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Review
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Biofortification of iron and zinc in rice and wheat

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ABSTRACT

Iron and zinc are critical micronutrients for human health. Approximately two billion people suffer from iron and zinc deficiencies worldwide, most of whom rely on rice (*Oryza sativa*) and wheat (*Triticum aestivum*) as staple foods. Therefore, biofortifying rice and wheat with iron and zinc is an important and economical approach to ameliorate these nutritional deficiencies. In this review, we provide a brief introduction to iron and zinc uptake, translocation, storage, and signaling pathways in rice and wheat. We then discuss current progress in efforts to biofortify rice and wheat with iron and zinc. Finally, we provide future perspectives for the biofortification of rice and wheat with iron and zinc.

Keywords: biofortification, hidden hunger, iron and zinc deficiency, malnutrition, micronutrient, rice, wheat

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INTRODUCTION

Iron and zinc are essential micronutrients for human health. As the co-factors of many critical enzymes, both iron and zinc are involved in numerous human metabolic processes. For example, iron-containing enzymes participate in oxidative metabolism, steroid hormone synthesis, and electron transport (Aggett, 2020), while zinc-containing enzymes play important roles in the biosynthesis and degradation of carbohydrates, lipids, proteins, and nucleic acids (Ryu and Aydemir, 2020). Besides being a co-factor of many enzymes, iron is a major component of the oxygen carrier hemoglobin. The effects of iron deficiency include anemia, impaired motor and cognitive development, increased risk of maternal mortality, and premature birth (Aggett, 2020). Young children and pregnant women are particularly vulnerable and severely affected by iron deficiency (Aggett, 2020). Zinc deficiency results in a weakened immune system, and stunted growth (Ryu and Aydemir, 2020). Approximately two billion people worldwide suffer from iron and/or zinc deficiency (Grebmer et al., 2014). This problem is particularly prevalent in low- and medium-income countries, where much of the population cannot afford foods that contain relatively high amounts of iron and zinc, such as meat and milk (Semba et al., 2022). Rice (*Oryza sativa*) and wheat (*Triticum aestivum*) are the dominant staple foods, feeding more than half of the human population of the world (Fitzgerald et al., 2009). Coincidentally, they are also the staple foods of most people who have iron and zinc deficiencies (Kiely et al., 2021; Satyavathi et al., 2021).

Over the past few decades, increasing crop yields to feed the growing human population has been a focus of crop breeding. While this has successfully generated modern crop cultivars with high yields, an unfortunate side effect has been low micronutrient contents in crops. In addition, milling removes the aleurone layer, which contains a large proportion of the iron and zinc; thus, the iron and zinc contents of polished rice and white flour are much lower than those of whole grains (Brier et al., 2015; Hensawang et al., 2020). For many people, rice and wheat provide sufficient dietary carbohydrates but not enough iron and zinc, resulting in malnutrition.

This malnutrition is also known as hidden hunger (Kiely et al., 2021; Satyavathi et al., 2021). The average iron concentration in current popular cultivars is 2 μg/g in polished rice and 30 μg/g in wheat grain. The average zinc concentration is 16 μg/g in polished rice and 25 μg/g in wheat grain (Bouis et al., 2011). Addressing iron and zinc deficiencies would require achieving target micronutrient concentrations of 15 μg/g iron in polished rice and 59 μg/g iron in wheat grain, as well as 28 μg/g zinc in polished rice and 38 μg/g zinc in wheat grain (Bouis et al., 2011).

Various approaches could be used to achieve these targets, including biofortification, which is the most promising, sustainable, and long-lasting. In this review, we describe the uptake, translocation, storage mechanisms, and signaling pathways of iron and zinc in rice and wheat. Based on these mechanisms, we then highlight current progress toward biofortification in rice and wheat and future perspectives in the field.

IRON UPTAKE, TRANSLOCATION, AND STORAGE IN RICE

Although iron is abundant in the earth's crust, it is mostly present as iron oxide minerals, which are insoluble. Plants have evolved two distinct strategies that allow them to take up sufficient iron: the reduction strategy (Strategy I), which is exhibited by all non-graminaceous plants, and the chelation strategy (Strategy II), which is exhibited by graminaceous plants, including rice and wheat (Liu et al., 2021b).

Strategy II involves Fe(III)-chelating molecules called phytosiderophores (PSs), which are a group of mugineic acids that are synthesized from deoxymugineic acid (DMA) in root cells. DMA is the product of a series of reactions catalyzed by NICOTIANAMINE SYNTHASE (NAS) (Higuchi et al., 1999), NICOTIANAMINE AMINOTRANFERASE (NAAT) (Takahashi et al., 1999), and DEOXYMUGINEIC ACID SYNTHASE (DMAS) (Bashir et al., 2006) that starts with S-adenosylmethionine. PSs are released to the rhizosphere by TOM1 (transporter of mugineic acid family phytosiderophores) (Nozoye et al., 2011) (Figure 1). In the rhizosphere, PSs chelate ferric iron. Then, the Fe(III)-PS complexes are transported into the root cells by YELLOW STRIPE1 (YS1) or YS1-LIKE transporters (Curie et al., 2001, 2009). However, unlike in other graminaceous plants, rice roots take up not only ferric iron, but also ferrous iron, which is the major form of iron in paddy soil. OsIRT1 and OsIRT2, two members of the ZIP transporter family, function in the uptake of ferrous iron in rice (Bughio et al., 2002; Ishimaru et al., 2006) (Figure 1).

