. In addition, IR68144 is able to retain most of the iron content (approximate 80%) after polishing for 15 minutes compared to other varieties [10]. Furthermore, consumption of IR68144 was reported to have improvement in iron status of women [38]. This rice cultivar can serve as a stepping stone for further transgenic enhancement [10].

Even though conventional breeding is able to develop high yield and semi dwarf IR68144, this approach alone in iron biofortification is insufficient in developing a sustainable agronomic plant in terms of yield and quality [39]. This is due to the possibility of inheriting undesirable traits from the parent line as the selection process is done based on the phenotypes and new traits can only be developed after performing extensive back crossing or wide crossing [40]. For instance, low phytic acid (PA) maize mutant (*lpa241*) has demonstrated its ability to reduce PA concentration by 90% in exchange of reduced germination rate by 30% [41]. Hence, conventional breeding is best coupled with other approach such as genetic engineering and agronomic practices to enhance iron content in grains [32, 37, 42, 43].

5. Iron biofortification via genetic engineering

The advancement of genetic engineering technologies allows the advancement in molecular field including the development of transgenic plants. Characterization and analysis of gene function are performed via genetic engineering by the manipulation of gene expression. These include introducing gene of interest from other closely-related organism, RNA interference (RNAi) gene silencing, and overexpression of gene of interest [18]. Genetic engineering technologies is able to provide a more efficient and reliable method to study the relationship between genotype and the phenotype as compared to agronomic and conventional plant breeding [44, 45]. As a result, genetic engineering is preferred as an alternative for biofortification to increase the iron content in rice grains. There are five different transgenic approaches (Table 1) and as well as combination of different transgenic approaches (Table 2), which have been attempted and successfully used to enhance the iron content in rice grain.

5.1. Enhancement of iron storage in rice via *ferritin* genes

Ferritin is an iron storage protein ubiquitously present in most organisms, which is capable to store up to 4500 iron atoms in a complex and nontoxic form [65, 66]. Iron complex in soybean ferritin is readily available for human body absorption via iron uptake mechanism in the intestine [46, 62]. Thus, the first approach in iron biofortification is to enhance the expression of ferritin by introducing soybean *ferritin* (*SoyferH1* and *SoyferH2*) genes into rice.

Approach	Genes-promoter used	Rice cultivar	Fold of Fe increase	References
Improving iron storage via <i>ferritin</i> genes	<i>OsGluB1</i> pro- <i>SoyferH1</i>	<i>Japonica</i> cv. Kitaake	1.5 fold (brown grain)	[48]
	<i>OsGluB1</i> pro- <i>SoyferH1</i>	<i>Japonica</i> cv. Kitaake	2 fold (polished grain)	[49]
	<i>OsGluB1</i> pro- <i>SoyferH1</i>	<i>Japonica</i> cv. Taipei 309	2.2 fold (brown grain)	[51]
	<i>OsGluB1</i> pro- <i>SoyferH1</i>	<i>Indica</i> cv. IR68144	3.7 fold (polished grain)	[50]
	<i>OsGluB4</i> pro- <i>SoyferH1</i>	<i>Indica</i> cv. IR64	3.4 fold (polished grain)	[64]
	<i>OsGluA2</i> pro- <i>Osfer2</i>	<i>Indica</i> cv. Pusa-Sugandh II	2.1 fold (polished grain)	[67]
Enhancing iron transport via <i>NAS</i> gene	35S pro- <i>OsNAS1, 2, 3</i>	<i>Japonica</i> cv. Nipponbare	4 fold (polished grain)	[2]
	Maize <i>Ubiquitin</i> pro- <i>OsNAS2</i>	<i>Japonica</i> cv. Kitaake	2.9 fold (polished grain)	[54]
	Maize <i>Ubiquitin</i> pro- <i>OsNAS3</i>	<i>Japonica</i> cv. Dongjin	2.9 fold (polished grain)	[55]
	35S pro- <i>HvNAS1</i>	<i>Japonica</i> cv. Tsukinohikari	2.3 fold (polished grain)	[74]
Enhancing iron influx via <i>OsYSL2</i> gene	<i>OsSUT1</i> pro- <i>OsYSL2</i>	<i>Japonica</i> cv. Tsukinohikari	4.4 fold (polished grain)	[75]
Enhancing iron uptake and translocation via <i>IDS3</i> gene	35S pro-barley 20-kb <i>IDS3</i> genome fragment	<i>Japonica</i> cv. Tsukinohikari	1.4 fold (polished grain)	[58]
	35S pro-barley 20-kb <i>IDS3</i> genome fragment	<i>Japonica</i> cv. Tsukinohikari	1.3 fold (brown grain)	[78]
Enhancing iron translocation via silencing <i>OsVITs</i> genes	<i>OsVIT1</i> or <i>OsVIT2</i> T-DNA insertion line	<i>Japonica</i> cv. Zhonghua11 and <i>Japonica</i> cv. Dongjin	1.4 fold (brown grain)	[59]
	<i>OsVIT2</i> T-DNA insertion line	<i>Japonica</i> cv. Dongjin	1.8 fold (polished grain)	[80]