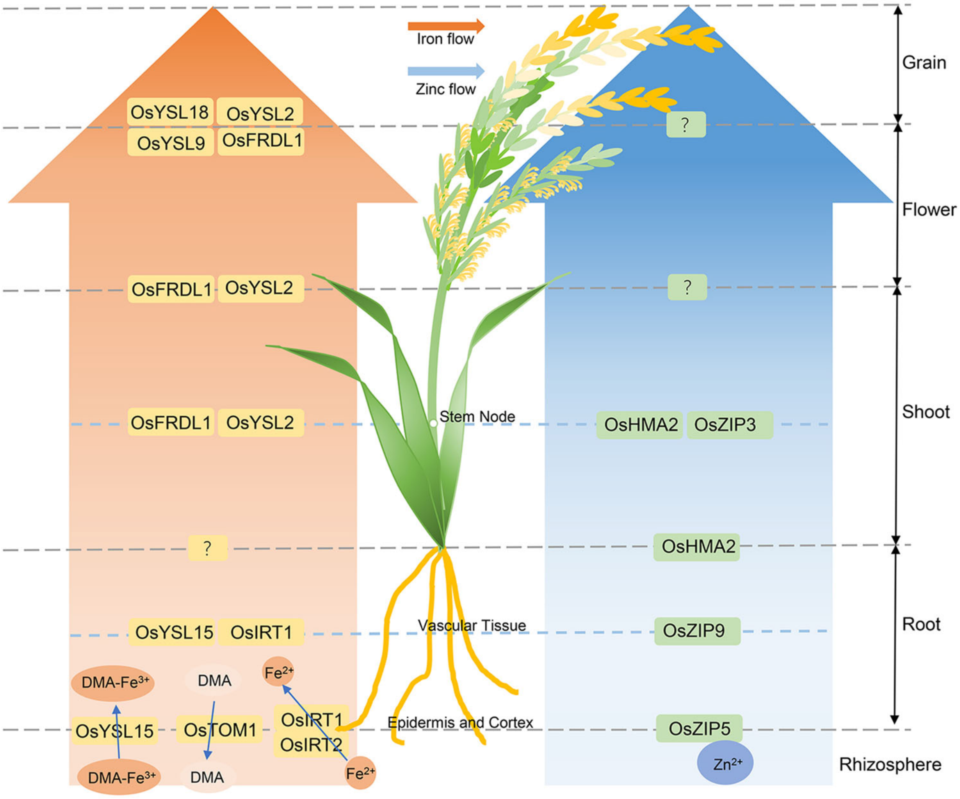

Figure 1. Uptake and translocation of iron and zinc in rice

Schematic of the critical proteins involved in the uptake and translocation of iron and zinc. The orange arrow indicates the iron flow and the blue arrow indicates the zinc flow.

In rice, the components of the Strategy II iron uptake system have been well studied. Rice has three *NAS* genes, six *NAAT* genes, and one *DMAS* gene. Among the six *NAAT* genes, *NAAT1* is the most important for the biosynthesis of DMA. A null mutant of *NAAT1* fails to produce DMA and cannot efficiently absorb Fe(III). A detailed analysis suggested that this *naat1* mutation results in strong stimulation of the Fe(II) acquisition system and leads to a significantly higher concentration of iron in both brown and polished *naat1* rice grown under water-logged field conditions (Cheng et al., 2007). This study also demonstrated the importance of the Fe (II) acquisition system in rice (Cheng et al., 2007). OsTOM1 is involved in the excretion of DMA into the soil, and OsYSL15 contributes to the uptake of the Fe(III)-DMA complexes (Lee et al., 2009a; Nozoye et al., 2011) (Figure 1). In contrast to the well-known process of iron uptake, only a few transporters involved in iron translocation have been identified. Among these transporters, the multidrug and toxic compound extrusion (MATE) transporter OsFRDL1 is responsible for the root-to-shoot translocation of the iron–citrate complex (Yokosho et al., 2009) (Figure 1). *OsFRDL1* is expressed in nodes and is required for the distribution of iron to grains (Yokosho et al., 2016) (Figure 1). Another protein, OsYSL2, transports iron and manganese in the phloem. Koike et al. (2004) established that *OsYSL2* is expressed in companion cells in roots, leaves, and leaf sheaths, and its expression is induced by iron deficiency. Additionally, *OsYSL2* is expressed in flowers and developing seeds. This expression pattern suggests that OsYSL2 functions in the phloem transport of iron and in iron accumulation in seeds (Koike et al., 2004). *Xenopus laevis* oocyte assays demonstrated that OsYSL2 transports Fe(II)-NA and Mn(II)-NA but not Fe(III)-DMA (Koike et al., 2004) (Figure 1). OsYSL18 was proposed to be involved in iron translocation in rice grains on the basis of its expression pattern (Figure 1). OsYSL18 transports Fe (III)-DMA but not Fe(II)-NA (Aoyama et al., 2009). Furthermore, OsYSL9 transports both Fe(II)-NA and Fe(III)-DMA (Senoura et al., 2017). Functional analysis suggested that OsYSL9 is involved in internal iron translocation, especially from the endosperm to the embryo (Senoura et al., 2017) (Figure 1).

Iron homeostasis is tightly controlled in plant cells, as excessive iron is toxic due to the generation of hydroxyl radicals. Ferritin, a globular protein, can store up to 4,500 iron atoms in a bioavailable and non-toxic form. Additionally, the vacuole is an important organelle for storage of excess iron in plants. Transporters located on the tonoplast regulate iron trafficking between the cytosol and vacuole to maintain iron homeostasis. Two iron transporters on tonoplast, OsVIT1 and OsVIT2, have been functionally characterized in rice. *osvit1* and *osvit2* mutants accumulated more iron and zinc in the seeds than did wild-type rice (Zhang et al., 2012). More importantly, polished grains of the *osvit2* mutant show elevated iron accumulation (Che et al., 2021). Moreover, they do not show yield penalty, suggesting that the *osvit2* mutation could potentially be exploited in breeding rice with high levels of iron in the grain.

KEY REGULATORS OF IRON DEFICIENCY RESPONSES IN RICE

Several transcription factors that play important roles in iron homeostasis in rice have been identified, such as the basic helix-loop-helix (bHLH) transcription factors OsbHLH156 (also known as OsFIT) and OsIRO2/OsbHLH56. Two groups independently showed that OsbHLH156 facilitates the nuclear localization of OsIRO2 and that these two transcription factors form a functional transcription activation complex that regulates iron uptake by initiating the expression of iron deficiency-inducible genes (Liang et al., 2020; Wang et al., 2020). Other bHLH transcription factors, including OsPRI1/OsbHLH60, OsPRI2/OsbHLH58, OsPRI3/OsbHLH59, and OsIRO3/OsbHLH63, also function in iron homeostasis in rice (Liu et al., 2021b). Besides the bHLH transcription factors, the IDEF1 and IDEF2 transcription factors are involved in rice iron homeostasis (Kobayashi et al., 2007, 2009, 2012; Ogo et al., 2008). IDEF1 belongs to the plant-specific transcription factor family ABI3/VP1 and specifically binds to the CATGC sequence in the iron deficiency-responsive cis-acting element IDE1 (Kobayashi et al., 2007). This transcription factor functions mainly in the early stages of the iron deficiency response by positively regulating *OsIRO2* and several other genes involved in iron homeostasis. Interestingly, IDEF1 plays a positive role in the iron deficiency response, likely through improving iron utilization rather than iron uptake (Kobayashi et al., 2007, 2009). IDEF1 can directly bind iron and zinc through histidine-asparagine and proline-rich regions, which are essential for IDEF1 function in iron homeostasis. Therefore, Kobayashi et al. (2012) proposed that IDEF1 is a cellular iron sensor. IDEF2 is a NAC transcription factor; it predominantly recognizes CA(A/C)G(T/C)(T/C/A)(T/C/A) within the iron deficiency-responsive element 2 (IDE2) (Ogo et al., 2008). Similar to IDEF1, IDEF2 is a positive regulator for iron homeostasis in rice and regulates the expression of *OsYSL2* and several other iron deficiency-inducible genes (Ogo et al., 2008).

In addition to these transcription factors, rice possesses many other critical regulators of the iron deficiency response, such as the ubiquitin ligases (OsHRZ1 and OsHRZ2) and iron deficiency-inducible peptide-coding genes (*OsIMA1* and *OsIMA2*) (Kobayashi et al., 2013, 2021). OsHRZ1 and OsHRZ2 are iron-binding hemerythrin motif-containing E3 ubiquitin ligases which negatively regulate the iron deficiency response. *OsHRZ* knockdown plants accumulate more iron in shoots and grains and show more tolerance for iron deficiency, along with higher expression levels of iron deficiency-inducible genes compared to wild-type plants under iron-sufficient conditions (Kobayashi et al., 2013). Given their iron-binding capacity, OsHRZs are also considered putative iron sensors (Kobayashi et al., 2013). OsHRZ1 interacts with and can degrade OsPRI1/OsbHLH60, OsPRI2/OsbHLH58, and OsPRI3/OsbHLH59 (Zhang et al., 2017, 2020). These proteins directly bind to the promoters of *OsIRO2/OsbHLH56* and *OsIRO3/OsbHLH63* and regulate the expression of these two genes (Zhang et al., 2017, 2020). Therefore, these regulators together form a OsHRZ1–OsPRI1/2/3–OsIRO2/3 signal transduction cascade in response to iron deficiency in rice (Zhang et al., 2017, 2020). The expression of HRZs is indirectly repressed by PHR2, which is the central regulator of phosphate signaling in rice (Guo et al., 2022). OsIMA1 and OsIMA2 are positive regulators in the iron deficiency response in rice. The expression of *OsIMA1* and *OsIMA2* is strongly induced by iron deficiency stress; positively regulated by IDEF1, OsPRI2/OsbHLH58, OsPRI3/OsbHLH59, and OsIMA1/2; and negatively regulated by HRZ (Kobayashi et al., 2021). Overexpression of *OsIMA1* or *OsIMA2* resulted in an increased tolerance for iron deficiency and increased accumulation of iron in the leaves and seeds (Kobayashi et al., 2021).