Table 1. Iron biofortification approach in rice targeting genes responsible for iron storage, iron transport, iron influx, iron uptake and translocation.

In soybean, there are two types of ferritin proteins, known as *SoyferH1* and *SoyferH2*, and both *ferritin* genes are controlled by endosperm specific promoters [47]. However, expression of multiple endosperm specific promoters (*Oryza sativa* Globulin (*OsGlb*) and *Oryza sativa* Glutelin (*OsGluB1*) promoters) did not produce a significant increase of iron concentration in

Genes-promoter used	Rice cultivar	Fold of Fe increase	References
<i>OsGlb1</i> pro- <i>Poferritin</i> 35S pro- <i>AtNAS1</i> <i>OsGlb</i> pro- <i>Afphtase</i>	<i>Japonica</i> cv. Taipei 309	6.3 fold (polished grain)	[23]
<i>OsGluB1</i> pro- <i>SoyferH2</i> <i>OsGlb1</i> pro- <i>SoyferH2</i> <i>HvNAS1</i> , <i>HvNAAT-A,-B</i> and <i>IDS3</i> genome fragments	<i>Japonica</i> cv. Tsukinohikari	4 fold (polished grain)	[77]
<i>MsENOD12B</i> pro- <i>AtIRT1</i> <i>OsGlb1</i> pro- <i>Poferritin</i> 35S pro- <i>AtNAS1</i> <i>OsGlb</i> pro- <i>Afphtase</i>	<i>Japonica</i> cv. Taipei 309	4.3 fold (polished grain)	[86]
Native <i>AtIRT1</i> pro- <i>AtIRT1</i> <i>OsGlb1</i> pro- <i>Poferritin</i> 35S pro- <i>AtNAS1</i>	<i>Japonica</i> cv. Nipponbare	4.7 fold (polished grain)	[66]
<i>OsGluB1</i> pro- <i>SoyferH2</i> <i>OsGlb1</i> pro- <i>SoyferH2</i> <i>OsAct</i> pro- <i>HvNAS1</i> <i>OsSUT1</i> pro- <i>OsYSL2</i> <i>OsGlb1</i> pro- <i>OsYSL2</i>	<i>Japonica</i> cv. Tsukinohikari	6 fold (brown grain)	[47]
<i>OsGluB1</i> pro- <i>SoyferH2</i> <i>OsGlb1</i> pro- <i>SoyferH2</i> <i>OsAct</i> pro- <i>HvNAS1</i> <i>OsSUT1</i> pro- <i>OsYSL2</i> <i>OsGlb1</i> pro- <i>OsYSL2</i>	Tropical <i>Japonica</i> cv. Paw San Yin	3.4 fold (polished grain)	[88]
<i>GluA2</i> pro- <i>SoyferH1</i> 35S pro- <i>OsNAS2</i>	IR64	6 fold (polished grain)	[69]

Table 2. Combinational of multiple transgene used for iron biofortification in rice.

rice grains when compared to transgenic rice with *ferritin* genes expression driven by single endosperm specific promoter [48]. On the other hand, the overexpression of soybean *ferritin* in rice has been demonstrated with at least twofold increase in iron concentration in endosperm compared to the wild-type rice [49–51, 64, 67].

Nevertheless, introducing *SoyferH2* into rice plants is preferred as *SoyferH1* is more susceptible to protease digestion causing alteration in structure in comparison to *SoyferH2*, which is more resistant to protease digestion [68, 69]. Interestingly, rice plants introduced with single soybean *ferritin* gene did not increase iron concentration in rice grain [48, 68]. This suggests that expressions of *ferritin* genes are dependent on soil composition and overexpression of

ferritin genes as a single transgene approach may be insufficient in combating iron deficiency [48, 70].