ZINC UPTAKE, TRANSLOCATION, AND STORAGE IN RICE

Zinc transporters are essential for zinc uptake in rice. OsZIP9, a well-characterized zinc transporter, contributes to zinc uptake in rice (Yang et al., 2020) (Figure 1). *OsZIP9* is expressed in the exodermis and endodermis of mature regions of the root (Huang et al., 2020a), and its coding protein possesses the highest zinc influx transport activity among the ZIP family proteins (Yang et al., 2020). *OsZIP9* expression is controlled not only by zinc deficiency in roots, but also by systemic signals of zinc status derived from the shoot. Knockout of *OsZIP9* results in greatly reduced zinc accumulation in roots, shoots, and grains under both hydroponic and paddy soil conditions (Yang et al., 2020). Overexpression of *OsZIP9* significantly increases the zinc contents in the aboveground tissues and brown rice (Tan et al., 2020). A trait association analysis suggested that the natural variation in *OsZIP9* expression levels is highly associated with zinc content in polished rice across different rice varieties (Yang et al., 2020). OsZIP5 also influences zinc uptake in rice (Lee et al., 2010a; Tan et al., 2020) (Figure 1). Based on the phenotypes of the knockout mutants *oszip5*, *oszip9*, and *oszip5oszip9*, OsZIP5 has functional redundancy with OsZIP9 but a relatively weaker effect (Tan et al., 2020). In addition to these members of the ZIP family, another zinc transporter OsZIP8 also controls zinc uptake in rice (Lee et al., 2010b).

Zinc transporters also contribute to zinc translocation in rice. *OsZIP3*, which is highly expressed in the nodes, regulates zinc translocation in rice (Sasaki et al., 2015) (Figure 1). Immunostaining and stable isotope assays suggested that OsZIP3 is responsible for unloading zinc from the xylem of enlarged vascular bundles and contributes to the preferential distribution of zinc to developing tissues of rice (Sasaki et al., 2015). In addition, OsHMA2, a P-type adenosine triphosphatase, is a major transporter of zinc from roots to shoots (Satoh-Nagasawa et al., 2012; Takahashi et al., 2012; Yamaji et al., 2013) (Figure 1). Similar to *OsZIP3*, *OsHMA2* is highly expressed in the nodes at the reproductive stage, suggesting that *OsHMA2* contributes to the preferential delivery of zinc to developing tissues (Yamaji et al., 2013). Interestingly, overexpression of *OsHMA2* significantly decreased grain cadmium content but did not affect zinc content of the grain (Takahashi et al., 2012). Notably, disruption of the C-terminal region of OsHMA2 reduced its affinity for the heavy metal cadmium but not for zinc (Satoh-Nagasawa et al., 2012). In addition, vacuolar sequestration is an important strategy to avoid zinc toxicity in rice. Cai et al. (2019) established that OsHMA3, a homolog of OsHMA2, is localized on tonoplast and mediates zinc sequestration into the vacuoles.

FUNCTIONALLY CHARACTERIZED GENES INVOLVED IN IRON AND ZINC HOMEOSTASIS IN WHEAT

In wheat, few genes involved in iron and zinc uptake, translocation, and storage have been functionally characterized, owing to the large, allohexaploid genome and the technical challenges in transforming wheat. Among these genes, *TaVIT2* has been identified as encoding a vacuolar iron transporter. Overexpression of *TaVIT2* with an endosperm-specific promoter results in a more than twofold increase in flour iron content (Connorton et al., 2017). Similar to OsHMA2, its wheat homolog, TaHMA2, is a zinc transporter that modulates the root-to-shoot zinc translocation. However, the functions of OsHMA2 and TaHMA2 are not identical. Overexpressing *OsHMA2* has no effect on the grain zinc content in rice, while overexpression of *TaHMA2* decreases the seed zinc content of transgenic wheat (Takahashi et al., 2012; Tan et al., 2013). Additionally, overexpression of *TaHMA2* disrupts zinc distribution in wheat grains: the grains of *TaHMA2*-overexpressing wheat exhibit a decreased zinc content in the ventral endosperm and an increased zinc content in the embryo and aleurone (Tan et al., 2013).

The function of ferritin genes in hexaploid wheat has also been characterized (Borg et al., 2012). Overexpression of *TaFer1-A* under the control of an endosperm-specific promoter results in a 50%–85% higher grain iron content (Borg et al., 2012). Qiao et al. (2019) isolated *TaCNR5* from common wheat and established that heterologous expression of *TaCNR5* in rice significantly increases the zinc concentration of brown rice. *TmNAS3* is an important nicotianamine synthase in *Triticum monococcum*. Overexpression of *TmNAS3* in wheat results in a more than onefold increase in grain iron concentration compared to that of the non-transgenic line (Wang et al., 2022). The NAC transcription factor NAM-B1, which is encoded by the causal gene of the *Gpc-B1* locus, was identified as an important regulator of iron and zinc homeostasis in wheat (Uauy et al., 2006). Functional NAM-B1 accelerates senescence and increases iron and zinc remobilization from leaves to developing grains (Uauy et al., 2006). *NAM* knockdown lines with reduced expression of all *NAM* family members have low grain iron and zinc concentrations (Waters et al., 2009). A transcriptome sequencing (RNA-seq) analysis of *NAM-B1/GPC1* loss-of-function mutants in tetraploid wheat suggested that NAM-B1/GPC1 regulation of iron and zinc transporters leads to the low grain iron and zinc contents of the *nam-b1/gpc1* mutant (Pearce et al., 2014).

PROGRESS IN THE BIOFORTIFICATION OF RICE AND WHEAT WITH IRON AND ZINC

Various approaches are used to combat iron and zinc malnutrition in humans, including fortification and biofortification. Fortification is an approach of supplement of iron and zinc salt to food. However, the effect of fortification is largely limited by the low bioavailability of iron and zinc and the low consumer acceptability of fortified food (Pyo et al., 2022). In contrast, biofortification is the most promising and sustainable approach and can be further categorized into agronomic biofortification, biofortification through conventional breeding, and biofortification through genetic modification.

Agronomic biofortification

Agronomic biofortification is a fertilizer-based approach that includes soil and foliar fertilizer application (Liu et al., 2017). Indeed, fertilizer application is a simple and fast-acting approach that can complement other biofortification approaches. However, soil fertilizer application is not efficient because many elements in soil can react with iron and zinc to produce insoluble forms and reduce their bioavailability (Ramzani et al., 2016). Foliar sprays are more efficient than soil applications (Zhang et al., 2010); however, they may result in iron and zinc toxicity in the leaves.

Numerous other factors can influence the effectiveness of agronomic biofortification, such as the form of fertilizers used, the application time point, soil iron and zinc contents, and the genotype of rice and wheat cultivars (Wei et al., 2012). Among the various forms of fertilizers, iron and zinc nanochelated fertilizers cause less leaf damage than conventional fertilizers, enhancing their effectiveness (Deshpande et al., 2017; Zhang et al., 2018b; Doolette et al., 2020; Fakharzadeh et al., 2020; Sun et al., 2020).

Biofortification through conventional breeding

In contrast to agronomic approaches, biofortification through conventional breeding offers an environmentally friendly and long-term solution (Figure 2A). However, conventional breeding is time-consuming, and could take several years or decades to breed an excellent variety with high grain iron and zinc contents.

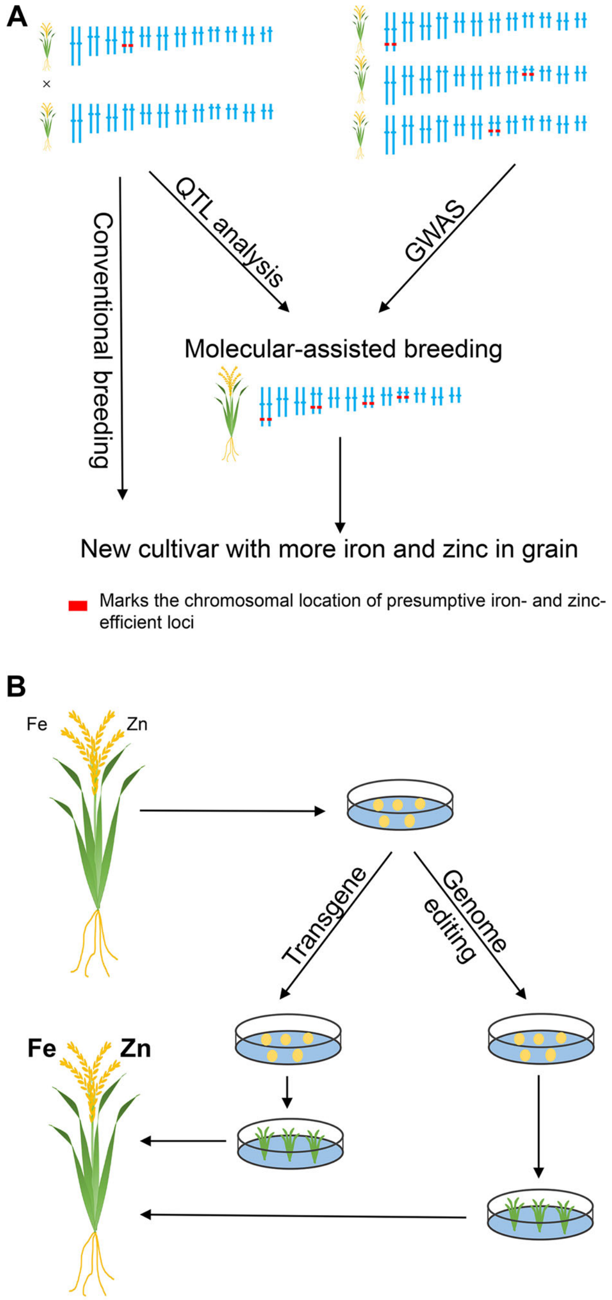

Figure 2. Different methods of biofortification
(A) Biofortification through conventional breeding. **(B)** Biofortification through genetic modification.