5.2. Enhancement of iron transport in rice via *NAS* genes

The second approach involves enhancing iron transport in the plant via overexpression of genes involved in biosynthesis of MA such as *nicotianamine synthase* (*NAS*). *NAS* is able to catalyze the synthesis of nicotianamine (NA) from *S*-adenosyl methionine [23]. NA, a natural metal chelators for Fe(II) and Fe(III), are found in all higher plants and involved with metal translocation and homeostasis in plants [47, 71–73].

Rice comprises of three *NAS* genes, *OsNAS1*, *OsNAS2*, and *OsNAS3*. These genes are involved in long-distance transportation in plants and each *NAS* gene is regulated at different parts of the plants in response to iron deficiency [9, 52]. Overexpression of *NAS* gene enhances MA secretion into the rhizosphere, and thus, increasing iron uptake into the plant via chelation-based strategy [23, 53, 72]. It has been demonstrated that overexpression of rice *OsNAS1*, *OsNAS2* and *OsNAS3* [2], *OsNAS2* [54], *OsNAS3* [55], and barley *HvNAS1* [74] genes are able to increase the iron content by more than twofold in polished grain.

5.3. Enhancement of iron influx into seeds via *OsYSL2* gene

A total of 18 different *YSL* (*yellow stripe-like*) genes were identified by Koike [73] in rice. 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 [73, 75]. *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, Koike [73] hypothesized that this transporter is involved in long-distance transport of iron-NA complexes via phloem in response to iron deficiency in rice plant.

Consistently, it was discovered that the iron influx into the rice endosperm could be controlled through iron nicotianamine transporter *OsYSL2* [60]. Ishimaru [75] successfully demonstrated that disruption of *OsYSL2* gene in rice decreased the iron content in both brown rice and polished grain by 18 and 39%, respectively with increased iron accumulation in roots as compared to wild-type rice. Moreover, Ishimaru [75] also able to increase the iron content in rice grain up to 4-fold in polished grain through enhanced expression of *OsYSL2* using the rice sucrose transporter (*OsSUT1*) promoter. However, overexpression of *OsYSL2* may cause opposite effect similar to *OsYSL2* gene silencing in transgenic rice such that the iron concentration in roots was found higher than in both shoot and rice grain. Undoubtedly, the expression of *OsYSL2* with *OsSUT1* promoter is a promising approach in iron biofortification of rice grains.

5.4. Enhancement of iron uptake and translocation via *IDS3* gene

As mentioned in Section 2.2, MAs are natural iron chelators, which are involved in translocation of iron from the rhizosphere into the plant by forming complexes with iron. Different sets of MAs genes were found in barley, which confers the ability to synthesize different types of MAs via biosynthetic pathway of MAs [76, 77]. In addition, the presence of iron deficiency

specific clone no. 2 (*IDS2*) and no. 3 (*IDS3*) in barley play an important role in combating iron deficiency [77]. The *IDS* genes enable the synthesis of different types of MAs via DMA and these genes are highly expressed in roots in response to iron deficiency [56]. On the contrary, rice lacks the ability to synthesize other types of MAs apart of DMA as rice does not contain both *IDS2* and *IDS3* genes. Having different sets of MAs enable barley become more tolerant to iron-deficient conditions as compared to rice.

Introducing *IDS3* gene from barley enables the synthesis and secretion of different types of MAs from transgenic rice into the rhizosphere [56]. 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 [57]. This may enhance iron translocation in rice in combating iron deficiency while increasing tolerance toward iron deficiency in rice plants. Furthermore, Masuda [58] and Suzuki [78] demonstrated that *IDS3* rice lines are able to increase Fe concentrations to 1.4 and 1.3-fold for both polished and brown grains respectively compared to wild-type rice when grown in either Fe-sufficient soil or Fe-deficient soil. Thus, presence of *IDS3* gene is able to enhance iron accumulation in rice grain even when it is grown in iron-sufficient soil and as well as enhancing tolerance toward iron deficiency.

5.5. Enhancement of iron translocation via *OsVIT* gene

Zhang [59] reported on the functional characterization of rice vacuolar iron transporter genes (*OsVIT1* and *OsVIT2*). These genes were found to be expressed ubiquitously in different parts of the plants at low levels but high level expression of *OsVIT* genes were detected in the flag leaves. These genes play an important role in transportation of Zn^{2+} and Fe^{2+} into vacuoles via tonoplast [79]. In addition, knockdown of *OsVIT* genes increases Fe and Zn accumulation in the rice grains significantly while decreases Fe and Zn accumulation in the flag leaves correspondingly [80]. Knockout of *OsVIT1* and *OsVIT2* genes were able to increase the iron content in rice grain by at least 1.4-fold [59, 80]. However, this approach is only applicable when the transgenic rice is grown in unpolluted soil. This is because studies had shown that accumulation of Cd^{2+} concentration was detected in rice when it is grown in polluted soil [59]. Hence, further understanding of regulatory mechanism is required to prevent toxic metal accumulation and to ensure the crops are safe for consumption.