The success of conventional breeding depends on sufficient germplasm variation, including major quantitative trait loci (QTL) and superior alleles. The iron content of rice grains ranged from 7.5 to 24.4 μg/g among 939 germplasms analyzed by the International Rice Research Institute (Grahama et al., 1999), and the zinc content ranged from 20 to 68 μg/g (Huang et al., 2020b). Liu et al. (2019) found that the grain iron content ranged from 32 to 63.1 μg/g in a hexaploid wheat mapping population. More recently, Liu et al. (2021a) reported that a grain zinc content ranging from 38.22 to 101.38 μg/g in a Chinese wheat mini core collection contained 246 germplasms. However, there have been few large-scale analyses of endosperm iron and zinc contents in rice and wheat (Zhang et al., 2018a; Suman et al., 2021). In a set of 698 rice germplasms, the concentrations of iron and zinc in the polished rice ranged from 0.9 to 9.1 μg/g and 5.8 to 29.6 μg/g, respectively (Zhang et al., 2018a). These studies suggested that it is possible to generate rice and wheat varieties with target levels of iron and zinc concentrations through conventional breeding because the iron and zinc concentrations of some germplasms are higher than the target levels of iron and zinc contents in rice and wheat (Bouis et al., 2011).

The variation in iron and zinc contents in rice and wheat grains indicates that these are quantitative genetic traits. Over the last few decades, many loci involved in iron and zinc accumulation in rice grains have been identified by QTL mapping and genome-wide association studies (GWASs). Ninety-three QTL associated with iron content have been identified over the 12 rice chromosomes, with the highest number located on chromosomes 1, 3, and 7, and 17 stable QTLs located on chromosomes 1–4, 6–8, 10 and 11. Importantly, 12 of these were the most prominent stable QTL across different seasons, locations, environments, and populations (Swamy et al., 2021). An association analysis of zinc content of 40 genotypes in three environments showed that single nucleotide polymorphisms located in three putative candidate genes on chromosomes 3 and 7 are associated with high zinc content in polished rice (Babu et al., 2020). An analysis of zinc contents in brown and polished rice from 190 recombinant inbred lines (RILs) in four environments identified two major QTL (*qZPR.1.1* and *qZPR.11.1*) for grain zinc content in polished rice on chromosomes 1 and 11, and a common major QTL (*qZBR.2.1* and *qZPR.2.1*) for zinc content in brown and polished rice was identified on chromosome 2 (Suman et al., 2021).

To explore the QTL for the zinc content of polished rice in wild rice, a QTL analysis was performed with the backcross RILs generated from *Oryza sativa* and the Australian wild rice *Oryza meridionalis*. Four QTLs were detected on chromosomes 2, 9, and 10. *qGZn9*, consisting of two tightly linked loci, *qGZn9a* and *qGZn9b*, had the largest effect on grain zinc content (Ishikawa et al., 2017). The authors also proposed that *qGZn9b* could be a valuable QTL for zinc biofortification through conventional breeding in rice. In addition to these multi-population studies, many association mapping studies have been performed to identify QTLs associated with both iron and zinc contents using a single population. A GWAS analysis with 144 multi-parent advanced generation intercross (MAGIC) plus lines detected seven loci for both iron and zinc contents in rice grains, among which *qZn7.1* was the most stable and consistently identified in all four environments (Descalsota et al., 2018). Six QTLs (*qFe1*, *qFe3*, *qFe6-1*, *qFe6-2*, *qFe7*, and *qFe10*) for iron content and four QTLs (*qZn1*, *qZn7*, *qZn9*, and *qZn12*) for zinc content in polished rice were identified from a set of 698 germplasms composed of two subsets of *indica* and *japonica* rice (Zhang et al., 2018a). From 485 germplasm lines, two novel QTL (*qFe3.3* and *qFe7.3*) for iron content and three novel QTL (*qZn2.2*, *qZn8.3*, and *qZn12.3*) for zinc content were detected in polished rice. Pradhan et al. (2020) suggested that 12 QTL (*qFe1.1*, *qFe3.1*, *qFe5.1*, *qFe7.1*, *qFe8.1*, *qFe3.3*, *qFe7.3*, *qZn6*, *qZn7*, *qZn2.2*, *qZn8.3*, and *qZn12.3*) could be used for biofortification through conventional breeding without yield penalty.

Numerous QTL analyses have sought to identify genomic regions associated with iron and zinc contents in wheat grain. A GWAS of 330 bread wheat lines revealed 39 markers associated with grain zinc content. The major QTL were identified on chromosomes 2 and 7 (Velu et al., 2018). Another GWAS of 246 wheat varieties discovered 16 genomic regions associated with grain zinc content on 11 chromosomes: 1B, 2B, 2D, 3A, 3D, 4A, 4B, 5A, 5D, 6B, and 7D. However, none of these regions contributed more than 6.6% of the variation (Liu et al., 2021a). A QTL mapping study in a hexaploid wheat mapping population detected nine QTL for grain iron content and 10 QTL for grain zinc content (Liu et al., 2019). Wild relatives of wheat also harbor a few QTL associated with grain iron and zinc contents. Several QTL studies of a mapping population derived from wild relatives of wheat have been conducted and have identified several major QTL for grain iron and zinc contents (Srinivasa et al., 2014; Velu et al., 2016; Crespo-Herrera et al., 2017; Krishnappa et al., 2017). These QTL are valuable resources for iron and zinc biofortification through conventional breeding.

In addition to the absolute contents, the bioavailability of iron and zinc in grain is important for biofortification. Phytic acid, a major storage form of phosphorus, has a high negative charge and can strongly bind to many cations, such as iron and zinc. Iron and zinc chelated by phytic acids cannot be absorbed by the gut, which dramatically reduces their bioavailability (Pramitha et al., 2021). Several low phytic acid mutants and QTL have been identified (Gyani et al., 2020; Wang et al., 2021). However, low phytic acid levels may not substantially boost the bioavailability of iron and zinc in polished rice and white flour, because the phytic acid is mainly accumulated in the aleurone layers, which is removed by milling (Williams, 1970; Tanaka et al., 1973).

Aleurone is the outer single-cell layer of a grain in which the majority of iron and zinc are accumulated. Increasing the thickness of aleurone layers could increase the iron and zinc contents in polished rice (Li et al., 2021). A large-scale screen recently identified two thick aleurone rice mutants, and their causal genes have since been identified (Liu et al., 2018; Li et al., 2021). The authors proposed that superior alleles generating thick aleurones are valuable for biofortification through conventional breeding.

In summary, many QTL and several superior alleles of grain with high iron and zinc contents have been characterized. These QTL and superior alleles, which dramatically increase the uptake and translocation of iron and zinc to the grain, could be pyramided through conventional breeding and/or molecular marker-assisted breeding to generate rice and wheat varieties with high grain iron and zinc contents.

Biofortification through genetic modification

In contrast to conventional breeding, biofortification through genetic modification is a more robust and quicker strategy to diminish human malnutrition worldwide. However, the biosafety of transgenics is a topic of intense debate (Figure 2B).

Transgenic approaches are important for genetic modifications. As rice is not only a staple food crop but also a model monocotyledonous plant, an efficient transformation protocol for rice has been developed, and transformation is relatively straightforward (Ishida et al., 2015). Therefore, most work involving biofortification through genetic modification has been performed in rice.

The aims of genetic modification for micronutrients biofortification include increasing the uptake of micronutrients from the soil, enhancing the translocation of micronutrients to the grain, especially to the endosperm, and increasing the bioavailability of micronutrients. These strategies may function synergistically and can be combined to achieve the best results. Numerous efforts have been made to biofortify rice with iron via transgenic approaches. Many of these trials employed transporter genes involved in iron uptake from the soil. The effects of overexpression of *OsIRT1*, which acquires ferrous iron from the soil, and *OsYSL15*, which acquires Fe (III)-DMA from the soil, on the iron content of the grain are very weak (Lee and An, 2009; Lee et al., 2009a). Rice lines overexpressing *OsIRT1* and *OsYSL15* showed a 13% and 30% increase, respectively, in grain iron content. To enhance the translocation of iron to grain, genes encoding the iron storage protein ferritin, nicotianamine synthase (NAS), and iron transporters were tested. Transformation of rice with the soybean (*Glycine max*) ferritin gene *SoyferH1* controlled by an endosperm-specific promoter, *GluB-1* or *Glb-1*, resulted in a threefold increase in the seed iron content (Goto et al., 1999; Qu le et al., 2005). Prussian blue staining revealed that the endosperm cells of transgenic rice grains accumulated significantly more iron than those of non-transgenic rice grains (Vasconcelos et al., 2003; Qu le et al., 2005). Overexpression of the rice endogenous ferritin gene *Osfer2* under the control of the endosperm-specific *GlutelinA2* (*OsGluA2*) promoter resulted in a 2.09-fold iron accumulation in polished rice (Paul et al., 2012). Overexpression of *NAS* genes (using either enhancer trap or transgenic strategy) increased the iron content in polished rice in different cultivars by 2- to 4-fold (Masuda et al., 2009; Lee et al., 2009b; Johnson et al., 2011). An analysis of grains through synchrotron X-ray fluorescence spectroscopy confirmed that *OsNAS2* overexpression results in a dramatic accumulation of iron and zinc in phosphorus-free regions of rice endosperm (Johnson et al., 2011). Moreover, overexpression of *NAS* increased not only the iron content in polished rice, but also its bioavailability because the iron chelated by NA can be digested and absorbed more easily, based on Caco2 cell assays (Zheng et al., 2010; Trijatmiko et al., 2016). Overexpression of the iron transporter gene *OsYSL2* driven by the promoter of *OsSUT1* resulted in a 4-fold increase in iron content in polished rice (Ishimaru et al., 2010).