5.6. Combinational of multiple transgenes

Multiple gene manipulation has been successfully carried out in rice. Wirth [23] has proven the synergism of three different genes expression with the increased of iron content in rice by 6-fold through introducing *Arabidopsis thaliana NAS1* (*AtNAS1*), *Phaseolus vulgaris ferritin* (*Poferritin*), and *Aspergillus fumigatus phytase* (*Aphytase*) genes into rice. The main purpose of introducing *phytase* genes is to reduce iron antinutrient phytate in rice. Some food may contain antinutrients like PA, which binds strongly to metal cations, such as iron and zinc, which render them insoluble [81]. Phytase is able to catalyze the hydrolysis of PA releasing the phosphate and chelated minerals [21]. Human digestive system lacks enzyme responsible for breakdown of such components [23]. Reducing antinutrients is a feasible approach to increase nutrient content in crops but it should be exercised with cautions due to many

antinutrients playing important roles in both plant metabolism and human diet [21, 32]. In plants, antinutrients involve in resistance toward pests, pathogens and abiotic stress and at the same time function as anticarcinogens in human diets [61, 63, 82–85]. For instance, PA is able to reduce the risk for both colon cancer and mammary cancer through its strong metal cations binding capabilities [63, 83]. Moreover, PA display antioxidant capability by acting as inhibitor of iron-mediated hydroxyl radical ($-OH$) formation in food and gastrointestinal tract, which would result in lipid peroxidation and tissue damage [83, 84]. On the other hand, antinutrient lectin was found to be responsible for plant defense system by exhibiting cytotoxic activities when ingested by pests and animals [85].

Masuda [77] has demonstrated that introducing a combination of different genes responsible for MA synthesis into rice (Fer-NAS-NAAT-IDS3 lines) and result in 4-fold increase of iron accumulation in endosperm. Likewise, transgenic line expressing *AtIRT1*, *Pvferritin*, *AtNAS1*, and *Afphytase* was shown to cause a 4-fold increase of iron accumulation in polished grain [66, 86]. The *OsYSL15* or *OsIRT1* genes are predominantly expressed in roots with enhanced expression in response to iron deficiency [86]. *OsIRT1* gene encode for Fe^{2+} transporter involved in both strategy I and II. Although overexpression of *OsIRT1* alone could increase the iron content in rice grain by 1.3-fold, *OsIRT1* has the potential to further enhance the iron content when it is expressed with other genes [87].

On the other hand, combination approaches were also demonstrated to increase the iron content in rice grain by 3.4- and 6-fold when introduced into Myanmar and Japanese rice cultivar respectively [47, 88]. Both *SoyFerH2* and *OsYSL2* were strongly expressed in transgenic rice due to the vector inserted contains two gene cassettes for each gene expression driven by different promoters for each gene cassettes (*OsSUT1* promoter-*OsYSL2*, *OsGlb* promoter-*OsYSL2*, *OsGluB1* promoter-*SoyferH2*, *OsGlb* promoter-*SoyferH2*). Interestingly, Trijatmiko [69] was able to develop transgenic rice expressing *OsNAS2* and *SoyferH1* genes result in 15 μg Fe/g increased (6-fold) in polished grain. In the transgenic rice line, the transgene construct was found to be inserted with inverted repeats in a single locus. This concludes that multiple transgene insertion was able to increase the iron concentration in rice [47, 69, 88]. However, transgene cassette with duplicated or inverted repeats of transgene may not be stable and inherited after several generations due to possibility of epigenetic silencing in transgenic plants [66, 89–91]. Hence, further investigation should be conducted to elucidate the stability of transgene or different approach to maintain multiple transgene over multiple generations.