Compared with the limited biofortification effect of transforming a single gene in rice as mentioned earlier, the simultaneous introduction of multiple genes results in greater biofortification. Trijatmiko et al. (2016) generated transgenic rice combining constitutive overexpression of *OsNAS2* and endosperm-specific expression of the soybean ferritin gene *SoyferH-1* in the cultivar IR64. The iron content of the polished rice of one single-insertion line was up to 7.5-fold increase compared to that of non-transgenic rice. Wirth et al. (2009) developed transgenic rice expressing three heterologous genes: the common bean (*Phaseolus vulgaris*) *ferritin* gene, the fungal (*Aspergillus fumigatus*) *phytase* gene driven by the rice endosperm-specific globulin promoter, and the *Arabidopsis* gene *AtNAS1* under the control of the constitutive 35S promoter. The iron contents in brown and polished transgenic rice were up to 2- and 6.3-fold that of the wild-type control, respectively. These results suggested that expression of these three genes enhanced iron translocation to the endosperm in the transgenic plants (Wirth et al., 2009). Similarly, Masuda et al. (2012) produced transgenic rice lines by simultaneously introducing three genes: the soybean *ferritin* gene *SoyferH2*, the barley (*Hordeum vulgare*) *nicotianamine synthase* gene *HvNAS1*, and the rice nicotianamine-metal transporter gene *OsYSL2*. *SoyferH2* expression was under the control of two endosperm-specific promoters (*OsGlb1* and *OsGluB1*), *HvNAS1* expression was under the control of the *OsActin1* promoter, and *OsYSL2* expression was under the control of the *OsSUT1* and *OsGlb1* promoters in the transgene cassettes. The iron concentration in the polished rice of the resulting transgenic lines was 6- and 4.4-fold higher than that of the non-transgenic lines when grown in the greenhouse and paddy field, respectively (Masuda et al., 2012).

In addition to iron biofortification, several studies of zinc biofortification in rice have been reported. Overexpression of *OsIRT1*, which encodes a transporter of both iron and zinc, increases the zinc concentration in mature seeds by 12%. As NA is also a critical chelator of zinc in rice, increasing NA biosynthesis would increase the zinc content of rice grains. Transgenic rice lines expressing *HvNAS1* driven by the 35S promoter and *OsACTIN1* promoter had 1.5- and 2.5-fold zinc contents in polished rice, respectively, compared to non-transgenic lines (Masuda et al., 2009). Overexpression of *OsNAS1-OsNAS3* under the control of different forms of the 35S promoter and the *UBIQUITIN1* promoter from maize in rice has been investigated by several groups. The transgenic lines had 2.2–2.9 times higher zinc contents than those of non-transgenic plants in polished rice (Lee et al., 2009b, 2011; Johnson et al., 2011).

In summary, most of the genes involved in iron and zinc uptake and homeostasis have been tested for applications in biofortification using transgenic approaches. The greatest increase in iron content in polished rice was achieved using a multi-transgenic approach, making this the most promising method for biofortification by genetic modification (Trijatmiko et al., 2016).

PERSPECTIVES

Although progress has been made in biofortifying rice and wheat with iron and zinc, many challenges remain and more genetic and technical resources are needed to further achieve this goal.

First, the molecular mechanisms underlying the translocation of iron and zinc to the endosperm of rice and wheat are unclear. The long-distance transport of both iron and zinc relies on the xylem; however, the xylem is discontinuous at the base of each seed in cereal plants. Key genes regulating the translocation of iron and zinc from the xylem to the grain via the phloem remain to be identified. These genes could help facilitate iron and zinc accumulation in grain. Both iron and zinc accumulate mainly in the aleurone layer of rice and wheat. However, few critical factors/genes controlling the translocation of iron and zinc from the aleurone layer to the endosperm have been identified. Identifying these factors/genes may greatly facilitate the biofortification of rice and wheat with iron and zinc.

Second, the development of genetic resources would advance biofortification research. Mutagenesis combined with non-destructive detection systems for iron and zinc is an important approach for creating elite germplasm with high endosperm iron and zinc contents. Wild relatives of rice and wheat should be screened for high endosperm iron and zinc contents; such plants could then be used for conventional breeding, transgenics, or *de novo* domestication. Pan-genome analysis is another method that could be used to discover genes or superior alleles that could be used for biofortification. Additionally, microbial genes that significantly increase the uptake, transport, and bioavailability of iron and zinc could be used for transgenically expressing in rice and wheat to biofortify iron and zinc.

Third, after identifying critical genes and factors, site-directed genome editing and marker-assisted breeding will likely be the most reliable technologies for biofortification of rice and wheat with iron and zinc. The first genome-edited tomato (*Solanum lycopersicum*) generated using the clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system was approved in Japan for commercial sale in December of 2020 and was not regulated as a genetically engineered product. Thus, site-directed genome editing might soon be a promising technology for efficiently generating new varieties. With the rapid development of CRISPR/Cas systems, many advances have been achieved, including prime editing, base editing, and protospacer adjacent motif-less editing. The CRISPR/Cas system provides a powerful tool for site-directed editing of the critical genes or factors involved in iron and zinc homeostasis to quickly generate new iron and zinc biofortified varieties of wheat and rice. This approach could significantly accelerate advances in biofortification, solving iron and zinc malnutrition issues worldwide. In addition to site-directed genome editing, pyramiding superior alleles and/or critical genes through marker-assisted breeding could also significantly accelerate the generation of new iron and zinc biofortified varieties of wheat and rice.

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AUTHOR CONTRIBUTIONS

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