6. Challenges and future prospect

Biofortification is a promising strategy for sustainable long-term approach in combating micronutrient deficiency but successful biofortification at the cost of the environmental damage is not acceptable. In agronomic practice, leaching is one of the main concerns in application of fertilizer as it will damage the environment, but most micronutrients are not susceptible to leaching as they are able to form a strong bond with the soil [13]. However, continuous application of micronutrient fertilizer may cause accumulation of these minerals which result in

toxicity. Excessive intake of iron may cause Fe^{2+} and Fe^{3+} to act as a catalyst to form noxious reactive oxygen species (ROS). ROS are strong oxidizing agents, which are able to cause detrimental effect on DNA, proteins, and lipids in plants [33]. Therefore, fertilization strategies should be devised and optimized to ensure adequate supply of iron for proper growth of agronomic plants while minimizing accumulation of iron [92]. For instance, the 4R Nutrient Stewardship principle (application of fertilizer at the right place, right rate, right time and right source) could be implemented with fertilizer application [34, 93]. Based on HarvestPlus breeding programs, the iron-biofortified rice are to meet a recommended target iron level which is approximate 30% of the estimated average requirement (EAR) or 15 $\mu\text{g/g}$ (dry weight) in polished grain [69, 94]. The recommended 30% EAR could be achieved via genetic engineering approaches listed in both **Tables 1** and **2**, however, the iron concentration in rice grain decreases when evaluated under field conditions as compared to iron concentration achieved in rice grown in greenhouse [47, 69]. This demonstrates that interactions between genetic and environment play an important role in iron concentration in rice grain [69, 95]. Field experiments should be included in evaluating iron levels in iron-biofortified rice for several growing seasons as evaluating iron levels in rice grown under strictly controlled environment conditions does not simulate the conditions when grown in the field [69, 94, 96, 97].

Biofortified crops still face strict regulatory hurdles and a lack of consumer acceptance, especially in Europe, even though there have been reports on improvement in nutritional status after consuming biofortified crops [16, 38, 98, 99]. For instance, golden rice, a product developed via genetic engineering in combating vitamin A deficiency, has been announced since early 2000, but it has yet to be seen in the market [100]. Although these transgenic plants has demonstrated its high nutritional content in combating micronutrient deficiency, but as far as public safety concern, additional regulations and more stringent monitoring are implemented onto transgenic crops before being available to the public compared to conventional breeding which is more widely accepted [3]. In addition, there are possibilities of irreversibility effect on health and environment due to the effects of GM crops on health and environment are not fully understood and not sustainable in the long run [100, 101]. Furthermore, there are possibilities of the transgene in biofortified crops survived through human digestion system which allows transgenic plant DNA such as antibiotic resistance genes to be transferred into small intestine microflora [102]. Therefore, additional researches from different disciplines are required in order to elucidate the effect of biofortified crops consumption on human health. This may appease public anxiety and to gain consumer acceptance [3]. On the other hand, the recent advancement of genetic engineering technologies, such as zinc-finger nucleases, TALENs and CRISPR-Cas9, could be a potential approach in iron biofortification, which allows efficient and effective gene editing without affecting the plant as a whole [18, 44, 45]. Moreover, gene-edited crops are subjected to different regulations and monitoring from government bodies and nongovernment organizations, which are not as stringent as genetically modified crops. As a result, gene-edited crops will have a higher consumer acceptance compared to conventional genetic engineering.

While iron biofortification in rice is a promising approach in combating iron deficiency, the success of biofortification is dependent on various factors and it requires the collaboration between different parties ranging from consumer, plant breeder, multilateral organizations, national governments, and researchers from various disciplines. Without the help and adoption from plant

breeders, biofortified crops are unable to be produced despite the crop has the potential to alleviate micronutrient deficiency. Hence, to gain plant breeder acceptance, biofortified crops should contain visible and favorable traits such as increased in yield, higher stress tolerant, disease resistance, and other important agronomic traits [10]. Plant breeders may be reluctant to produce the biofortified crops with the potential income risk if the consumer does not adopt with the new crop variety especially with biofortified crops having their sensory characteristics altered such as the color and taste [12]. Some biofortified crops have been introduced for production and accepted by the public in some countries despite the change in sensory characteristics [14]. These biofortified crops are orange flesh sweet potato, orange maize, yellow cassava, iron pearl millet, and iron beans. Consumer acceptance on biofortified crops is not easy and achievable in a short duration of time but it can be accomplished through thoroughly planned strategies such as spreading knowledge among the people, raising awareness of micronutrient deficiency, creating new market opportunities, and creating a demand on biofortified variety [103]. On the contrary, the success of iron biofortification would results in improved nutritional value of micronutrient-deficient affected areas in developing countries and as a first step toward improving nutritional status worldwide.

Acknowledgements

The authors thank Putra Grant (GP/2017/9572000) from Universiti Putra Malaysia and Fundamental Research Grant Scheme (FRGS/1/2014/SG05/MOSTI/1) from Ministry of Higher Education, Malaysia for the support.

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